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Detection of extended-spectrum beta-lactamases in Gram-negative bacilli from seawater in Algiers port (Algeria)

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ABSTRACT/RESUME

Abstract: *The antibiotics resistance of aquatic environmental bacteria is an established fact; however, in order to understand its origin and development, it is necessary to elucidate its mechanisms. Basing on the importance of beta-lactams in therapy and extended-spectrum beta-lactamases (ESBLs) as a resistance mechanism, the objective of this work was to investigate ESBLs in Gram negative bacilli recovered from seawaters of Algiers port. Twelve isolates, identified as Citrobacter freundii (n=4), Escherichia coli (n=1), Aeromonas hydrophila (n=2), Vibrio fluvialis (n=4) and Bordetella sp (n=1), were found producers of ESBLs CTX-M-3, CTX-M-15, PER-1, VEB-1, SHV_{2a} and oxacillinase OXA-101. bla_{CTX-M} and bla_{PER-1} genes were transferred in association with self-transferable plasmids of approximately >70kb and 138 kb, respectively. The class - integrons were present in 7 isolates and were transferable from 3 isolates. The insertion sequence ISEcp1B was found genetically linked to bla_{CTX-M} genes. This study described different types of ESBLs in marine water which is related to anthropogenic inputs. This is an emerging concern around the world and requires implementation of serious resistance monitoring in environment.*

I. Introduction

Beta-lactams are the most varied and widely used antibiotics, and the production of beta-lactamases is one of the most common mechanisms of bacterial resistance. Extended-spectrum beta-lactamases (ESBLs) are plasmid-mediated enzymes which confer resistance to the expanded-spectrum cephalosporins and aztreonam, and which have emerged worldwide since several years. ESBLs were involved in many outbreaks and related to enhanced morbidity and mortality rates. Classically, ESBLs have evolved from point mutations affecting old enzymes TEM-1, TEM-2 and SHV-1. However, other types of ESBLs have been reported, such as PER, VEB, TLA-1, GES/IBC, SFO-1, BES-1, CTX-M [1]. The CTX-M beta-lactamases are the most widespread enzymes with a growing prevalence [1]. Environmental contamination with pathogenic and resistant bacteria as well as by antibiotics and other agents may contribute to the development and spread of resistance genes [2-4]. Indeed, the aquatic environments constitute a privileged system for horizontal resistance-genes exchanges mediated by plasmids, transposons and integrons [2,4-5]. The passage of antibiotic resistance genes from aquatic bacteria to human and animal pathogens has been demonstrated [1, 2, 4], this is an emerging concern which requires evaluation of this resistance in human-related

aquatic environments. Most studies on environmental antibiotic resistance have only focused on its evaluation [6,7], while few is known about mechanisms, genes and mobile genetic elements implicated specially in marine water [8,9]. With the importance of beta-lactams in therapy and ESBLs as prevalent resistance mechanism, the aim of this study was to investigate the ESBLs in Gram negative bacilli recovered from Algiers port seawater

II. Materials and methods

II.1. Bacterial isolates

Six samples of seawater were collected in February 2008 at the docks 22 and 25 of Algiers port which received urban effluents. biological (BOD) oxygen demand of water at the sampling sites were 100 mg O_2/l and 80 mg O_2/l respectively for dock 25 and dock 22; this high values demonstrate that the sample sites are subject to pollution. Samples were taken directly from the docks waters at 20 m from the edge and a depth of about 20 cm using sterile glass bottles of 250 ml and they were transported in cold bags at 4°C to the laboratory within 6 hours. The selection of cefotaxime resistant isolates was done on Hecktoen agar plate supplemented with 32 µg/ml of cefotaxime which correspond to resistance critical concentration according to the guidelines of the French Society of Microbiology [10]. Cefotaxime resistant isolates were screened for extended-spectrum beta-lactamases production by using the Double-Disc Synergy Test (DDST) [11]. The DDST positive isolates were identified by using API 20E and API 20NE systems (Biomerieux France). The clonally relationships between ESBL producing strains were analyzed by Enterobacterial repetitive consensus PCR (ERIC- PCR) using primer ERIC2 [12]. Cycling conditions were as follows: 3 min at 95°C, 40 cycles of 30s at 92°C, 1 min at 52°C and 8 min at 65°C and final extension of 16 min at 65°C. Fingerprints were visually compared and the patterns differing by at least one amplification band were classified as different.

II.2. Antimicrobial susceptibility

Antibiograms were done on Mueller-Hinton agar plates with the disc-diffusion method and MICs were determined by agar-dilution method according to the guidelines of the French Society of Microbiology [10]. Antibiotic disks were purchased from BioRad (Marnes-la-Coquette, France). Escherichia coli ATCC 25922 was used as a control strain.

II.3. Conjugation experiments and plasmid analysis

Mating experiments were performed as previously described [12] with different recipients: *E. coli* BM21 (nalidixic acid resistant), *E. coli* BM21 (sodium azide resistant) and *E. coli* J53 (rifampicin resistant). Selective agents were used at the following concentrations: cefotaxime (4 µg/ml) and nalidixic acid (50 µg/ml), or sodium azide (300 µg/ml), or rifampin (150 µg/ml). The transconjugants selected were subjected to antimicrobials susceptibility, DDST and PCR analysis. Plasmid DNA was extracted by alcalin lysis method as previously described [13], plasmid size was estimated by comparison with plasmid size standards from *E. coli* strain V517 (54 - 35 - 5.6 - 5,1 - 3.9 - 3 - 2.7 - 2.1 kb), pIP 173 (127 kb), pIP2073 (54 kb), pRK 2013 (48 kb) and pBR 322 (4.36 kb).

II.4. Characterization of ESBL-encoding genes and genetic environment

The identification of ESBL genes was carried out by PCR as previously described using universal primers of CTX-M, TEM, SHV, PER, VEB, GES and specific primers for CTX-M groups (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, CTX-M-25) and OXA-10 [12, 14,15] (Table 1). The PCR products were sequenced and the nucleotide sequences and deduced-protein sequences were analysed with the BLAST and FASTA programs of the National Center for Biotechnology Information (http://www.ncbi.nlm.nhi.gov).

Detection of class 1 integrons was performed by PCR using primers derived from conserved regions 5'CS and 3'CS under following conditions [16] (Table 1): 1 cycle of 94 °C for 5 min, 35 cycles of 94°C for 45 s, 55 °C for 1 min, 72°C for 2 min, and 1 cycle of 72°C for 10 min. Primers couples sulI1F/CTX-MB, CTX-MA/sulI1R, sulI1F/OXA-10R and OXA-10F/sulI1R were used to explore the possible linkage between class 1 integrons and $bla_{\text{CTX-M}}$ or bla_{OXA} The amplification was performed with 6 U of Taq polymerase under the following conditions: one cycle of 94°C for 5 min, 35 cycles of 94°C for 45 s, 60°C for 1 min, 72°C for 4 min and one final cycle of 72°C for 10 min. [17]. *E. coli* TN19 containing an association of $bla_{\text{CTX-M-2}}$ with class 1 integrons was used as control [18].

Detection of IS*Ecp*1B sequence was performed by PCR using primers derived from the IS*Ecp*1B transposase gene (Table 1) under following conditions: 1 cycle of 94°C for 5 min, 30 cycles 94°C for 30 s, 58°C for 30 s and 1 final cycle of 72°C for 10 min. The screen for a genetic linkage between IS*Ecp*1B and ESBL genes was conducted by PCR using a combination of PROM+/CTXMB primers (Table 1), under following conditions: 1 cycle 94°C for 5 min, 35 cycles 94°C for 45 s, 56°C for 30 s, 72°C for 2 min, and 1 final cycle of 72°C for 10 min [19]. *E. coli* TN03, carrying ISEcp1-like element upstream $bla_{\text{CTX-M-15}}$ gene was used as control [18].

Table 1. *Primers used in this study*

Primers	Sequences (5' →3')	Gene types	Size (pb)	annealing temperature	References
TEM low TEM up	CCAATGCTTAATCAGTGAGG ATGAGTATTCAACATTTCCG	*bla*$_{TEM}$	858	52°C	(46)
SHV low SHV up	GATTTGCTGATTTCGCTCGG TTATCTCCCTGTTAGCCACC	*bla*$_{SHV}$	795	50°C	(44)
CTX-MA CTX-MB	CGCTTTGCGATGTGCAG ACCGCGATATCGTTGGT	*bla*$_{CTX-M}$	550	55°C	(7)
CTX-M1low CTX-M1 up	TTGGTGACGATTTTAGCCGC GGTTAAAAAATCACTGCGTC	*bla*$_{CTX-M-1}$ cluster	550	55°C	(24)
CTX-M2 low CTX-M2 up	TGGGTTACGATTTTCGCCGC ATGATGACTCAGAGCATTCG	*bla*$_{CTX-M-2}$ cluster	870	55°C	(24)
CTX-M9 low CTX-M9 up	CCCTTCGGCGATGATTCTC ATGGTGACAAAGAGAGTGCA	*bla*$_{CTX-M-9}$ cluster	865	55°C	(22)
CTX-M-8 low CTX-M-8 up	CGCTCCACATTTTTTAGAATTA ATACTTCAGCCACACGGATTC	*bla*$_{CTX-M-8}$ cluster	891	55°C	(24)
CTX-M-25low CTX-M-25up	TGGGTTACGATTTTCGCCGC ATGATGACTCAGAGCATTCG	*bla*$_{CTX-M-25}$ cluster	870	55°C	(24)
PER-1 F PER-1 B	AATTTGGGCTTAGGGCAGAA ATGAATGTCATTATAAAAGC	*bla*$_{PER-1}$	925	55°C	(24)
OXA-10 F OXA-10 B	TCTTTCGAGTACGGCATTAGC CCAATGATGCCCTCACTTTCC	*bla*$_{OXA-10}$	756	55°C	(24)
VEB-1F VEB-1B	CGACTTCCATTTCCCGATGC GGACTCTGCAACAAATACGC	*bla*$_{VEB-1}$	643	55°C	(24)
GES-1A GES-1B	ATGCGCTTCATTCACGCAC CTATTTGTCCGTGCTCAGG	*bla*$_{GES-1}$	846	55°C	(24)
IS*Ecp1*A IS*Ecp1*B	GCAGGTCTTTTTCTGCTCC TTTCCGCAGCACCGTTTGC	IS*Ecp1B* transposase	527	58°C	(40)
PROM+	TGCTCTGTGGATAACTTGC	Right part of IS*Ecp1B* including -35 and -10 promoter sequences	-	56°C	(40)
3'CS 5'CS	AAGCAGACTTGACCTGAT GGCATCCAAGCAGCAAGC	Class 1 integrons	Variable	55°C	(12)
SulI1-F Sull1-R	TCAGACGTCGTGGATGTCG CGAAGAACCGCACAATCTCG	*sulI1*	346	55°C	(36)

III. Results

Forty isolates were selected on Hecktoen medium supplemented with cefotaxime, of which twelve were tested positive for synergy test, auguring production of ESBLs. These isolates were identified as *Citrobacter freundii* (n=4), *E. coli* (n=1), *Aeromonas hydrophila* (n=2), *Vibrio fluvialis* (n=4) and *Bordetella sp* (n=1). ERIC-PCR typing of isolates showed unrelated patterns. Antibiogram and MICs showed a resistance to cefotaxime (12/12, MICs: 32-512 mg/L), ceftriaxone (12/12, MICs: 32-1024 mg/L), ceftazidime (9/12, MICs: 4-2048 mg/L), aztreonam (9/12, MICs: 8->256 mg/L), cefepime (3/12, MICs: 2-128 mg/L) and cefpirome (9/12, MICs: 2-128 mg/L), and total susceptibility to imipenem (0/12, MICs: 0,5-4 mg/L). Associated resistances to aminoglycosides, sulfonamides, trimethoprim and quinolones were observed for most isolates (Table 2).

PCR amplification and nucleotide sequencing identified ESBLs as CTX-M-15 in *C. freundii* (C5, C24 et C27), CTX-M-3 in *C. freundii* (C31) and *E. coli* (E32), SHV_{2a} in *A. hydrophila* (A9), OXA-101 in *V. fluvialis* (V12 , V34), PER-1 in *V. fluvialis* (V8 et V35) and in *A. hydrophila* (A7) and VEB-1 in *Bordetella sp* (B33). bla_{TEM-1} gene was found in CTX-M producing *C. freundii* (C5, C27 et C31) and *E. coli* (E32), in OXA-101 producing *V. fluvialis* (V12) (Table 2).

The plasmid profiles analysis showed that the isolates contained 1 to 5 plasmids whose size varies from about 4 kb to 159 kb. Mating assays carried out on the 12 ESBL isolates allowed the transfer of bla_{CTX-M} genes from *Citrobacter* and *E. coli* isolates and of bla_{PER-1} gene from *V. fluvialis* (V8) and *A. hydrophila* (A7) isolates in association with self-transferable plasmids of approximately >70 kb for bla_{CTX-M} and 138 kb for bla_{PER-1}. bla_{TEM-1} gene has cotransferred with bla_{CTX-M} genes. Aminoglycosides, sulfonamides and trimethoprim resistances have cotransferred with ESBL phenotypes. The ESBLs SHV_{2a}, VEB-1, OXA-101 were not transferred in spite of the presence of large or medium plasmids in isolates (Table 2).

The class 1 integrons were detected in 7 of 12 isolates including *C. freundii*, *E. coli*, *V. fluvialis* (V34) and *A. hydrophila (*A9) isolates, and in some transconjugants. The insertion-sequence IS*Ecp1B* was found in 6 isolates of *C. freundii* CTX-M+, *E. coli* CTX-M+ and *V. fluvialis* OXA-101+ (V12) (Table 2). No association between class 1 integrons and ESBL genes has been found, while the isolates CTX-M positive yielded PCR products with PROM+/CTX-MB primers of approximately 1100 bp for CTX-M-3 producers and 1000 bp for CTX-M-15 producers, indicating a genetic linkage between bla_{CTX-M} genes and IS*Ecp1B.*

IV. Discussion

Results showed that among the 40 isolates growing in the presence of cefotaxime, 12 were producing ESBL. These isolates showed resistance to penicillins, oxyimino-cephalosporins and aztreonam, and susceptibility to imipenem, characteristic features of ESBLs [1]. However, peculiarities in resistance patterns were noted, particularly the resistance of *C. freundii, A. hydrophila* and *V. fluvialis* to cefoxitin due to chromosomal AmpC cephalosporinase. Very few works have concerned ESBLs in marine environment; however, our results are consistent with studies conducted in different aquatic environments which conclude that the drug resistance is emerging dramatically in environmental bacteria in correlation with human impact [2, 4, 8, 20, 21] and the prevalence of ESBLs-producing bacteria is notable, suggesting a global expansion of these enzymes as well in clinic than in environment [8, 20, 22- 24]. The association of ESBLs production with the resistance to aminoglycosides, sulfonamides and tetracycline, noticed in our isolates, was reported for the clinical isolates in Algeria [15, 25], and in the world [26]. This suggests that genes encoding these resistances are carried by the same genetic elements as previously reported [1,15,20,26]. This concomitant resistance to several classes of antibiotics increases the risks of therapeutic failure.

The ESBLs produced by our isolates are mainly represented by CTX-M-3 and CTX-M-15 which are the most prevailing in the Algerian hospitals [15, 27] and worldwide [1]. To our knowledge, there is few works which have described CTX-M in the marine water environment [8, 9]. CTX-M-15 differs from CTX-M-3 by a Gly240Asp substitution which increases its hydrolytic activity against ceftazidime [28], this observation is also valid for our isolates. It is the first time that SHV-2a was detected in Algeria, in *A. hydrophila* species and in seawater. However, it was already described SHV2a in *K. pneumoniae* from companion animals in Italy [29]. Ceftazidimase ESBLs PER-1 and VEB-1, and oxacillinase OXA-101 were detected in this study in environmental species. PER-1 and VEB-1 were previously described in environmental isolate of *Aeromonas sp* recovered from the Seine river [30]. In Algeria, PER-1 and VEB-1 were reported in Enterobacter clinical isolates [25, 27] whereas OXA-101 was detected for the first time in Algeria and in *V. fluvialis* species; indeed, this enzyme was detected in clinical strain of *Citrobacter freundii* [31]. ESBL genes detected in this study are the same which prevail among clinical strains; they would have been transferred to native strains from allochthonous strains of anthropogenic inputs. bla_{CTX-M} and bla_{PER-1} genes were carried by selftransferable plasmid of >70 kb and 138kb,

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Table 2. *Resistance phenotypes, ESBL genes, mobile elements of ESBL-positive strains isolated from marine water of port*

Isolate DDST+	Resistance phenotype	β-lactamase genes	Transferred genes	Plasmid Content (kb)	Transferred plasmids	Cotransferred resistances	IS*Ecp1*	Integrons
C. freundii (C5)	AMX-TIC-PIP-CTX-CRO-CAZ-ATM-FOX-CPO-K-TM-GM-AN-SSS-TMP-NA-CIP-TE-C	CTX-M-15 TEM	CTX-M-15 TEM	85 -10.9	85	K-TM- GM- SSS-TE	+(*)	+
C. freundii (C24)	AMX-TIC-PIP-CTX-CRO-CAZ-ATM-FOX-CPO-FOX-K-TM-GM-AN-SSS-TMP-NA-CIP-TE-C	CTX-M-15	CTX-M-15	85-67.3-54-48.6-10.9	85-67.3-54-48.6-10.9	K-GM-SSS-TMP- NA -TE-C	+(*)	+(t)
C. freundii (C27)	AMX-TIC-PIP-CTX-CRO-CAZ-ATM-FOX-CPO-K-TM-GM-AN-SSS-TMP-NA-CIP-TE-C	CTX-M-15 TEM	CTX-M-15 TEM	127-85-67.7-5.1	85-5.1	K-SSS	+(*)	+
C. freundii (C31)	AMX-TIC-PIP-CTX-CRO-ATM-FOX-CPO-K-TM -GM-AN-SSS-TMP-NA-CIP-TE-RA	CTX-M-3 TEM	CTX-M-3 TEM	85-13.4	85	K –TM-GM-SSS-TMP	+(*)	+(t)
E. coli (E32)	AMX-TIC-PIP-CRO-GM-AN-SSS-TMP-TE-RA	CTX-M-3 TEM	CTX-M-3 TEM	141-70-54	70	GM-SSS-TMP	+(*)	+(t)
A. hydrophila (A9)	AMX-TIC-PIP-CTX-CRO-CAZ-FOX-K-TM-SSS-TMP-NA-CIP-C-TE	$SHV_{2\ a}$	NT	159-6.1-5.6-4.14	NT	-	-	+
A. hydrophila (A7)	AMX-TIC-PIP-CTX-CRO-CAZ-ATM-FEP-CPO-FOX-K-TM-GM-SSS-TMP-NA-CIP-C	PER-1	PER-1	138-12	138-12	K-TM-GM-SSS-TMP-C	-	-
V. fluvialis (V8)	AMX-TIC- PIP-CTX-CRO-CAZ-ATM-CPO-K-TM-GM-SSS-RA	PER-1	PER-1	138-12	138-12	K-TM-GM-SSS-RA	-	-
V. fluvialis (V35)	AMX-TIC-PIP-CTX-CRO-CAZ-ATM-FEP-CPO-FOX-K-TM-GM-SSS-TMP-NA-TE-RA	PER-1	NT	138-20	NT	-	-	-
V. fluvialis (V12)	AMX-TIC-PIP-FOX-K-TM-GM-SSS-NA-CIP-C-TE-RA	OXA-101 TEM	NT	34	NT	-	+	-
V. fluvialis (V34)	AMX-TIC-PIP-CTX-CRO-CAZ-ATM-CPO-K-SSS -CIP-C-TE-RA	OXA-101	NT	24.5-6.6-4.83	NT	-	-	+
Bordetella sp (B33)	AMX-TIC-PIP-CTX-CRO-CAZ-ATM-FEP-CPO-K –TM-GM-AN -TMP-NA-CIP-C-TE	VEB-1	NT	128	NT	-	-	-

ESBL: extended spectrum beta-lactamases; +: presence, - absence; (*): association between IS*Ecp1* and ESBL gene; (t): transferable integrons; NT: no transfer; ND: not determined; AMX: amoxicillin, TIC: ticarcillin, PIP: piperacillin, CTX: cefotaxime, CRO: ceftriaxone, CAZ: ceftazidime, ATM: aztreonam, FOX: cefoxitin, FEP: cefepime; CPO: cefpirome, K: kanamycin, TM: tobramycin, GM: gentamicin, AN: amikacin, SSS: sulfonamides, TMP: trimethoprim, NA: nalidixic acid, CIP: ciprofloxacin, C: chloramphenicol, TE: tetracycline, RA: rifampin.

respectively. The encoding of CTX-M-15 and CTX-M-3 by plasmids of >70 kb was reported in Algerian clinical and environmental isolates [15, 20, 27]. While, the CTX-M recovered at marine E. coli was encoded by plasmid of 54 Kb [8]. *bla*$_{\text{TEM-1}}$ and resistance determinants to aminoglycosides and sulfonamides have cotransferred with ESBLs genes and carried by the same plasmids, this closely association has been observed by many authors [8, 15, 20]. The plasmids transfer between phylogenetically distant bacteria reflects their ubiquitous nature, therefore their ability to spread. The presence of plasmid bands of similar size in different strains may be indicative of transfer in situ. Antibiotics resistance mediated by plasmids was described in marine environment and it has been shown that plasmid transfer between bacteria occurs in a variety of natural habitats [2].

Integrons and insertion sequences provide an additional means of mobility to resistance genes. In our isolates, IS*Ecp*1B insertion sequence was found upstream of *bla*$_{\text{CTX-M}}$, this genetic organization was described [12] and IS*Ecp*1B would be implicated in the mobility and expression of *bla*$_{\text{CTX-M}}$ [19]. The intercalary regions between IS*Ecp*1B and *bla*$_{\text{CTX-M-3}}$ or *bla*$_{\text{CTX-M-15}}$ were found different, this result is in agreement with several studies [18, 20]. In contrast, in a study conducted on clinical isolates in Algeria, this intercalary region was found to have the same organization for both blaCTX-M-15 and blaCTX-M-3 [15]. These results suggest that the evolution mechanism which led to the emergence of these ESBL genes in the environment is different from that which prevailed in the clinical context in Algeria [20].

Class 1 integrons were detected in the majority of our isolates and are carried, in certain cases, by the plasmid encoding CTX-M; however, there is not a direct linkage between them. This result is in agreement with what was reported on the genetic environment of CTX-M of cluster 1, conversely to other CTX-M clusters [18]. The same finding was observed for OXA-101 which, however, was described as encoded by cassette genes [31]. The role of class 1 integrons in conferring antibiotic resistance to bacteria is well documented in clinical and aquatic environments [16,21,32], this is consistent with our results on seawater.

V. Conclusion

This work strengthens data on antibiotic resistance in the marine environment and it is the first work devoted different types to ESBLs in this aquatic environment. The emergence of ESBLs, class 1 integrons and IS*Ecp*1B in seawater in Algeria are demonstrated. The dumping of pollutants into aquatic environments results in widespread of antibiotic resistance. There is potential risk that resistance genes may be transferred into a wide range of aquatic environmental bacteria, when significant human infection with these organisms is also increasing. We are witnessing in the environment to a similar situation if not more critical than that observed in clinical, this requires implementation of serious resistance monitoring in environment.

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