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CTX-M-15-producing *Escherichia coli* and the pandemic clone O25b-ST131 isolated from wild fish in Mediterranean Sea

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Extended-spectrum β-lactamases (ESBL) are now the main resistance mechanism observed in all *Enterobacteriaceae* species. Among these ESBL, CTX-M enzymes are the most widespread in *Escherichia coli* both in nosocomial and in the community setting. Moreover, the diffusion of the pandemic clone designated CTX-M-15-producing *E. coli* O25b:H4-ST131 has exacerbated this situation. This clone has been described in environment, food, and animals [1]. Even though this clone has been recently observed in Swiss lakes [2], no report has described the presence of the pandemic clone ST131 in wild fish in the sea.

From March 2012 to February 2013, gut and gills samples from different fish caught in the Mediterranean Sea far from Béjaia (Algeria) were prospectively and randomly collected. All the fish were sampled by opening the gut using a sterile scalpel after washing the gut surface with sterile saline and then swabbing the contents. To screen for oxyimino-cephalosporin-resistant (OCR) *E. coli*, samples were placed in 1 mL sterile 0.9% saline and then vortexed. From this suspension, 100 μL was plated on two MacConkey agar dishes supplemented with cefotaxime (2 μg/mL) or ceftazidime (2 μg/mL) incubated for 24 hours at 37 °C under aerobic conditions. A single colony of *E. coli* was selected for further study. Bacterial genus and species were identified by conventional methods (API 20E; BioMérieux, Marcy l'Étoile, France). Susceptibility to antimicrobial agents was tested by disk diffusion assay on Mueller-Hinton agar, and ESBL production was screened by the double-disk synergy test according to recommendations of the Antibiotic Committee of the French Society for Microbiology (http://www.sfm-microbiologie.org). Plasmid DNA was extracted from the isolates using the EZ1 DNA Tissue kit on the BioRobot EZ1 extraction platform (QIAGEN, Courtaboeuf, France). The genes coding for ESBL (bla_{TEM}, bla_{SHV}, and bla_{CTX-M}) were detected by PCR using specific primers and identified by sequencing the PCR products [3]. We used a triplex PCR specific for the CTX-M-15 producing *E. coli* O25b:H4-ST131 clone as described previously [4]. The *qnrA, qnrB, qnrS, aac(6′)-Ib-cr* (identified by *BtsCI* digestion), and *qepA* genes were screened by PCR in all of the quinolone-resistant isolates and identified by sequencing [5,6]. The *aac(3′)-IV*, *aac(3′)-II*, *aac(3)-III*, *ant(2″)-I*, and *aac(3′)-I* genes were screened by PCR. Phylogenetic grouping and the O group of the ESBL-producing *E. coli* isolates were determined by PCR-based methods developed by Clermont *et al.* [7]. For all multi-drug-resistant *E. coli*, the genetic relationship was studied using the DiversiLab system (bioMérieux), a semi-automated fingerprinting tool based on repetitive sequence-based PCR (Rep-PCR), by following the manufacturer's instructions. Isolates with identical strain patterns were considered indistinguishable if their similarity percentage was ≥95%. Moreover, multi-locus sequence typing (MLST) was carried out according to the Institut Pasteur's MLST scheme (http://www.pasteur.fr/mlst) and the Achtman scheme (http://mlst.ucc.ie/mlst/dbs/Ecoli).

Of the 300 samples analyzed, 22 (7.3%) OCR *E. coli* isolates were detected. They were isolated from guts (*n* = 14) and gills (*n* = 8). All the isolates were resistant to cefotaxime, 90.9% to ceftazidime, and 72.7% to cefepime. Co-resistances to non-β-lactams were also observed: nalidixic acid (86.4%), ofloxacin (77.3%), ciprofloxacin (68.2%), co-trimoxazole (54.5%), kanamycin (36.4%), gentamycin (27.3%), netilmicin (22.7%), and amikacin (4.5%). All the isolates were susceptible to carbapenems (Table 1).

The 22 isolates produced an ESBL. The prevalence of CTX-M-producing *E. coli* within OCR *E. coli* isolates was 72.7% (*n* = 16). CTX-M-15 was the mainly CTX-M-encoding gene (93.8%) in our study. One strain harboured *bla*CTX-M-9 group gene. The six remaining strains (27.3%) carried TEM-24 enzymes (Table 1).

To study the plasmidic co-resistance, we screened the presence of *qnr, aac6′-Ib-cr*, and *qepA* genes. The prevalence of *qnr* genes was 36.4% of ESBL-producing *E. coli* strains. *qnrB* and *qnrS* were identified in 2 (9.1%) and 6 (27.3%) strains, respectively. The *qnrB* gene was detected in only the two CTX-M-15-producing isolates, whereas the *qnrS* determinant was found in

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TABLE 1. Characteristics of ESBL-producing *Escherichia coli* isolated from wild fish in Mediterranean Sea near Béjaia, Algeria

Strains	Fish species	Resistance phenotype	Phylogenetic group	Sequence type Achtman	β-lactamase content	PMQR content and associated resistance gene
ES1	*Boops boops*	CTX CAZ FEP NAL OFX CIP	A	ST471	CTX-M-15	–
ES2	*Sardina pilchardus*	CTX CAZ FEP	A	ST132	CTX-M-15	–
ES3	*Sarpa salpa*	CTX CAZ FEP	A	ST398	CTX-M-15	–
ES4	*Sarpa salpa*	CTX	B1	ST398	CTX-M-15	–
ES5	*Sarpa salpa*	CTX CAZ FEP KNM NAL	B1	ST 398	CTX-M-15	*aac(3′)-IV*
ES6	*Sarpa salpa*	CTX CAZ FEP NAL OFX CIP SXT	A	ST 37	CTX-M-15	*qnrS*
ES7	*Trachurus trachurus*	CTX CAZ NAL OFX CIP SXT	D	ST 31	TEM-24	*qnrS*
ES8	*Trachurus trachurus*	CTX CAZ NAL OFX CIP SXT	A	ST 471	TEM-24	–
ES9	*Pagellus acarne*	CTX FEP NAL SXT	B2	ST 8	CTX-M groupe 9	–
ES10	*Pagellus acarne*	CTX CAZ FEP NET AKN GMI KNM NAL OFX CIP SXT	D	ST 477	CTX-M-15	*aac6′-Ib-cr; qnrS; aac(3′)-IV; aac(3′)-II; aac(3′)-I; aac(3)-III*
ES11	*Pagellus acarne*	CTX CAZ FEP NET GMI KNM NAL OFX CIP SXT	B2	ST 131	CTX-M-15	*aac6′-Ib-cr; qnrB; aac(3′)-IV; aac(3′)-II; aac(3)-III*
ES12	*Pagellus acarne*	CTX CAZ NAL OFX CIP	A	ST 471	CTX-M-15	–
ES13	*Pagellus acarne*	CTX CAZ FEP NET GMI KNM NAL OFX CIP SXT	B2	ST131	CTX-M-15	*aac6′-Ib-cr; qnrB; aac(3′)-IV; aac(3′)-II; aac(3)-III; ant(2″)-I*
ES14	*Pagellus acarne*	CTX CAZ FEP NAL OFX CIP	A	ST 471	CTX-M-15	–
ES15	*Pagellus acarne*	CTX CAZ FEP NET GMI KNM NAL OFX CIP SXT	D	ST477	CTX-M-15	*aac6′-Ib-cr; qnrS; aac(3′)-II; aac(3)-III*
ES16	*Pagellus acarne*	CTX CAZ FEP NAL OFX CIP	A	ST471	TEM-24	*aac6′-Ib-cr; qnrS*
ES17	*Pagellus acarne*	CTX CAZ NAL OFX CIP SXT	B1	ST 21	TEM-24	–
ES18	*Pagellus acarne*	CTX CAZ FEP GMI KNM NAL OFX CIP SXT	A	ST66	TEM-24	–
ES19	*Sardina pilchardus*	CTX CAZ FEP KNM NAL OFX CIP	D	ST31	CTX-M-15	*qnrS; aac(3′)-IV; aac(3)-III; ant(2″)-I*
ES20	*Sardina pilchardus*	CTX CAZ NET GMI NAL OFX	A	ST 74	TEM-24	–
ES21	*Sardina pilchardus*	CTX CAZ FEP KNM NAL OFX CIP SXT	A	ST 471	CTX-M-15	*aac(3′)-IV*
ES22	*Sardina pilchardus*	CTX CAZ FEP NAL OFX SXT	A	ST 471	CTX-M-15	–

ESBL, extended-spectrum β-lactamase; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; NET, netilmicin; GMI, gentamicin; KNM, kanamycin; OFX, ofloxacin; CIP, ciprofloxacin; NAL, nalidixic acid; SXT, co-trimoxazole; PMQR, plasmid-mediated quinolone resistance.

four CTX-M-15-producers and two TEM-24-producers. The prevalence of *aac6′-Ib-cr* was 22.7% of the strains. No isolates carried *qnrA* or *qepA* genes. Resistance to aminoglycoside was associated with the presence of *aac(3′)-IV* (n = 6, 27.3%), *aac(2′)-III* (n = 5, 22.7%), *aac(3′)-II* (n = 4, 18.2%), *ant-(2″)-I* (n = 2, 9.1%), and *aac(3′)-I* (n = 1, 4.5%) genes. Finally, 14 (63.6%) of the studied isolates harboured multiple resistant genes (Table 1).

The 22 ESBL-producing isolates scattered among all four major phylogenetic groups described in *E. coli*: 59.1% (n = 13) belonged to A, 18.2% (n = 4) to D, 13.6% (n = 3) to B2, and 9.1% (n = 2) to B1. Three CTX-M-15-producing strains displayed serogroup O25. Among them, two (ES11, ES13) were B2 phylogroup and belonged to the intercontinental *E. coli* clone O25b:H4-ST131. They harboured multiple antibiotic resistance genes. The remaining O25 positive strain was of phylogroup D.

Rep-PCR revealed a great genomic diversity of the ESBL-producing *E. coli* isolates as they were classified into 14 profiles. However, Rep-PCR also displayed five clusters (>95% similarity) of isolates, including two or more isolates (defined as major clonal groups), C_V (n = 5; phylogenetic group A), C_{II} and C_{III} (each n = 2; D), C_I (n = 2; B2), and C_{VI} (n = 2; A). The residual profiles contained single isolates (9 Rep-PCR patterns). The five major clonal groups belonged, respectively, to sequence types ST471 (n = 7), ST31 (n = 2), ST131 (n = 2), ST398 (n = 3), and ST477 (n = 2), and the others were mainly of ST132, ST37, ST8, ST21, ST74, and ST66 (Table 1). No seasonal link between strains could be demonstrated.

This report documents for the first time that wild fish in the Mediterranean Sea are reservoirs of plasmid-mediated oxy-imino-cephalosporin-, aminoglycoside-, and quinolone-resistant *E. coli*, including pandemic clone B2-O25b:H4-ST131. We could suggest that these isolates were most likely derived from contamination of the fish from human sewage via river water and a growing amount of waste from land urban, industrial, and agricultural operation discharged untreated into the sea near the coast in the regions of the Mediterranean Sea. Our findings favour the hypothesis that natural environments are potent reservoirs of multi-drug-resistant bacteria and associated genes. Moreover, efforts should be made to more closely control the antibiotic use and untreated waste, as this represents the major reservoir of resistance genes and a human threat for the future.

Transparency declaration

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