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Spread of $bla_{CTX\text{-}M\text{-}15}$-Producing *Enterobacteriaceae* and OXA-23-Producing *Acinetobacter baumannii* Sequence Type 2 in Tunisian Seafood

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ABSTRACT Bivalves are filter-feeding animals and markers of bacterial pollution. We report a massive spread of $bla_{CTX\text{-}M\text{-}15}$ through dominant *Escherichia coli* and *Klebsiella pneumoniae* lineages and/or plasmid subtypes (F31:A4:B1) as well as the presence of OXA-23-producing *Acinetobacter baumannii* sequence type 2 (ST2) in seafood, highlighting a direct risk for the consumer. These findings should urge authorities to consider hospital effluents, and also farm and urban effluents, as important sources of extended-spectrum-beta-lactamase (ESBL)/carbapenemase producers that filter-feeding animals can concentrate and further spread to humans.

KEYWORDS seafood, ESBL, carbapenemase, *E. coli*, *Acinetobacter*, OXA-23, CTX-M-15, IncF, plasmid

Bivalves are filter-feeding animals that host high concentrations of microbial communities that may promote horizontal gene transfer (1), and their low trophic level as primary consumers enables them to further disseminate microorganisms along the food chain. Therefore, they are relevant markers of biological pollution, and human waste was accused as the source of detected multidrug resistant (MDR) *Escherichia coli* in bivalves in Norway and KPC-3-producing *E. coli* sequence type 167 (ST167) in mussels in Tunisia (2, 3). The goal of this study was to clarify the burden caused by bacteria resistant to broad-spectrum cephalosporins from wastewater effluents (including hospital, urban, and farm effluents) on shellfish intended for human consumption.

Three categories of bivalves were sampled in unrelated markets over three different time periods in Tunisia. First, 400 specimens of depurated mussels (*Mytilus galloprovincialis*) collected in the Bizerte lagoon were purchased in five different batches (containing 40, 50, 95, 105, and 110 pieces, respectively) between January and March 2015. Second, 36 oysters (*Crassostrea gigas*) from Bizerte were purchased on a single day in March 2016. Third, three batches (30, 68, and 83 pieces) of scallops (*Placopecten magellanicus*) from the Monastir lagoon (several hundred kilometers south from Bizerte) were purchased between April and May 2016. All bivalves were aseptically transported at 4°C to the laboratory and were immediately processed. After removal of shell debris and algae under tap water, bivalves were dried, disinfected (70% ethanol), opened using a sterilized scalpel, and disposed in tubes containing peptone salt broth for 24 h at 37°C. Each tube contained either

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one (for oysters and scallops) or a pool of five (for mussels) bivalve sample(s). Overnight cultures were streaked on selective MacConkey agar plates containing 4 mg/liter cefotaxime, and one colony per morphology per plate was picked up. Extended-spectrum cephalosporin-resistant (ESC-R) bacteria were identified in all batches of mussels (52 positive tubes out of the 80 tested), 3/36 oysters (8.3%), and 26/181 scallops (14.4%). Identification (Vitek-2; bioMérieux, Marcy l'Etoile, France) and typing (XbaI-pulsed-field gel electrophoresis [PFGE]) were performed on all isolates. For one representative of each PFGE profile per sampling date, resistance genes, clones, and plasmids were characterized more deeply by PCR and sequencing, phylogrouping (for *E. coli*), S1-PFGE, Southern blot, multilocus sequence typing (MLST) (according to the following websites: http://bigsdb.pasteur.fr/klebsiella/klebsiella.html for *K. pneumoniae*; https://pubmlst.org/bigsdb?db=pubmlst_mlst_seqdef for *E. coli*; and https://pubmlst.org/abaumannii/ for *A. baumannii*) and plasmid typing (using plasmid MLST [pMLST] and the IncF formula according to the following website: https://pubmlst.org/plasmid/) (Table 1).

Two *A. baumannii* isolates were incidentally isolated (since our study design was first intended to identify extended spectrum beta-lactamase [ESBL]/pAmpC producers) in one mussel (2015) and one oyster (2016) from the same city, Bizerte. Both isolates belonged to ST2 and produced the OXA-23 carbapenemase. OXA-23-producing *A. baumannii* strains have been widely reported in North African hospitals (Algeria, Tunisia, and Libya), and OXA-23-producing *A. baumannii* ST2 was reported in Tunis, which is close to Bizerte (4). Several hospitals in Bizerte have their effluents discharged into the Bizerte Lagoon, where mussel and oyster farms are located, which makes seafood contamination by hospitals activities very likely. The same polluted situation is observed in the Monastir Lagoon.

A dominant CTX-M-15 epidemiology was also observed in different species of *Enterobacteriaceae* (Table 1). $bla_{\text{CTX-M-15}}$ was identified in one *Citrobacter freundii* isolate and in one ST394 and two ST101 *K. pneumoniae* isolates; the last lineage widely spreads $bla_{\text{CTX-M-15}}$ in North Africa (5). CTX-M-15 was also produced by 80% ($n = 53/66$) of the ESBL-producing *E. coli* heavily contaminating mussels (46/80 ESBL-positive tubes), scallops (18/181 ESBL-positive animals), and oysters (1/36). Interestingly, ST617, ST410, and ST131 *E. coli* strains, all three widely recognized in humans, were dominant. As an example, ST617 was found in 22/53 ESBL-positive samples in mussels and in 9/18 in scallops. Only two other STs (ST88 and ST155) were sporadically identified. These data again suggest a human origin of the seafood contamination and a massive ESBL load not even diluted by the aquatic environment. Moreover, ST617 and ST410 were recovered at different periods and places in Tunisia, suggesting a wide prevalence in the environment (6–11).

Surprisingly, hybridizations on S1-PFGE gels revealed a unique $bla_{\text{CTX-M-15}}$-bearing IncF plasmid of the F31:A4:B1 formula in all ST617 and ST410 isolates and in one ST131 isolate. This very same $bla_{\text{CTX-M-15}}$ IncF plasmid has regularly been identified in ST617 and ST410 in numerous animal species, foodstuffs, and countries worldwide (12–16), and in two ST44 and ST131 clinical isolates in the United States (17), thereby suggesting that this plasmid is a major genetic support for the dissemination of $bla_{\text{CTX-M-15}}$.

Thus, we first report OXA-23-producing *A. baumannii* ST2 in seafood, highlighting a direct risk for the consumer. We also show a massive spread of $bla_{\text{CTX-M-15}}$ in seafood, particularly through dominant *E. coli* and *K. pneumoniae* lineages and/or plasmid subtypes (F31:A4:B1). These data strongly suggest a contamination of human origin, even though other sources cannot be excluded. These findings should urge authorities to set up monitoring programs for the surveillance of MDR bacteria (including those resistant to carbapenems) and to consider hospital effluents, but also farm and urban effluents, as sources of ESBL/carbapenemase producers that filter-feeding animals can concentrate and further spread to humans through the food chain.

TABLE 1 Characteristics of *Enterobacteriaceae* (one representative per lineage and sampling date) recovered from bivalve mollusks

Animal host	Sampling date	Batch no. (no. of pieces)	No. of positive tubes/total no. of tubes[a]	Isolate no.	No. of clonal isolates	Bacterial species	Phylogroup	Sequence type[b]	Phenotype	ESBL/ carbapenemase gene	ESBL-carrying plasmid	Plasmid subtype
Mussel	15 January 2015	1 (105)	21/21	40610	21	*E. coli*	A	ST617	ESBL	$bla_{CTX-M-15}$	IncF	F31:A4:B1
	27 January 2015	2 (50)	2/10	40631	2	*E. coli*	A	ST617	ESBL	$bla_{CTX-M-15}$	IncF	F31:A4:B1
	04 February 2015	3 (40)	2/8	40633	2	*E. coli*	B2	ST131	ESBL	$bla_{CTX-M-27}$	IncF	F31:A4:B1
	07 March 2015	4 (110)	22/22	40637	20	*E. coli*	A	ST410	ESBL	$bla_{CTX-M-15}$	IncF	F31:A4:B1
				40639	1	*E. coli*	A	ST88	ESBL	$bla_{CTX-M-1}$	IncF	F18:A−:B1
				40656	1	*K. pneumoniae*		ST394	ESBL	$bla_{CTX-M-15}$	IncF	Fk7:A−:B−
	25 March 2015	5 (95)	5/19	40660	2	*K. pneumoniae*		ST101	ESBL	$bla_{CTX-M-15}$	IncF	Fk1:A−:B−
				40662	1	*E. coli*	A	ST10	ESBL	$bla_{CTX-M-15}$	IncY	
				40663	2	*A. baumannii*		ST2	Carba	bla_{OXA-23}		
Oyster	12 March 2016	1 (36)	3/36	43728	1	*A. baumannii*		ST2	Carba	bla_{OXA-23}		
				43729	1	*E. coli*	A	ST410	ESBL	$bla_{CTX-M-15}$	IncF	F31:A4:B1
Scallop	14 April 2016	1 (30)	8/30	43731	8	*E. coli*	B2	ST131	ESBL	$bla_{CTX-M-14}$	IncF	F29:A−:B10
	17 May 2016	2 (68)	8/68	43734	8	*E. coli*	A	ST617	ESBL	$bla_{CTX-M-15}$	IncF	F31:A4:B1
	25 May 2016	3 (83)	10/83	43735	8	*C. freundii*		ND	ESBL	$bla_{CTX-M-15}$	IncHI1	
				43736	1	*E. coli*	A	ST617	ESBL	$bla_{CTX-M-15}$	IncF	F31:A4:B1
				43737	1	*E. coli*	B1	ST155	ESBL	$bla_{CTX-M-1}$	IncF	F18:A−:B1

[a]For mussels, each tube was a pool of five individuals. For oysters and scallops, each piece was analyzed individually.
[b]ST, sequence type; ND, not determined.

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