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First description of two OXA-23-mediated carbapenem resistance in Sequence Type 2 *Acinetobacter baumannii* isolates in *Pagellus acarne* fished in the Mediterranean Sea afar Bejaia (Algeria)

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Running Head: OXA-23-producing *A. baumannii* in wild fish

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Abstract

To determine the occurrence of carbapenem-resistant *Acinetobacter baumannii* in fish fished in Mediterranean Sea near the Bejaia coast (Algeria), we studied 300 gills and gut samples randomly and prospectively collected during 1 year.
After screening on selective agar media, using PCR arrays and whole genome sequencing, we reported for the first time two OXA-23-producing *A. baumannii* strains belonging to the widespread ST2/International Clone II and harbouring aminoglycoside-modifying enzymes (*aac(6')*-Ib and *aac(3')*-I genes).

Keywords: *Acinetobacter baumannii*, Carbapenemase, *bla*$_{OXA-23}$, aminoglycoside-modifying enzymes, fish, Mediterranean Sea.

Acinetobacter baumannii is an opportunistic aerobic non-fermentative Gram-negative rod found ubiquitously in the environment (1). This bacterium emerged as an important cause of nosocomial infections, most notably ventilator-associated pneumonia and bacteremia associated with high mortality, urinary tract infections, and endocarditis (2). Moreover it is highly capable of developing resistance to antimicrobial agents (1).
Over the last 10 years, an increase in carbapenem-resistant *A. baumannii* strains has been observed worldwide; in particular, we could note a high prevalence in the different countries in South of Europe (3). The most common mechanism of carbapenem resistance in *Acinetobacter* species is the production of acquired carbapenem-hydrolysing OXA-type class D ®-lactamases (4). They are represented worldwide by six gene clusters: intrinsic chromosomal OXA-51-like, of which there are over 70 variants and the acquired OXA-23-like, OXA-24/40-like, OXA-58-like, OXA-143-like and OXA-235- like β -lactamases (5-7).
Worldwide dissemination of the OXA-23-producing *A. baumannii* is now well established notably among Algerian hospitals in recent years (8-10). Whilst *A. baumannii* is isolated from patients and hospital environmental sources during outbreaks, the reservoir outside of the hospital is not well delineated.
Several investigators have suspected that the survival of *A. baumannii* in the environment (in particular in water) could contribute to the transmission of the organism during outbreaks (3). Moreover recent reports have also described the presence of carbapenem-resistant *Acinetobacter* spp. from animals.
The OXA-23 carbapenemase has been found in *Acinetobacter* spp. from cattle, horses and cat (11-13). The NDM-1 has been also reported in *Acinetobacter* spp. from food animal origin (chicken and pig farms, respectively) in China (14,15). However, knowledge about carbapenemase-producing *Acinetobacter* spp. of animal origin remains very limited, making it difficult to assess its impact on public health.
Between 1 March 2012 and 28 February 2013, we randomly and prospectively screened a total of 300 samples from different fish fished in the Mediterranean Sea (2 km from Bejaia coast, Algeria). Sampling was carried out from *Sardina pilchardus* (n= 62), *Engraulis encrasicolus* (n= 38), *Trachurus trachurus* (n= 45), *Sarpa salpa* (n= 60) *Pagellus acarne* (n= 55) and *Boops boops* (n= 40).
The gills and gut of each fish sample were collected by opening the gut using a sterile scalpel following washing the gut surface with sterile saline. Samples were placed in 1 mL sterile 0.9% saline and then vortexed. Cultures were inoculated by streaking 100 μ L of the suspensions onto Mac Conkey agar plates supplemented with 2 μ g/mL of ceftazidime and incubated 24h at 37° C under aerobic conditions. One colony per sample was retained for further investigation.
Bacterial identification was carried out using MALDI-TOF MS (Vitek MS, BioMérieux).
Susceptibility testing was performed by disk-diffusion procedure (BioRad) and E-tests (BioMérieux) according to the recommendations of the Antibiotic Committee of the French Society for Microbiology (http://www.sfm-

microbiologie.org).
Isolates were screened for carbapenemase production using the modified Hodge test (MHT) (16) and by the imipenem- EDTA method (17). Multiplex PCR detection and sequencing of genes that encode carbapenem68 hydrolysing class D ®-lactamases was used (18). The presence of genes encoding the aminoglycoside-modifying enzymes (AMEs) was also performed using PCR (19).
Finally the genetic relationship was investigated by rep-PCR (using DiversiLab system) and MLST (20).
Plasmid electroporation assays were performed in *A. baumannii* ATCC19606. To identify the clonal lineage of the OXA-23-producing *A. baumannii* isolates, we used PCR previously described (21). Genomic DNA of *A. baumannii* IM2 was sequenced using a NextSeq 500 Illumina® platform by Helixio, St-Beauzire (France).
Of the 300 samples analysed, two *A. baumannii* isolates IM1 and IM2 (0.7%) were recovered from two fish (*Pagellus acarne*). The two isolates were resistant to almost all the antibiotics tested including carbapenems. They remained susceptible only to amikacin and netilmicin (Table 1).
The phenotypic assays showed that the two isolates were positive according to the MHT but the activity of β -lactamases was not inhibited by EDTA. PCR detection showed that the two *A. baumannii* strains harbored the naturally occurring *bla*$_{\text{OXA-51}}$-like gene and the acquired OXA-carbapenemase *bla*$_{\text{OXA-23}}$-like gene. Sequencing confirmed that the isolates harbored ®-lactamase OXA-23.
Moreover we detected the presence of *aac(6')-Ib* and *aac(3')-I* genes in the two isolates. These two isolates and different human clinical strains isolated in French (Languedoc-Roussillon, southern France) and Algerian (Annaba, eastern Algerian) shared the same genotype (Figure 1).
The assays to transfer plasmid by electroporation were unsuccessful. MLST assigned the isolates to sequence type ST2.
Multiplex PCR to identify clonal lineages was positive for group 1 showing that the strains belonged to the widespread ST2/International Clone-II. The whole genome sequencing of IM2 isolate highlighted that the transposon Tn*2006* was the vehicle of the *bla*$_{\text{OXA-23}}$ resistant gene. The 5292 bp of this transposon were found into the genomic DNA of the IM2 isolate (in position 2759078 after a hypothetical protein) confirming the chromosomal insertion of Tn*2006* (Genbank accession number: KU168371).

This study highlighted for the first time that wild fish in Mediterranean Sea can be contaminated with carbapenem- and aminoglycoside-resistant *A. baumannii* belonging to the worldwide clone ST2. The emergence and spread of several outbreak or sporadic *A. baumannii* strains producing OXA-23-like enzymes have been reported around the world and were assigned to international clonal lineages I 98 or II (4).
During a long period, the *bla*$_{\text{OXA-58}}$ carbapenemase gene has been predominated among carbapenem-resistant *A. baumannii* isolates in various Mediterranean countries. Since 2009, a replacement of *bla*$_{\text{OXA-58}}$ gene with *bla*$_{\text{OXA-23}}$ gene has been reported and it became the most prevalent carbapenemase-encoding gene circulating in the Mediterranean region (5). The replacement of OXA-58 by OXA-23 might be explained by the selective advantage associated with the higher carbapenemase activity of OXA-23 (22).
Recently, different reports showed the dissemination of multidrug resistant pathogenic bacteria in food products and in food-producing animals (5,23,24). Comparison of human and animal carbapenem-resistant *Acinetobacter* isolates is important to enhance the knowledge of the potential routes of transfer of these bacteria and resistance genes in different ecosystems. Few studies were published describing the dissemination of carbapenem-resistant *Acinetobacter* isolates in food animals and wild animals (11-15).
All these points and the clonality between a panel of clinical strains showed the possible exchange place between the *A. baumannii* populations in infected humans and water. 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 Mediterranean Sea.
In conclusion, our study highlighted that OXA-23-producing *A. baumannii* may be isolated from wild animals in rare cases. These findings emphasize the ability of these isolates to spread in the environment. More studies should be performed in the future to track the evolution of carbapenem-resistant *Acinetobacter* isolates and their frequencies in different ecosystems.

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Potential conflicts of interest
All authors: No reported conflicts.

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Figure 1. Dendrogram of Rep-PCR and MLST of the two OXA-23-producing *Acinetobacter baumannii* strains isolated from wild fish in Mediterranean Sea, 3 representative human clinical strains isolated from a French hospital and 3 representative human clinical strains isolated from an Algerian hospital.
For the purpose of predicting different clones, the top match feature at 95% similarity was used.

209 Table 1. Characteristics of carbapenem-resistant *A. baumanii* isolated from wild fish in the Mediterranean Sea (2 km from Bejaia
210 coast, Algeria).

Strains	Fish specimen	Isolation date (m/d/y)	Organ	Resistance Phenotype Antibiotics (MIC, µg/mL)	Sequence type	Transposon	β-lactamase content	Associated resistance gene
IM1	*Pagellus acarne*	11/04/2012	Gills	CTX (32), CAZ (32), FEP (32), IMP (>32), MEM (32), DOR (>32), AMK (2), GEN (16), KAN(>32), NET (1), OFX (>32), CIP (>32), SXT (8)	ST2	Tn*2006*	OXA-23, OXA-51	*aac(6')*-Io, *aac(3')*-I
IM2	*Pagellus acarne*	04/14/2012	Gut	CTX (32), CAZ (32), FEP (32), IMP (>32), MEM (32), DOR (>32), AMK (2), GEN (16), KAN(>32), NET (1), OFX (>32), CIP (>32), SXT (8)	ST2	Tn*2006*	OXA-23, OXA-51	*aac(6')*-Io, *aac(3')*-I

211 **m/d/y**, month, day, year; **CTX**, cefotaxime; **CAZ**, ceftazidime; **FEP**, cefepime; **IMP**, imipenem; **MEM**, meropenem; **DOR**, doripenem; **AMK**, amikacine;
212 **GEN**, gentamicine; **KAN**, kanamycine; **NET**, netilmycine; **OFX**, ofloxacine; **CIP**, ciprofloxacine; **SXT**, cotrimoxazole