

Epidemiological Description and Detection of Antimicrobial Resistance in Various Aquatic Sites in Marseille, France

Sabah El-Sawalhi, Océane Revol, Amanda Chamieh, Alexandre Lacoste, Alexandre Annessi, Bernard La Scola, Jean-Marc Rolain, Isabelle Pagnier

▶ **To cite this version:**

Sabah El-Sawalhi, Océane Revol, Amanda Chamieh, Alexandre Lacoste, Alexandre Annessi, et al.. Epidemiological Description and Detection of Antimicrobial Resistance in Various Aquatic Sites in Marseille, France. Microbiology Spectrum, 2023, 11 (2), pp.e0142622. 10.1128/spectrum.01426-22 . hal-04536967

HAL Id: hal-04536967

https://amu.hal.science/hal-04536967v1

Submitted on 8 Apr 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution 4.0 International License

AMERICAN SOCIETY FOR MICROBIOLOGY | Microbiology Spectrum

RESEARCH ARTICLE

Epidemiological Description and Detection of Antimicrobial Resistance in Various Aquatic Sites in Marseille, France

Sabah El-Sawalhi,[a,b] Océane Revol,[a,b] Amanda Chamieh,[a,b] Alexandre Lacoste,[c] Alexandre Annessi,[c] Bernard La Scola,[a,b] Jean-Marc Rolain,[a,b] Isabelle Pagnier[a,b]

[a]Aix-Marseille Université, IRD, APHM, MEPHI, Faculté de Médecine et de Pharmacie, Marseille CEDEX 05, France
[b]IHU Méditerranée Infection, Marseille CEDEX 05, France
[c]Bataillon des Marins Pompiers de Marseille, CIS BMPM, Marseille, France

ABSTRACT Antibiotic resistance is a worldwide public health concern and has been associated with reports of elevated mortality. According to the One Health concept, antibiotic resistance genes are transferrable to organisms, and organisms are shared among humans, animals, and the environment. Consequently, aquatic environments are a possible reservoir of bacteria harboring antibiotic resistance genes. In our study, we screened water and wastewater samples for antibiotic resistance genes by culturing samples on different types of agar media. Then, we performed real-time PCR to detect the presence of genes conferring resistance to beta lactams and colistin, followed by standard PCR and gene sequencing for verification. We mainly isolated *Enterobacteriaceae* from all samples. In water samples, 36 Gram-negative bacterial strains were isolated and identified. We found three extended-spectrum β-lactamase (ESBL)-producing bacteria—*Escherichia coli* and *Enterobacter cloacae* strains—harboring the CTX-M and TEM groups. In wastewater samples, we isolated 114 Gram-negative bacterial strains, mainly *E. coli*, *Klebsiella pneumoniae*, *Citrobacter freundii* and *Proteus mirabilis* strains. Forty-two bacterial strains were ESBL-producing bacteria, and they harbored at least one gene belonging to the CTX-M, SHV, and TEM groups. We also detected carbapenem-resistant genes, including NDM, KPC, and OXA-48, in four isolates of *E. coli*. This short epidemiological study allowed us to identify new antibiotic resistance genes present in bacterial strains isolated from water in Marseille. This type of surveillance shows the importance of tracking bacterial resistance in aquatic environments.

IMPORTANCE Antibiotic-resistant bacteria are involved in serious infections in humans. The dissemination of these bacteria in water, which is in close contact with human activities, is a serious problem, especially under the concept of One Health. This study was done to survey and localize the circulation of bacterial strains, along with their antibiotic resistance genes, in the aquatic environment in Marseille, France. The importance of this study is to monitor the frequency of these circulating bacteria by creating and surveying water treatments.

KEYWORDS aquatic environment, carbapenemase, ESBL, antibiotic resistance

Antimicrobial resistance is a worldwide and multifaceted public health concern, as demonstrated by the spread and emergence of multidrug-resistant (MDR) organisms (1). The increase in MDR infections may be attributed to the increasing prevalence of β-lactamases, including extended-spectrum β-lactamase (ESBL) enzymes, metallo-β-lactamases, and carbapenemases. This subsequently confers resistance to third-generation cephalosporins and then carbapenems (2).

It has been demonstrated that antibiotic resistance may be a process that is transferred between humans, animals, and the environment, especially water (3), giving rise to the concept of One Health. The One Health concept takes on a cooperative and multidisciplinary

Editor Sébastien P. Faucher, McGill University
Copyright © 2023 El-Sawalhi et al. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license.
Address correspondence to Isabelle Pagnier, isabelle.pagnier@univ-amu.fr.
The authors declare no conflict of interest.
Received 26 April 2022
Accepted 22 December 2022
Published 28 March 2023

approach that aims to outline the interrelatedness and optimizes the health outcomes among humans, animals, and their environment (4).

Aquatic environments are a reservoir of antibiotic-resistant bacteria (5) that have increased especially in the presence of antimicrobials (6). Freshwater, mostly rivers and lakes, are well-known environments for the spread and evolution of antibiotic-resistant bacteria (7). Seawater environments also play a major role in the spread and circulation of antibiotics and antibiotic-resistant bacteria (8).

In addition, wastewater also leads to a high and dangerous exposure of bacterial resistance in the environment (9). It has been shown that wastewater provides suitable conditions for the bacterial community to grow, thus leading to the spread of antibiotic resistance (10). The circulation of *Escherichia coli* isolates producing ESBLs has been reported in several aquatic environments, mostly wastewater (11). In a study conducted on wastewaters in Ivry-sur-Seine in France, several *E. coli* strains were found to harbor genes, including from the CTX-M and TEM groups (12). In addition, the presence of the CTX-M-1, CTX-M-14, CTX-M-15, and SHV-12 genes was detected in some ESBL-producing bacteria isolated from the wastewater of a hospital located in the city of Besançon, also in France (13). Another study, conducted on hospital wastewater in Clermont-Ferrand, revealed the presence of carbapenemase genes in several genera, notably *Stenotrophomonas*, *Aeromonas*, *Acinetobacter*, and *Pseudomonas* spp. (14). The detected antibiotic resistance genes (ARGs) were NDM, VIM, GES, and OXA-48. In addition, colistin resistance genes, such as the *mcr-1*, *mcr-3*, *mcr-4*, and *mcr-5* genes, have been detected in bacterial strains isolated from water (5).

Due to the abundance of bacteria in aquatic environments, it is important to determine whether they are multidrug resistant. Through this demonstration of an integrated One Health approach, we aim to screen several water and wastewater sample sites in Marseille for the presence of ARGs. Consequently, this environmental survey will allow us to identify new ARGs that may be transmissible among humans, animals, and the environment.

RESULTS

Between October and November 2020, we collected 14 samples from seven different aquatic sites (Fig. 1) in Marseille, France. These sites included seawater (W1-Vieux Port, W2-Plage des Catalans, W3-Plage de L'Huveaune, and W6-L'Estaque), freshwater (W4-Ruisseau des Aygalades and W7-L'Huveaune), and drinking water from one of the major sources in Marseille (W5-Canal de Marseille).

We isolated a total of 36 Gram-negative bacteria (Table 1). From the saltwater sites, we identified *Serratia marcescens* ($n = 3$), *Klebsiella oxytoca* ($n = 2$), *Pseudomonas putida* ($n = 2$), *Citrobacter braakii* ($n = 1$), *Acinetobacter ursingii* ($n = 2$), and *Brevundimonas diminuta* ($n = 1$). We did not detect any ARGs in these isolates. From freshwater sites, we identified *Enterobacter cloacae* ($n = 5$), *Escherichia coli* ($n = 2$), *Pseudomonas putida* ($n = 3$), *Citrobacter freundii* ($n = 1$), and *Serratia liquefaciens* ($n = 1$). Two isolates obtained from W7 were ESBL producers as determined by antimicrobial susceptibility testing. Real-time and standard PCR testing showed that one *E. coli* isolate harbored a TEM-98 gene, while another *E. cloacae* isolate harbored a TEM-214 gene (Table 1). Interestingly, the source of the drinking water from the Canal de Marseille was rich in diversity and the quantity of isolates. We identified *Enterobacter cloacae* ($n = 3$), *Stenotrophomonas maltophilia* ($n = 3$), *Pseudomonas putida* ($n = 2$), *Citrobacter gillenii* ($n = 1$), *Escherichia coli* ($n = 1$), and *Pseudomonas thivervalensis* ($n = 1$). We detected the presence of CTX-M-15 and CTX-M-17 in an *E. coli* isolate (Table 1).

From the wastewater samples, we isolated 114 Gram-negative bacterial strains on different types of media. Table S3 in the supplemental material shows the details of the Gram-negative bacterial strains isolated from the MP1, MP2, MP3, and MP4 membranes of the wastewater samples. A total of 43 strains of the isolated bacteria harbored ARGs (Table 2). Whole-genome sequencing was only performed for the isolated bacterial strains harboring carbapenem-resistant genes. These isolates were *E. coli* strains: CMP2c, CMP4c, EMP4c, EMP4d, and EMP4e.

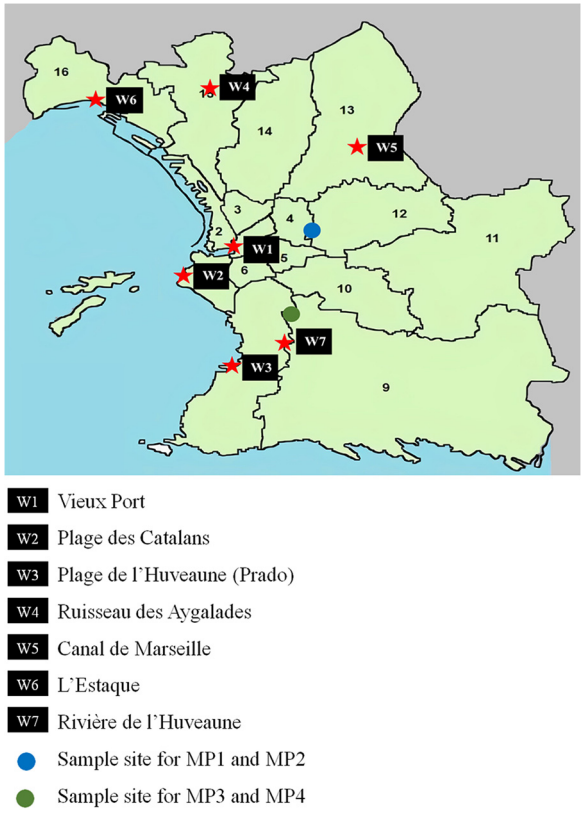

FIG 1 Water sampling sites in Marseille. The red stars represent the sampling site in each area. The blue circle represents the sampling site of the combined sewers. The green circle represents the sampling site of the separated sewers.

For the MP1 wastewater samples, three bacterial strains were positive for ESBLs. One *E. coli* strain was positive for both CTX-M-1 and TEM. Another *E. coli* strain harbored TEM and a strain of *K. pneumoniae* had an SHV.

For the MP2 wastewater samples, nine ESBL-producing bacterial strains were isolated. Three strains of *E. coli* had a CTX-M-1 gene, and two strains of *E. coli* had a CTX-M-9 gene. In addition, six strains of *E. coli*, one strain of *C. youngae*, and one strain of *P. mirabilis* had the TEM gene. Moreover, one of the isolated *E. coli* strains was resistant to Ertapenem, and it was positive following the β-CARBA test (Bio-Rad, Hercules, CA). The KPC-2 gene was detected in this bacterial strain, which explains its production of carbapenemase.

For the MP3 wastewater samples, 10 bacterial isolates were positive for ESBLs. Two *E. coli* strains harbored the CTX-M-1 gene, three *K. pneumoniae* strains had a SHV gene, and six *E. coli* strains and one *P. mirabilis* strain had a TEM gene.

For the MP4 wastewater results, 20 bacterial strains were positive for ESBLs, and three bacterial strains were positive for carbapenemases. Four strains of *E. coli* and one strain of *K. pneumoniae* were positive for the CTX-M-1 gene. Five strains of *E. coli* and one strain of *K. pneumoniae* were shown to have a CTX-M-9 gene. For the SHV gene, three strains of *E. coli* and three strain of *K. pneumoniae* had this gene. In addition, nine strains of *E. coli*, two strains of *P. mirabilis*, one strain *C. freundii*, and one strain of *K. pneumoniae* were shown to have a TEM gene. As for the carbapenemases, three isolates of *E. coli* showed resistance to ertapenem. All of these strains were positive following the β-CARBA test, with one of them (EMP4c) harboring the KPC-2 gene, another strain (EMP4d) harboring the OXA-48 gene, and the third strain (EMP4e) harboring the NDM-5 gene. One strain of *E. coli* (CMP4c) was shown to harbor the OXA-10, CTX-M-9, and *mcr-9* genes. The *mcr-9* analysis is ongoing since this is the first time that this gene has been found in an *E. coli* strain isolated from wastewater in France (data not shown).

TABLE 1 Identified Gram-negative bacteria isolated from water samples (n = 36)

Sampling site (code)	Gram-negative isolates (no.)	Isolate code	ARG(s)[a]
Vieux-Port (W1)	*Citrobacter braakii* (1)	W1a	
	Klebsiella oxytoca (1)	W1b	
	Serratia marcescens (1)	W1c	
	Pseudomonas putida (1)	W1d	
Plage des Catalans (W2)	*Klebsiella oxytoca* (1)	W2a	
	Serratia marcescens (1)	W2b	
	Pseudomonas putida (1)	W2c	
Plage de l'Huveaune (W3)	*Serratia marcescens* (1)	W3a	
Ruisseau des Aygalades (W4)	*Enterobacter cloacae* (2)	W4a	
		W4b	
	Escherichia coli (1)	W4c	
	Pseudomonas putida (2)	W4d	
		W4e	
Canal de Marseille (W5)	*Citrobacter gillenii* (1)	W5a	
	Enterobacter cloacae (3)	W5b	
		W5c	
		W5d	
	Escherichia coli (1)	W5e	CTX-M-15, CTX-M-17
	Serratia liquefaciens (1)	W5f	
	Pseudomonas putida (2)	W5g	
		W5h	
	Pseudomonas thivervalensis (1)	W5i	
	Stenotrophomonas maltophilia (3)	W5j	
		W5k	
		W5l	
L'Estaque (W6)	*Acinetobacter ursingii* (2)	W6a	
		W6b	
	Brevundimonas diminuta (1)	W6c	
L'Huveaune (W7)	*Citrobacter freundii* (1)	W7a	
	Enterobacter cloacae (3)	W7b	
		W7c	
		W7d	TEM-214
	Escherichia coli (1)	W7e	TEM-98
	Serratia liquefaciens (2)	W7f	
		W7g	
	Pseudomonas putida (1)	W7h	

[a]ARG(s), antibiotic resistance gene(s).

DISCUSSION

This descriptive study is among the first to describe the various Gram-negative bacterial isolates identified in different water sites around Marseille, France and to report the presence of ARGs, most importantly carbapenemases such as NDM, KPC, and OXA-48.

Interestingly, our detection of common ESBL genes such as CTX-M and TEM β-lactamases in *E. coli* and *E. cloacae* strains came from the drinking water canal and a river (Table 1). Similar results were found in rivers located in the province of Sichuan in China, where CTX-Ms were found mostly in *Enterobacteriaceae* species (15). Another study showed a high detection of TEM genes present in *Enterobacteriaceae* strains isolated from rivers in Podhale in southern Poland (16). The canal in Marseille (W5) is a watercourse that passes through 36 communes in the city, and the Huveaune river (W7) passes through several communes. This may explain the presence of ESBL-producing bacteria in these aquatic sites, since they have a long course and so are bound to be contaminated.

On the other hand, all the wastewater sample isolates contained the CTX-M and TEM genes. Similar studies have reported the presence of CTX-M-1 and CTX-M-9 genes in *E. coli* from wastewater in Japan and the Netherlands (17, 18). CTX-M and TEM are

TABLE 2 ARGs present in the isolates of the wastewater samples ($n = 43$)[a]

Membrane	Isolate	Isolate code[b]	Antibiotic resistance gene(s)
MP1	*E. coli*	MMP1a	TEM-206
	E. coli	CMP1a	CTX-M-15, TEM-214
	K. pneumoniae	MMP1c	SHV-187
MP2	*C. youngae*	MMP2a	TEM-214
	E. coli	MMP2f	TEM-214
	E. coli	CMP2a	CTX-M-27
	E. coli	CMP2b	CTX-M-27, TEM-214
	E. coli	CMP2c*	CTX-M-3, TEM-98, kPC-2
	E. coli	CMP2d	CTX-M-15, TEM-214
	E. coli	CMP2e	CTX-M-15, TEM-214
	E. coli	CMP2f	TEM-206
	P. mirabilis	LMP2b	TEM-217
MP3	*E. coli*	MMP3c	TEM-206
	E. coli	MMP3d	TEM-214
	E. coli	MMP3f	TEM-206
	K. pneumoniae	MMP3i	SHV-89
	P. mirabilis	MMP3j	TEM-206
	E. coli	MMP3l	TEM-206
	E. coli	CMP3b	CTX-M-15, TEM-206
	E. coli	CMP3c	CTX-M-15, TEM-206
	K. pneumoniae	CMP3d	SHV-12
	K. pneumoniae	CMP3e	SHV-160
MP4	*E. coli*	MMP4i	TEM-206
	E. coli	MMP4j	TEM-206
	E. coli	MMP4k	TEM-206
	E. coli	MMP4l	SHV-101, TEM-214
	K. pneumoniae	MMP4m	SHV-101
	C. freundii	CMP4b	TEM-2
	E. coli	CMP4c*	CTXM-9, OXA-10, *mcr-9*
	E. coli	CMP4d	CTXM-15, CTXM-125, TEM-214
	E. coli	CMP4e	CTXM-15, CTXM-126, TEM-206
	E. coli	CMP4f	SHV-12
	E. coli	CMP4g	SHV-12
	E. coli	CMP4h	CTXM-15
	E. coli	CMP4i	CTXM-15, CTXM-17, TEM-214
	K. pneumoniae	CMP4k	CTX-M-15, CTX-M-125, SHV-52, TEM-206
	E. coli	EMP4b	TEM-2
	E. coli	EMP4c*	KPC-2
	E. coli	EMP4d*	TEM-214, OXA-48
	E. coli	EMP4e*	CTXM-126, NDM-5
	P. mirabilis	CoMP4b	TEM-2
	K. pneumoniae	LMP4a	SHV-36
	P. mirabilis	LMP4b	TEM-2

[a]ARG(s), antibiotic resistance gene(s).
[b]*, Bacterial strains subjected to whole-genome sequencing.

the most frequently found ESBLs in humans (19, 20). Moreover, TEM genes have been detected in poultry meat samples (21) and in wastewater released into rivers (22). SHV genes were detected in the *Enterobacteriaceae* strains isolated from wastewater in Germany (23) and in the wastewater from several hospitals in South Africa (24). SHV-101 was found in one strain of *E. coli* and one strain of *K. pneumoniae*. To date, no description of this gene has been given.

We detected the KPC-2, NDM-5, and OXA-48 genes in different *E. coli* strains in this study. KPC-2 was described and detected in KPC-producing *K. pneumoniae* clinical strains isolated in Chilean hospitals (25), and the NDM-5 gene was detected from wastewater in Japan (26). The OXA-48 gene was described to be highly present in *E. coli* strains in Mediterranean countries (27). This gene was detected clinically in France (28) and also in animals, such as cats and dogs (29). The presence of these carbapenemases in Marseille wastewater is alarming due to their circulation in various sites across the city.

From the MP4 wastewater samples, we detected an *mcr-9* gene in a strain of *E. coli* (CMP4c; data not shown). This gene was first detected in *Salmonella enterica* serotype Typhimurium, isolated from a patient in Washington State, and it was shown to have the ability to lower susceptibility to colistin (30). It was detected in bacterial strains isolated exclusively from humans (5). This gene was not previously described in wastewater and has not yet been clinically detected in Marseille.

Marseille is a big city with at least three large university hospitals and several smaller ones. In addition, the port of this city is a major shipping, transport, and immigration portal in France. In terms of the MP4 wastewater, a high number of bacterial isolates were identified, since the separated sewers are mainly located near all the hospitals of the city. This may explain the abundance of ARG and is simultaneously of concern because it implies a high level of circulating ARGs, specifically ESBL, in this community. Since it is known that ESBL is quasi-endemic, the detection of carbapenemase and *mcr* genes in the sewers is a warning sign to monitor their carriage in healthy, nonhospitalized individuals in the community. Overall, a high prevalence of ARGs was found in separated sewers and wastewater in the city of Marseille, France.

Conclusions and future perspectives. We are aware there are some limitations to our work, such as the need to increase our sample size and sampling frequency for a more complete monitoring. However, that may be done at a later stage with dedicated policies and resources. In addition, for example, the Huveaune river and the Marseille canal are both sites which extend over a large area and cut through various communes. This may increase the risk of possible contamination of these waters, leading to our results.

Nonetheless, our brief surveillance period allowed us to identify ARGs that were not previously described in water, as well as to establish a current epidemiologic inventory of ARGs in the water in Marseille. ESBL-producing and carbapenem-resistant Gram-negative bacteria are usually implicated in severe infections in humans.

We highlight a significant circulation of antibiotic-resistant bacteria in ecosystems which are in close contact with human activities, implying possible emerging community outbreak. We are now facing a public health challenge that is best dealt with by continuous, local surveillance to anticipate the spread of MDR bacteria, especially under the One Health umbrella. A lot remains to be explored, starting with adapting routine surveillance of water sites as a tool to track antimicrobial resistance, and creating and revising existing water treatment policies.

MATERIALS AND METHODS

Sample collection. We collected 14 water samples from different water sampling sites (W1 to W7) in Marseille, France (Fig. 1), between October and November 2020. These water sites included seawater (W1-Vieux Port, W2-Plage des Catalans, W3-Plage de L'Huveaune, and W6 L'Estaque), freshwater (W4-Ruisseau des Aygalades and W7-L'Huveaune), and drinking water from one of the major sources in Marseille (W5-Canal de Marseille). Canal de Marseille water is treated with ferric chloride to make a decantation, and it does not contain any disinfectant. One liter of water was collected randomly from each of these sites. Samples were collected in sterile flasks and were transferred immediately to the laboratory to be placed at −80°C for later use.

In addition, four different wastewater samples collected from Marseille in March and April 2021 (Fig. 1) were added to the study. Two types of sampling were performed: one was a grab sample, in which the water is collected once, and the other is a composite sample, in which the water is collected over a period of time, 24 h in this case. Two grab samples (MP1 and MP3) were collected by the Bataillon des Marins Pompiers de Marseille (BMPM) from two different districts of Marseille: the National and the Belle-de-Mai. The composite sampling of wastewater was done by automatic ASP-Station 2000 RPS20B vacuum samplers (Endress Hauser, Huningue, France). This type of sampler allows the filling of a refrigerated flask of 20 L per 24 h of wastewater collected from 8 a.m. to 8 a.m. This process was automated from the Marseille municipal sewage system (Service d'Assainissement Marseille Métropole [SERAMM]). Samples were drawn from a combined network (composed of central Marseille wastewater and rainwater) for MP1 and MP2 and from a separated network (sewage only, from the entire city) for MP3 and MP4.

The geographical distances between the samples are presented in Table S1 in the supplemental material. All samples were transferred to the IHU Méditerranée Infection by BMPM, at 4°C, and were processed 1 h after sampling.

Sample filtration. We filtered 1 L of each of the water samples using a filtration ramp device and 0.2-μm-pore-size membranes (Nuclepore Track-Etch polycarbonate membrane; Whatman, Buckinghamshire, UK).

TABLE 3 Primers used in real-time PCR and standard PCR

PCR type	Primer	Sequence (5′–3′)[a]	Reference
Real-time PCR	CTX-M-a	F: CGGGCRATGGCGCARAC	36
		R: TGCRCCGGTSGTATTGCC	
		P: CCARCGGGCGCAGYTGGTGAC	
	CTX-M-B	F: ACCGAGCCSACGCTCAA	36
		R: CCGCTGCCGGTTTTATC	
		P: CCCGCGYGATACCACCACGC	
	TEM	F: TTCTGCTATGTGGTGCGGTA	36
		R: GTCCTCCGATCGTTGTCAGA	
		P: AACTCGGTCGCCGCATACACTATTCTCAGA	
	SHV	F: TCCCATGATGAGCACCTTTAAA	36
		R: TCCTGCTGGCGATAGTGGAT	
		P: TGCCGGTGACGAACAGCTGGAG	
	VIM	F: CACAGYGGCMCTTCTCGCGGAGA	37
		R: GCGTACGTYGCCACYCCAGCC	
		P: 6-FAM-AGTCTCCACGCACTTTCATGACGACCGCGTCGGCG-TAMRA	
	NDM	F: GCGCAACACAGCCTGACTTT	38
		R: CAGCCACCAAAAGCGATGTC	
		P: 6-FAM-CAACCGCGCCCAACTTTGGC-TAMRA	
	KPC	F: GATACCACGTTCCGTCTGGA	39
		R: GGTCGTGTTTCCCTTTAGCC	
		P: 6-FAM-CGCGCGCCGTGACGGAAAGC-TAMRA	
	OXA-23	F: TGCTCTAAGCCGCGCAAATA	40
		R: TGACCTTTTCTCGCCCTTCC	
		P: FAM-GCCCTGATCGGATTGGAGAACCA-TAMRA	
	OXA-24	F: CAAATGAGATTTTCAAATGGGATGG	40
		R: TCCGTCTTGCAAGCTCTTGAT	
		P: FAM-GGTGAGGCAATGGCATTGTCAGCA-TAMRA	
	OXA-48	F: TCTTAAACGGGCGAACCAAG	37
		R: GCGTCTGTCCATCCCACTTA	
		P: 6-FAM-AGCTTGATCGCCCTCGATTTGG-TAMRA	
	OXA-58	F: CGCAGAGGGGAGAATCGTCT	40
		R: TTGCCCATCTGCCTTTTCAA	
		P: FAM-GGGGAATGGCTGTAGACCCGC-TAMRA	
	mcr-1	F: GCAGCATACTTCTGTGTGGTAC	41
		R: ACAAAGCCGAGATTGTCCGCG	
		P: FAM-GACCGCGACCGCCAATCTTACC-TAMRA	
	mcr-2	F: CTGTGCCGTGTATGTTCAGC	42
		R: TTATCCATCACGCCTTTTGAG	
		P: VIC-TGACCGCTTGGGTGTGGGTA-TAMRA	
	mcr-3	F: TGAATCACTGGGAGCATTAGGGC	42
		R: TGCTGCAAACACGCCATATCAAC	
		P: FAM-TGCACCGGATGATCAGACCCGT-TAMRA	
	mcr-4	F: GCCAACCAATGCTCATACCCAAAA	42
		R: CCGCCCCATTCGTGAAAACATAC	
		P: FAM-GCCACGGCGGTGTCTCTACCC-TAMRA	
	mcr-5	F: TATCCCGCAAGCTACCGACGC	42
		R: ACGGGCAAGCACATGATCGGT	
		P: FAM-TGCGACACCACCGATCTGGCCA-TAMRA	
	mcr-8	F: TCCGGGATGCGTGACGTTGC	43
		R: TGCTGCGCGAATGAAGACGA	
		P: FAM-TCATGGAGAATCGCTGGGGGAAAGC-TAMRA	
Standard PCR	CTX-M-1	F: CCCATGGTTAAAAAATCACTGC	36
		R: CAGCGCTTTTGCCGTCTAAG	
	CTX-M-9	F: GCGCATGGTGACAAAGAGAGTGCAA	36
		R: GTTACAGCCCTTCGGCGATGATTC	
	TEM	F: ATGAGTATTCAACATTTCCGTG	42
		R: TTACCAATGCTTAATCAGTGAG	
	SHV	F: ATTTGTCGCTTCTTTACTCGC	42
		R: TTTATGGCGTTACCTTTGACC	
	NDM	F: CATTTGCGGGGTTTTTAATG	38
		R: CTGGGTCGAGGTCAGGATAG	

(Continued on next page)

TABLE 3 (Continued)

PCR type	Primer	Sequence (5′–3′)[a]	Reference
	KPC	F: ATGTCACTGTATCGCCGTCT	38
		R: TTTTCAGAGCCTTACTGCCC	
	OXA-48	F: TTGGTGGCATCGATTATCGG	44
		R: GAGCACTTCTTTTGTGATGGC	

[a]R, reverse; F, forward; P, probe.

Filtration membranes were used for bacterial isolation. Each membrane was incubated in tryptic soy broth (TSB) tubes at 37°C for 72 h for bacterial enrichment.

For the wastewater samples, prefiltration was conducted for the grab sample from the National district (MP1). This was performed using filtration papers with 10- to 20-μm porosity (FPLIS33; Labelians, Nemours, France). This was followed by filtration using 5-μm (TMTP04700; Merck Millipore) and 0.1-μm (VCWP04700; Merck Millipore) filtration membranes. The 0.1-μm filtration membranes were stored at 4°C in sterile water. Membranes were incubated in TSB tubes at 37°C for 72 h.

The three other wastewater samples were centrifuged at 700 $\times$ *g* for 10 min. The supernatants were then filtered using 5-μm nitrocellulose membranes (TMTP04700; Merck Millipore). The 5-μm filtration membranes were stored at 4°C in sterile water. The membranes were incubated in TSB tubes at 37°C for 72 h.

DNA extraction. From the 14 water samples and the 4 wastewater samples, we prepared genomic DNA by incubating a volume of samples from the TSB tubes at 56°C overnight using a EZ1 DNeasy blood tissue kit (Qiagen GmbH, Hilden, Germany). DNA extraction was performed following the manufacturer's protocol. We then performed real-time PCR to detect the presence of specific ARGs. The primers used were those encoding genes for ESBLs (TEM, SHV, CTX-a, and CTX-b), genes for carbapenemases (OXA-23, OXA-58, OXA-24, OXA-48, NDM, KPC, and VIM), and colistin-resistant genes (*mcr*-1 to *mcr*-5 and *mcr*-8) (Table 3).

Bacterial culture. We inoculated 20 μL of each of the TSB tubes on different types of agar: Columbia agar + 5% sheep blood, MacConkey agar, MacConkey agar + ertapenem (0.5 μg/mL), MacConkey agar + cefotaxime (1 μg/mL), and LBJMR (Lucie Bardet Jean-Marc Rolain) agar plates containing 4 μg/mL colistin and 100 μg/mL vancomycin (31). The used concentrations were based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. We then incubated the plates for 24 h at 37°C. The colonies were identified using matrix-assisted laser desorption ionization–time of flight mass spectrometry (Bruker Daltonik, Bremen, Germany), as described previously (32).

Antimicrobial susceptibility test. We used the disc diffusion method (in line with the EUCAST recommendations) to determine the antibiotic susceptibility of growing bacteria. The keyhole effect between a third- or fourth-generation cephalosporin and clavulanic acid implied the detection of ESBLs. We performed the Etest method (bioMérieux, Marcy-l'Etoile, France) to determine the MICs of Imipenem and Ertapenem. We used the unitary MIC microdilution method (Biocentric, Bandol, France) to determine the MIC of colistin, and the β-CARBA test to determine the presence of carbapenemase.

Molecular and genomic analysis. We performed qPCR and standard PCR to confirm our results on the bacterial strains to investigate ESBL genes (CTX-M, SHV, and TEM), carbapenemase genes (NDM, VIM, KPC, OXA-23, OXA-24, OXA-48, and OXA-58), and colistin-resistant genes (*mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, *mcr-5*, and *mcr-8*) (Table 3). We then performed gene sequencing of the positive samples using a BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). The NCBI reference sequence database was used to detect antibiotic-resistant genes present in the isolates. Table S2 shows the reference sequence numbers of the detected ARGs.

The genomes of five carbapenem-resistant *E. coli* strains were sequenced using MiSeq technology (Illumina, Inc., San Diego, CA) applying a paired-end strategy. The Galaxy Europe platform (https://usegalaxy.eu/) was then used for analysis. The SPAdes algorithm was used to assemble the genomes (33), and Prokka was used for their annotation (34). ARGs were identified using the Resfinder database with Abricate (Galaxy version 1.0.1) (35). The sequenced genomes have been deposited under BioProject number PRJNA856496 in the NCBI database. All sequenced bacterial strains are available from the CSUR collection at the IHU laboratory with specific CSUR numbers (Q6629, Q6631, Q6632, Q6633, and Q6634) for each sample, and they are stored at −80°C.

Data availability. The sequenced genomes have been deposited under BioProject number PRJNA856496 in the NCBI Database.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.05 MB.

ACKNOWLEDGMENTS

We thank Linda Hadjadj for help in the laboratory. We thank TradOnline for English corrections.

We declare that we have no competing interests.

J.-M.R. and I.P. designed the study. S.E.-S., O.R., and A.C. drafted the manuscript. J.-M.R., I.P., and B.L.S. revised the manuscript. S.E.-S., A.L., and A.A. collected the samples.

S.E.-S. performed microbiology analyses. All authors have read and approved the final manuscript.

This study was supported by the French Government under the Investissements d'Avenir (Investments for the Future) program managed by the Agence Nationale de la Recherche (ANR [National Agency for Research]; reference Méditerranée Infection 10-IAHU-03). This study was supported by the Région Provence Alpes Côte d'Azur and European ERDF PRIMI funding.

REFERENCES

1. Davies J, Davies D. 2010. Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev 74:417–433. https://doi.org/10.1128/MMBR.00016-10.
2. Blair JMA, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJV. 2015. Molecular mechanisms of antibiotic resistance. Nat Rev Microbiol 13:42–51. https://doi.org/10.1038/nrmicro3380.
3. Hernando-Amado S, Coque TM, Baquero F, Martínez JL. 2019. Defining and combating antibiotic resistance from One Health and Global Health perspectives. Nat Microbiol 4:1432–1442. https://doi.org/10.1038/s41564-019-0503-9.
4. Mackenzie JS, Jeggo M. 2019. The One Health approach: why is it so important? Tropical Med 4:88. https://doi.org/10.3390/tropicalmed4020088.
5. Khedher MB, Baron SA, Riziki T, Ruimy R, Raoult D, Diene SM, Rolain J-M. 2020. Massive analysis of 64,628 bacterial genomes to decipher water reservoir and origin of mobile colistin resistance genes: is there another role for these enzymes? Sci Rep 10:5970. https://doi.org/10.1038/s41598-020-63167-5.
6. Figueira V, Serra E, Manaia CM. 2011. Differential patterns of antimicrobial resistance in population subsets of *Escherichia coli* isolated from waste- and surface waters. Sci Total Environ 409:1017–1023. https://doi.org/10.1016/j.scitotenv.2010.12.011.
7. Nnadozie CF, Odume ON. 2019. Freshwater environments as reservoirs of antibiotic resistant bacteria and their role in the dissemination of antibiotic resistance genes. Environ Pollut 254:113067. https://doi.org/10.1016/j.envpol.2019.113067.
8. Smaldone G, Marrone R, Cappiello S, Martin GA, Oliva G, Cortesi ML, Anastasio A. 2014. Occurrence of antibiotic resistance in bacteria isolated from seawater organisms caught in Campania Region: preliminary study. BMC Vet Res 10:161. https://doi.org/10.1186/1746-6148-10-161.
9. Kumar A, Pal D. 2018. Antibiotic resistance and wastewater: correlation, impact and critical human health challenges. J Environ Chem Eng 6:52–58. https://doi.org/10.1016/j.jece.2017.11.059.
10. Ben Said L, Jouini A, Alonso CA, Klibi N, Dziri R, Boudabous A, Ben Slama K, Torres C. 2016. Characteristics of extended-spectrum β-lactamase (ESBL)- and pAmpC beta-lactamase-producing *Enterobacteriaceae* of water samples in Tunisia. Sci Total Environ 550:1103–1109. https://doi.org/10.1016/j.scitotenv.2016.01.042.
11. Diwan V, Chandran SP, Tamhankar AJ, Stalsby Lundborg C, Macaden R. 2012. Identification of extended-spectrum -lactamase and quinolone resistance genes in *Escherichia coli* isolated from hospital wastewater from central India. J Antimicrob Chemother 67:857–859. https://doi.org/10.1093/jac/dkr564.
12. Drieux L, Haenn S, Moulin L, Jarlier V. 2016. Quantitative evaluation of extended-spectrum β-lactamase-producing *Escherichia coli* strains in the wastewater of a French teaching hospital and relation to patient strain. Antimicrob Resist Infect Control 5:9. https://doi.org/10.1186/s13756-016-0108-5.
13. Bréchet C, Plantin J, Sauget M, Thouverez M, Talon D, Cholley P, Guyeux C, Hocquet D, Bertrand X. 2014. Wastewater treatment plants release large amounts of extended-spectrum β-lactamase-producing *Escherichia coli* into the environment. Clin Infect Dis 58:1658–1665. https://doi.org/10.1093/cid/ciu190.
14. Ory J, Bricheux G, Robin F, Togola A, Forestier C, Traore O. 2019. Biofilms in hospital effluents as a potential crossroads for carbapenemase-encoding strains. Sci Total Environ 657:7–15. https://doi.org/10.1016/j.scitotenv.2018.11.427.
15. Zhang L, Ma X, Luo L, Hu N, Duan J, Tang Z, Zhong R, Li Y. 2020. The prevalence and characterization of extended-spectrum β-lactamase- and carbapenemase-producing bacteria from hospital sewage, treated effluents and receiving rivers. IJERPH 17:1183. https://doi.org/10.3390/ijerph17041183.
16. Lenart-Boroń A. 2017. Antimicrobial resistance and prevalence of extended-spectrum beta-lactamase genes in *Escherichia coli* from major rivers in Podhale, southern Poland. Int J Environ Sci Technol 14:241–250. https://doi.org/10.1007/s13762-016-1155-4.
17. Tanaka H, Hayashi W, Iimura M, Taniguchi Y, Soga E, Matsuo N, Kawamura K, Arakawa Y, Nagano Y, Nagano N. 2019. Wastewater as a probable environmental reservoir of extended-spectrum-β-lactamase genes: detection of chimeric β-lactamases CTX-M-64 and CTX-M. Appl Environ Microbiol 85:e01740-19. https://doi.org/10.1128/AEM.01740-19.
18. Blaak H, Lynch G, Italiaander R, Hamidjaja RA, Schets FM, de Roda Husman AM. 2015. Multidrug-resistant and extended spectrum beta-lactamase-producing *Escherichia coli* in Dutch surface water and wastewater. PLoS One 10:e0127752. https://doi.org/10.1371/journal.pone.0127752.
19. Batchelor M, Hopkins K, Threlfall EJ, Clifton-Hadley FA, Stallwood AD, Davies RH, Liebana E. 2005. bla_{CTX-M} genes in clinical *Salmonella* isolates recovered from humans in England and Wales from 1992 to 2003. Antimicrob Agents Chemother 49:1319–1322. https://doi.org/10.1128/AAC.49.4.1319-1322.2005.
20. Dandachi I, Chaddad A, Hanna J, Matta J, Daoud Z. 2019. Understanding the epidemiology of multi-drug resistant Gram-negative bacilli in the Middle East using a One Health approach. Front Microbiol 10:1941. https://doi.org/10.3389/fmicb.2019.01941.
21. Yasir M, Qureshi AK, Kensarah EA, Bibi F, Al-Zahrani IA, Abd El Ghany M, Azhar EI. 2021. Draft genome sequence of colistin-resistant and extended-spectrum β-lactamase (ESBL)-producing multidrug-resistant *Escherichia coli* isolated from poultry meat. J Glob Antimicrob Resist 27:112–114. https://doi.org/10.1016/j.jgar.2021.08.014.
22. Yu X, Zhang M, Zuo J, Shi X, Tang X, Chen L, Li Z. 2018. Evaluation of antibiotic resistant lactose fermentative opportunistic pathogenic *Enterobacteriaceae* bacteria and bla_{TEM-2} gene in cephalosporin wastewater and its discharge receiving river. J Environ Manage 228:458–465. https://doi.org/10.1016/j.jenvman.2018.09.043.
23. Homeier-Bachmann T, Heiden SE, Lübcke PK, Bachmann L, Bohnert JA, Zimmermann D, Schaufler K. 2021. Antibiotic-resistant *Enterobacteriaceae* in wastewater of abattoirs. Antibiotics 10:568. https://doi.org/10.3390/antibiotics10050568.
24. Ekwanzala MD, Dewar JB, Kamika I, Momba MNB. 2019. Tracking the environmental dissemination of carbapenem-resistant *Klebsiella pneumoniae* using whole-genome sequencing. Sci Total Environ 691:80–92. https://doi.org/10.1016/j.scitotenv.2019.06.533.
25. Barría-Loaiza C, Pincheira A, Quezada M, Vera A, Valenzuela P, Domínguez M, Lima CA, Araya I, Araya P, Prat S, Aguayo C, Fernández J, Hormazábal JC, Bello-Toledo H, González-Rocha G. 2016. Molecular typing and genetic environment of the bla_{KPC} gene in Chilean isolates of *Klebsiella pneumoniae*. J Glob Antimicrob Resist 4:28–34. https://doi.org/10.1016/j.jgar.2016.01.001.
26. Sekizuka T, Inamine Y, Segawa T, Kuroda M. 2019. Characterization of NDM-5- and CTX-M-55-coproducing *Escherichia coli* GSH8M-2 isolated from the effluent of a wastewater treatment plant in Tokyo Bay. Infect Drug Resist 12:2243–2249. https://doi.org/10.2147/IDR.S215273.
27. Djahmi N, Dunyach-Remy C, Pantel A, Dekhil M, Sotto A, Lavigne J-P. 2014. Epidemiology of carbapenemase-producing *Enterobacteriaceae* and *Acinetobacter baumannii* in Mediterranean countries. Biomed Res Int 2014:1–11. https://doi.org/10.1155/2014/305784.
28. Pantel A, Richaud-Morel B, Cazaban M, Bouziges N, Sotto A, Lavigne J-P. 2016. Environmental persistence of OXA-48-producing *Klebsiella pneumoniae* in a French intensive care unit. Am J Infect Control 44:366–368. https://doi.org/10.1016/j.ajic.2015.09.021.
29. Dandachi I, Chabou S, Daoud Z, Rolain J-M. 2018. Prevalence and emergence of extended-spectrum cephalosporin-, carbapenem-, and colistin-

resistant Gram-negative bacteria of animal origin in the Mediterranean basin. Front Microbiol 9:2299. https://doi.org/10.3389/fmicb.2018.02299.
30. Carroll LM, Gaballa A, Guldimann C, Sullivan G, Henderson LO, Wiedmann M. 2019. Identification of novel mobilized colistin resistance gene *mcr-9* in a multidrug-resistant, colistin-susceptible *Salmonella enterica* serotype Typhimurium isolate. mBio 10:e00853-19. https://doi.org/10.1128/mBio.00853-19.
31. Bardet L, Le Page S, Leangapichart T, Rolain J-M. 2017. LBJMR medium: a new polyvalent culture medium for isolating and selecting vancomycin and colistin-resistant bacteria. BMC Microbiol 17:220. https://doi.org/10.1186/s12866-017-1128-x.
32. Seng P, Drancourt M, Gouriet F, La Scola B, Fournier P-E, Rolain JM, Raoult D. 2009. Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin Infect Dis 49:543–551. https://doi.org/10.1086/600885.
33. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19:455–477. https://doi.org/10.1089/cmb.2012.0021.
34. Seemann T. 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30:2068–2069. https://doi.org/10.1093/bioinformatics/btu153.
35. Blankenberg D, Von Kuster G, Bouvier E, Baker D, Afgan E, Stoler N, Taylor J, Nekrutenko A, Galaxy Team. 2014. Dissemination of scientific software with Galaxy ToolShed. Genome Biol 15:403. https://doi.org/10.1186/gb4161.
36. Koudokpon H, Dougnon V, Hadjadj L, Kissira I, Fanou B, Loko F, Bankole HS, Diene S, Rolain J-M. 2018. First sequence analysis of genes mediating extended-spectrum beta-lactamase (ESBL) bla-TEM, SHV- and CTX-M production in isolates of *Enterobacteriaceae* in Southern Benin. Int J Infect https://doi.org/10.5812/iji.83194.
37. Al Bayssari C, Diene SM, Loucif L, Gupta SK, Dabboussi F, Mallat H, Hamze M, Rolain J-M. 2014. Emergence of VIM-2 and IMP-15 carbapenemases and inactivation of *oprD* gene in carbapenem-resistant *Pseudomonas aeruginosa* clinical isolates from Lebanon. Antimicrob Agents Chemother 58:4966–4970. https://doi.org/10.1128/AAC.02523-13.
38. Diene SM, Bruder N, Raoult D, Rolain J-M. 2011. Real-time PCR assay allows detection of the New Delhi metallo-β-lactamase (NDM-1)-encoding gene in France. Int J Antimicrob Agents 37:544–546. https://doi.org/10.1016/j.ijantimicag.2011.02.006.
39. Mathlouthi N, Al-Bayssari C, El Salabi A, Bakour S, Ben Gwierif S, Zorgani AA, Jridi Y, Ben Slama K, Rolain J-M, Chouchani C. 2016. Carbapenemases and extended-spectrum β-lactamases producing *Enterobacteriaceae* isolated from Tunisian and Libyan hospitals. J Infect Dev Ctries 10:718–727. https://doi.org/10.3855/jidc.7426.
40. Mesli E, Berrazeg M, Drissi M, Bekkhoucha SN, Rolain J-M. 2013. Prevalence of carbapenemase-encoding genes including New Delhi metallo-β-lactamase in *Acinetobacter* species, Algeria. Int J Infect Dis 17:e739–e743. https://doi.org/10.1016/j.ijid.2013.02.024.
41. Chabou S, Leangapichart T, Okdah L, Le Page S, Hadjadj L, Rolain J-M. 2016. Real-time quantitative PCR assay with TaqMan probe for rapid detection of MCR-1 plasmid-mediated colistin resistance. New Microbes New Infect 13:71–74. https://doi.org/10.1016/j.nmni.2016.06.017.
42. Touati M, Hadjadj L, Berrazeg M, Baron SA, Rolain JM. 2020. Emergence of *Escherichia coli* harbouring *mcr-1* and *mcr-3* genes in North West Algerian farmlands. J Glob Antimicrob Resist 21:132–137. https://doi.org/10.1016/j.jgar.2019.10.001.
43. Nabti LZ, Sahli F, Ngaiganam EP, Radji N, Mezaghcha W, Lupande-Mwenebitu D, Baron SA, Rolain J-M, Diene SM. 2020. Development of real-time PCR assay allowed describing the first clinical *Klebsiella pneumoniae* isolate harboring plasmid-mediated colistin resistance mcr-8 gene in Algeria. J Glob Antimicrob Resist 20:266–271. https://doi.org/10.1016/j.jgar.2019.08.018.
44. Poirel L, Bonnin RA, Nordmann P. 2012. Genetic features of the widespread plasmid coding for the carbapenemase OXA-48. Antimicrob Agents Chemother 56:559–562. https://doi.org/10.1128/AAC.05289-11.