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Spread of *mcr-1*-carrying Enterobacteriaceae in sewage water from Spain

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Objectives: The mobile colistin resistance gene *mcr-1* has been identified worldwide in human and animal sources, while its occurrence in the environment is still largely unknown. The aim of this study was to investigate the presence of *mcr-1*-harbouring Enterobacteriaceae in water samples obtained from rivers and waste water treatment plants in the area of Barcelona, Spain.

Methods: The presence of *mcr-1* was detected by PCR. Bacterial identification was performed via MALDI-TOF MS. Resistance to colistin was determined by a broth dilution method. The epidemiological relationship between the positive isolates was assessed with PFGE and ST was determined by MLST. Plasmid characterization was performed by transformation experiments, antimicrobial susceptibility testing and incompatibility group PCR.

Results: Thirty MDR isolates bearing *mcr-1*, 29 *Escherichia coli* (ST632 and ST479) and 1 *Klebsiella pneumoniae* (ST526), were identified in sewage from two different waste water treatment plants, whereas the gene was not found in river water. All isolates, including the *K. pneumoniae*, harboured $bla_{\text{CTX-M-55}}$ and $bla_{\text{TEM-1}}$. *mcr-1* was in all cases associated with an IncI2 plasmid, which only conferred resistance to colistin. *mcr-1* was harboured by two predominant *E. coli* clones that were found in both waste water treatment plants.

Conclusions: This study showed a high occurrence of *mcr-1* in the sewage of Barcelona, mainly due to the dissemination of two *E. coli* pulsotypes that are circulating in the population. The presence of *mcr-1* in the environment is a cause for concern, and suggests high prevalence of *mcr-1* in the community.

Introduction

Since its discovery in 1949, colistin (polymyxin E) has been widely used in veterinary medicine, while its application in humans was restricted due to its toxicity.[1] However, as a consequence of the emergence of XDR bacteria, colistin has become a last-resort therapeutic agent against life-threatening infections.[2]

After the first identification of *mcr-1*, the unique transferable resistance mechanism to colistin in China by Liu *et al.*,[3] this gene has been described in Enterobacteriaceae from both humans and animals (livestock and food) worldwide.[4] However, it has only been detected in two *Escherichia coli* isolates from environmental sources in Switzerland and Malaysia.[5,6]

In this work, we identified a high occurrence of *mcr-1* in Enterobacteriaceae present in sewage water in Barcelona, Spain.

Materials and methods

Bacterial isolation

The samples were collected from July to November 2013 from river and sewage water in the area of Barcelona. Eight samples were taken from two Spanish rivers, the Cardener and the Llobregat. From each river, samples were taken from water (three samples) and sediments (one sample) at two locations and different dates. For analysis, 10 mL of river water was filtered through a cellulose membrane (0.45 μm pore size), after which the filter was placed on MacConkey agar plates (Oxoid Ltd, Basingstoke, UK) without colistin antibiotic pressure. Likewise, 5 g of sediment from each river was homogenized 1/10 in PBS, centrifuged for 5 min at 300 **g** and the resulting supernatant was then plated onto MacConkey agar without colistin. Five sewage samples were obtained at different dates from two waste water treatment plants (WWTPs) located in Baix Llobregat; three samples were obtained from El Prat and two samples were obtained from Gavá. Samples

were 10-fold diluted in PBS and plated onto MacConkey agar without colistin. Up to 10 lactose-positive colonies from each sampling site and date were analysed, to avoid repetitive isolation of the same clone. From all these, finally 90 isolates from sewage and 105 from river, distributed from different sites and dates, were used for this study.

Bacterial identification, PCR and susceptibility testing

The bacterial species were subsequently identified via MALDI-TOF MS (VISAVET, Madrid, Spain). The presence of the *mcr-1* gene was detected via PCR from each colony as described by Liu *et al.*[3] These primers amplified both *mcr-1* and *mcr-1.2*.[7] The antibiotic resistance level was assessed by MIC using commercial Sensititre EUVSEC plates (Trek Diagnostics Inc., Westlake, OH, USA). The results were interpreted following the EUCAST guidelines for Enterobacteriaceae.[8] Multiplex PCR was performed for the detection of ESBLs and plasmid-mediated AmpC β-lactamases as previously described.[9]

Strain typing

The clonal relatedness of the *mcr-1*-positive isolates was determined via PFGE as previously described.[10] Briefly, DNA plugs were digested using XbaI restriction enzyme (Takara Bio Inc., Japan) for 14 h. PFGE was undertaken on a CHEF-DR III (Bio-Rad, Hercules, CA, USA) using the following parameters: running time 22 h, temperature 14°C, field strength 6 V/cm^2, angles 120°, initial pulse time 2.2 s, final pulse time 63.8 s. Moreover, MLST analysis was performed following the protocols described on the Pasteur MLST web site (*E. coli* and *Klebsiella pneumoniae* MLST, www.pasteur.fr/mlst) and Warwick MLST web site (*E. coli* MLST, www.warwick.ac.uk/mlst/).

Plasmid characterization

Plasmid DNA was extracted from one isolate per pulsotype using a Plasmid Midi Kit (Qiagen Inc., CA, USA). Transformations were then carried out using electrocompetent *E. coli* DH5α as the recipient.[11] The resulting transformants were selected on BHI agar plates with colistin (2 mg/L) and screened for *mcr-1* by PCR.[3] The antibiotic resistance profile of the transformants was determined according to EUCAST (see above). A PCR-based replicon typing kit (Diatheva, Fano, Italy) was used to classify the plasmids according to their incompatibility group.

Results

A total of 195 isolates were obtained from the river and sewage sampling. MALDI-TOF revealed the presence of the following Enterobacteriaceae: *E. coli*, *K. pneumoniae*, *Citrobacter freundii*, *Citrobacter koseri*, *Enterobacter kobei*, *Enterobacter cloacae*, *Enterobacter ludwigii* and *Enterobacter asburiae*. None of the 105 isolates collected from the rivers was positive for *mcr-1*. On the contrary, 30 of the 90 isolates recovered from the sewage of both WWTPs were positive for *mcr-1*. All the *mcr-1*-positive Enterobacteriaceae were *E. coli* except one identified as *K. pneumoniae*.

Antibiotic susceptibility testing showed that the 30 isolates exhibited resistance to many antibiotics such as quinolones, aminoglycosides and β-lactams, including third-generation cephalosporins (Table 1). All isolates harboured $bla_{\text{CTX-M-55}}$ and $bla_{\text{TEM-1}}$. Interestingly one of the isolates containing the *mcr-1* gene, BB1459, was susceptible to colistin. The *E. coli* PFGE results revealed two predominant patterns, pulsotype I (12 isolates) and pulsotype II (15 isolates), present in the two WWTPs [Table 1 and Figure S1 (available as Supplementary data at *JAC* Online)]. MLST was performed in representative *E. coli* isolates from the different pulsotypes and the *K. pneumoniae* isolate (Figure S1). Twenty-eight *E. coli* (pulsotypes I, II and III) belonged to the same infrequent ST, namely ST1196 following the Warwick MLST scheme, ST632 according to the Pasteur web site. Both ST were recently described and related to nosocomial infections caused by carbapenemase-producing *E. coli*.[12,13] The non-typeable *E. coli* BB1459 was identified as ST224 or ST479, Warwick and Pasteur schemes, respectively, and the *K. pneumoniae* as ST526.

Plasmids harbouring *mcr-1* from one *E. coli* isolate per pulsotype (corresponding to pulsotypes I, II and III and the non-typeable isolate BB1459) and the *K. pneumoniae* isolate were successfully transformed in *E. coli* DH5α. All the transformed plasmids belonged to the incompatibility group IncI2 and no coexistence with other antibiotic resistance was observed (Table 1). Although BB1459 was susceptible to colistin, its transformant was resistant to colistin (MIC = 4 mg/L), confirming the functionality of the gene.

Discussion

The discovery of new less toxic drugs, such as aminoglycosides, led to colistin use being restricted to topical treatments in human medicine, while in veterinary medicine the use of colistin was indicated for treatment as well as for the prevention of infections.[2] The alarming rise of antibiotic resistance along with the scarcity of new drugs is resulting in a lack of effective antibiotics.[14] As such, colistin has been reintroduced in human medicine as a last-resort antibiotic despite its toxicity.[3] Until last year, the only known resistance mechanism to colistin was mediated by chromosomal mutations. As mutational mechanisms of resistance are only transmitted vertically, their dissemination has been more confined.[3] Nevertheless, Liu *et al.*[3] recently described the first plasmid-mediated colistin resistance gene, *mcr-1*, which greatly facilitates the propagation of colistin resistance by horizontal gene transfer. Since then, the presence of *mcr-1* has been reported in many different countries and species, and from a variety of origins.[4] Our study highlights the elevated prevalence of *mcr-1* in different bacterial species recovered without colistin selection from sewage water around Barcelona.

Aquatic environments play an important role in the dissemination of antibiotic resistance, acting as a reservoir where phenomena such as acquisition, transmission and genetic evolution of resistance genes are frequent.[15] The presence of *mcr-1* in water, and therefore in the environment, not only facilitates the spread of colistin-resistant clones into the population, but also favours the transmission of this gene among different species such as pathogenic *K. pneumoniae*.

Recently, Prim *et al.*[16] reported plasmid-mediated colistin resistance in 15 clinical strains of *E. coli* isolated from patients of the Hospital de la Santa Creu i Sant Pau in Barcelona. Their study underlines the genetic diversity of *mcr-1*-bearing clones in the hospital.[16] In our study, the colistin resistance gene was present in two predominant *E. coli* clones, two unique *E. coli* as well as in a *K. pneumoniae*. The predominant pulsotypes were isolated from two WWTPs, one (Gavá) serving an area consisting of a number of cities and towns, with a population of 370 000 inhabitants, and the other (el Prat) collecting the sewage from the south of Barcelona city, corresponding to 2 000 000 inhabitants. Because sewage waters comprise a mixture from numerous individuals, our data provide an optimal representation of what is circulating within the human population. In all isolates positive for *mcr-1*, selected in the

Table 1. Epidemiological data and MICs for the 30 isolates positive for *mcr-1* obtained from sewage plants in Barcelona, Spain

			MIC (mg/L)											
Name	Pulsotype	Source	TMP	NAL	CIP	TET	CHL	AMP	CTX	CAZ	MEM	GEN	TGC	CST
BB1418	I	Gavá	>**32**	>**128**	>**8**	<2	<8	>**64**	>**4**	**4**	<0.03	>**32**	<0.25	**4**
BB1422	I	Gavá	>**32**	>**128**	>**8**	<2	<8	>**64**	>**4**	>**8**	<0.03	>**32**	1	**4**
BB1427	I	Gavá	>**32**	>**128**	>**8**	>**64**	>**128**	>**64**	>**4**	**8**	<0.03	>**32**	0.5	**4**
BB1430	I	Gavá	>**32**	>**128**	>**8**	>**64**	>**128**	>**64**	>**4**	**8**	<0.03	>**32**	0.5	**4**
BB1433	I	Gavá	>**32**	>**128**	>**8**	>**64**	>**128**	>**64**	>**4**	**8**	<0.03	>**32**	<0.25	**4**
BB1437	I	El Prat	>**32**	>**128**	>**8**	>**64**	>**128**	>**64**	>**4**	**8**	<0.03	>**32**	1	**4**
BB1438	I	El Prat	>**32**	>**128**	>**8**	<2	<8	>**64**	>**4**	**4**	<0.03	>**32**	2	**4**
BB1450	I	Gavá	>**32**	>**128**	>**8**	>**64**	>**128**	>**64**	>**4**	**4**	<0.03	>**32**	<0.25	**4**
BB1451	I	Gavá	>**32**	>**128**	>**8**	>**64**	>**128**	>**64**	>**4**	**8**	<0.03	>**32**	<0.25	**4**
BB1452	I	Gavá	>**32**	>**128**	>**8**	>**64**	>**128**	>**64**	>**4**	**4**	<0.03	>**32**	0.5	**4**
BB1455	I	Gavá	>**32**	>**128**	>**8**	>**64**	>**128**	>**64**	>**4**	**8**	<0.03	>**32**	<0.25	**4**
BB1456	I	Gavá	>**32**	>**128**	>**8**	>**64**	>**128**	>**64**	>**4**	**8**	<0.03	>**32**	0.5	**4**
BB1420	II	Gavá	>**32**	>**128**	>**8**	>**64**	>**128**	>**64**	>**4**	**4**	<0.03	>**32**	<0.25	**4**
BB1461	II	Gavá	>**32**	>**128**	>**8**	<2	<8	>**64**	>**4**	**4**	<0.03	>**32**	<0.25	**4**
BB1428	II	Gavá	>**32**	>**128**	>**8**	<2	<8	>**64**	>**4**	**4**	<0.03	>**32**	<0.25	**4**
BB1429	II	Gavá	>**32**	>**128**	>**8**	>**64**	>**128**	>**64**	>**4**	**4**	<0.03	>**32**	1	**4**
BB1431	II	Gavá	>**32**	>**128**	>**8**	<2	<8	>**64**	>**4**	**8**	<0.03	>**32**	<0.25	**4**
BB1434	II	Gavá	>**32**	>**128**	>**8**	>**64**	>**128**	>**64**	>**4**	**8**	<0.03	>**32**	<0.25	**4**
BB1435	II	El Prat	>**32**	>**128**	>**8**	<2	<8	>**64**	>**4**	**4**	<0.03	>**32**	0.5	**4**
BB1436	II	El Prat	>**32**	>**128**	>**8**	>**64**	>**128**	>**64**	>**4**	**4**	<0.03	>**32**	1	**8**
BB1447	II	Gavá	>**32**	>**128**	>**8**	>**64**	>**128**	>**64**	>**4**	**8**	<0.03	>**32**	<0.25	**4**
BB1448	II	Gavá	>**32**	>**128**	>**8**	<2	<8	>**64**	>**4**	**4**	<0.03	>**32**	<0.25	**4**
BB1449	II	Gavá	>**32**	>**128**	>**8**	>**64**	>**128**	>**64**	>**4**	**4**	<0.03	>**32**	0.5	**4**
BB1453	II	Gavá	>**32**	>**128**	>**8**	>**64**	>**128**	>**64**	>**4**	**8**	<0.03	>**32**	<0.25	**4**
BB1454	II	Gavá	>**32**	>**128**	>**8**	<2	<8	>**64**	>**4**	**8**	<0.03	>**32**	<0.25	**4**
BB1457	II	Gavá	>**32**	>**128**	>**8**	>**64**	>**128**	>**64**	>**4**	**4**	<0.03	>**32**	<0.25	**4**
BB1458	II	Gavá	>**32**	>**128**	>**8**	>**64**	>**128**	>**64**	>**4**	**8**	<0.03	>**32**	0.5	**4**
BB1425	III	Gavá	>**32**	>**128**	>**8**	<2	<8	>**64**	>**4**	**4**	<0.03	>**32**	0.5	**4**
BB1459	non-typeable	Gavá	>**32**	>**128**	>**8**	>**64**	<8	>**64**	>**4**	>**8**	<0.03	>**32**	0.5	<1
BB1460[a]	ND	Gavá	>**32**	>**128**	>**8**	<2	<8	>**64**	>**4**	**8**	<0.03	>**32**	<0.25	**4**
BB1438T	transformant	—	<0.25	**64**	<0.06	<2	<8	2	<0.25	<0.5	<0.03	<0.5	<0.25	**4**
BB1460T	transformant	—	<0.25	**64**	<0.06	<2	<8	2	<0.25	<0.5	<0.03	<0.5	<0.25	**4**
BB1459T	transformant	—	<0.25	**64**	<0.06	<2	<8	2	<0.25	<0.5	<0.03	<0.5	<0.25	**4**
BB1428T	transformant	—	<0.25	**64**	<0.06	<2	<8	2	<0.25	<0.5	<0.03	<0.5	<0.25	**4**
BB1425T	transformant	—	<0.25	**64**	<0.06	<2	<8	2	<0.25	<0.5	<0.03	<0.5	<0.25	**4**
DH5α	—	laboratory	<0.25	**64**	<0.06	<2	<8	2	<0.25	<0.5	<0.03	<0.5	<0.25	<1

TMP, trimethoprim; NAL, nalidixic acid; CIP, ciprofloxacin; TET, tetracycline; CHL, chloramphenicol; AMP, ampicillin; CTX, cefotaxime; CAZ, ceftazidime; MEM, meropenem; GEN, gentamicin; TGC, tigecycline; CST, colistin; ND, not determined.
Resistance is highlighted in bold.
[a]*K. pneumoniae*.

absence of colistin, the gene was located on an IncI2 plasmid conferring resistance exclusively to colistin. Our results support the notion that a plasmid bearing *mcr-1* is circulating among the population of Barcelona predominantly within two clones, and that it has been transferred to other *E. coli* as well as to other species such as *K. pneumoniae*.

Surveillance of resistance is essential to mitigate the threat of antimicrobial resistance.[17] Because colistin was not used to select the *mcr-1* isolates, our approach allowed detection of a *mcr-1*-positive isolate that was nevertheless susceptible to colistin. *mcr-1* was harboured on a plasmid that we were able to transform, thereby conferring resistance to colistin. Hence, *mcr-1* can act as a silent antimicrobial resistance gene, evading phenotypic detection, which causes treatment failure and favours its dissemination.[18,19] This isolate has a different PFGE pattern compared to all the others. Thus, the genetic context of *mcr-1* seems to be responsible for this phenotype. In this sense, we could speculate that either the cell wall structure or the copy number of the *mcr-1* IncI2 plasmid could be the underlying cause of this phenomenon.

The absence of *mcr-1*-bearing isolates in rivers could be due: (i) to the lack of colistin-treated animals/humans contaminating the rivers in these sites; and (ii) to the lower enterobacterial density in river water as compared to sewage. As well as the epidemiological observation of *mcr-1*-positive isolates circulating in the population, the high prevalence of *mcr-1*-positive isolates in sewage is a risk factor promoting the dissemination of this resistance mechanism to environmental water and other ecosystems since sewage treatment processes reduce, but do not remove completely, the antibiotic-resistant bacteria from the final effluent.[20] To safeguard the future of colistin, it is essential to adapt a multidisciplinary 'One Health' strategy that should include control measures from the source (i.e. regulating use in human and veterinary medicine) to the sewage (i.e. improving water treatment protocols).

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Transparency declarations

None to declare.

Author contributions

C. M. O. and J. F. D.-B. designed the study, performed the experiments, analysed and interpreted the results and wrote the draft manuscript. C. M. O. and J. F. D.-B. contributed equally to this work. W. C.-C. and M. M. collected the samples, provided the isolates for this study, and revised and approved the final version of the manuscript. B. G.-Z. conceived, designed and coordinated the study, and revised and approved the final version of the manuscript.

Supplementary data

Figure S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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