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Short Communication

Microbiological surveillance of plasmid mediated colistin resistance in human *Enterobacteriaceae* isolates in Romagna (Northern Italy): August 2016–July 2017

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ABSTRACT

Objectives: To start a surveillance program to investigate the possible diffusion of mobilized colistin resistance genes in *Enterobacteriaceae* strains isolated in the Unit of Microbiology of the Great Romagna Hub Laboratory.
Methods: All the colistin resistant *Enterobacteriaceae*, isolated from August 1st 2016 to July 31st 2017, were prospectively evaluated for *mcr-1* and *mcr-2*. Backdated survey of *mcr-3*, *mcr-4* and *mcr-5* was performed on the same group of isolates. Species identification was achieved by Vitek MS and the antibiotic susceptibility testing was performed both with Vitek-2 and Sensititre systems. Colistin resistant isolates were screened by PCR for the presence of the plasmid-mediated colistin resistance genes and amplicons were verified by sequencing. All mcr-1 positive isolates were subjected to MLST analysis.
Results: Over the total of 19053 isolates belonging to *Enterobacteriaceae*, 90 were colistin resistant. The presence of *mcr-1* was detected in 26 *Escherichia coli*. The overall prevalence of *mcr-1* was 0.14%. The *mcr-1* positive *E. coli* strains were assigned to 13 distinct sequence types (STs) according to MLST.
Conclusions: The prospective epidemiological survey carried out in our study gave a glimpse of the plasmid-mediated colistin resistance dissemination in Romagna. Since the prevalence rate of carbapenem resistant *Enterobacteriaceae* (CRE) in some hospital wards in our area is alarming, we underline the importance of a Surveillance Program to monitor the spread of the plasmid-mediated colistin resistance genes into MDR Gram-negative bacteria.

Enterobacteriaceae are responsible for a significant number of infections and deaths worldwide, and the prevalence of antibiotic resistance continues to rise in these bacteria (World Health Organization, 2014). Colistin is nowadays reported as one of the last line antimicrobial drugs active against multidrug-resistant gram-negative bacteria (MDR). This renewed therapeutic role is now potentially hampered by four plasmidic genes *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5* that mediate the resistance to colistin (Liu et al., 2016; Xavier et al., 2016; Yin et al., 2017; Carattoli et al., 2017; Borowiak et al., 2017).

In July 2016 we were informed by the Institute for Experimental Veterinary Medicine of Lombardy and Emilia-Romagna that a *Salmonella enterica* isolated in January 2015 from a 3 year old patient in our Laboratory, was bearing the *mcr-1* gene. This isolate was included in a retrospective analysis performed on human and veterinary *Salmonella* spp. isolates in Emilia Romagna (Carnevali et al., 2016). A surveillance program to investigate the possible diffusion of *mcr-1* and *mcr-2* genes in humans was promptly started. All the colistin resistant *Enterobacteriaceae,* prospectively isolated from clinical specimens from August 1st 2016 to July 31st 2017 were evaluated. A specific analysis for *mcr -3, mcr-4* and *mcr-5* was performed on the same colistin resistant isolates in later stages.

The bacteria identification was carried out by the standard procedure (Vitek-MS; BioMérieux, Marcy l'Etoile, France). The minimum inhibitory concentrations (MICs) were determined with the Vitek 2 (BioMérieux; AST-card N201) according to The European Committee on Antimicrobial Susceptibility Testing (EUCAST) rules. Out of a total of 19053 non-duplicate

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Enterobacteriaceae (*E. coli* n. = 12441; 65% of overall isolates), 91 strains displayed colistin MICs of ≥ 2 mg/L. In 90 cases the resistance was confirmed through a microdilution method (TREK Sensititre custom panel GNX2F; Thermo-Fisher TREK Diagnostic Systems, Cleveland, OH, USA) as recommended by a joint EUCAST and CLSI subcommittee (www.eucast.org/ast_of_bacteria/warnings/). These 90 strains included *E. coli* (n = 63), *Klebsiella pneumoniae* (n = 22), *Enterobacter cloacae* (n = 4) and *Hafnia alvei* (n = 1). The isolates were first screened for *mcr-1* and *mcr-2* genes by PCR. PCR assays were performed as described previously (Liu et al., 2016; Xavier et al., 2016) *mcr-1* was detected in 26 isolates, all *E. coli* (26/12441, 0.21% overall for this species), whereas no *mcr-2* was found. Confirmation and allele identification were carried out by bidirectional DNA sequencing. The amplified sequences were 100% homologous with the phosphoethanolamine transferase sequence reported worldwide (Liu et al., 2016). *mcr-3*, *mcr-4* and *mcr-5* were discovered after the start of our surveillance program. In order to implement the *mcr*-screening, the 90 colistin resistant strains collected from August 1st 2016 to July 31st 2017 were tested for *mcr-3*, *mcr-4*, *mcr-5*. PCR assays were performed as reported previously (Yin et al., 2017; Carattoli et al., 2017; Borowiak et al., 2017): none of the three genes was detected.

Out of 26 *mcr-1* positive strains, 16 were isolated from outpatients while 10 were from hospitalized patients. These were cultured from urine in 21 cases, from blood in 2 cases, in 2 cases from a wound swab, and in 1 case from bronchial aspirate. MICs for colistin ranged from 4 mg/L (n = 4) to 8 mg/L (n = 22) and the isolates were susceptible to the majority of antimicrobial agent tested. Table 1 shows the details about MICs of the individual strains.

In addition, two strains (3O/RA; 19O/CE) showed also an extended-spectrum β-lactamase phenotype (ESβLs). Key antibiotic resistance markers were investigated by multiplex tandem PCR CRE assay (AusDiagnostics, Mascot – NSW, Australia) among *mcr-1* positive *E. coli* isolates and on the strain of *S. enterica* isolated in 2015: CTX-M group 1 target was detected on 19O/CE strain, according to the phenotypic antimicrobial susceptibility.

Out of the *Enterobacteriaceae* isolates, the colistin resistance rate was 0.47%, about a quarter of the one reported by a large study of strains isolated worldwide from 2014 to 2015 (SENTRY Program) (Castanheira et al., 2016). The overall prevalence rate of *mcr-1* positive enterobacterial strains (0,14%) is similar to that observed by the Program. The prevalence of *mcr-1* among colistin-resistant *E .coli* isolates was 41%, more elevated than that registered by SENTRY.

All the 26 *mcr-1* harboring *E. coli* were subjected to multilocus sequence types (MLST) analysis. PCR amplification and sequencing were performed following the protocols specified at the *E. coli* MLST website (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli). The analyzed strains were assigned to 13 distinct sequence types (STs), notably 8 strains belonged to 8 different STs, as detailed in Table 1. Additionally, the finding of several *mcr-1* carrying *E. coli* isolates belonging to STs associated with environment, animals, food and feed products according to the MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/GetTableInfo_htm) is in agreement with a likely nonhuman origin of the *mcr-1* determinant.

One important question refers to the potential role of food-producing animals in the epidemiology of *Enterobacteriaceae* carrying the *mcr-1* gene. Colistin has been widely used for decades in veterinary medicine both as a prophylactic agent and for treatment of food-producing animals. In 2013, polymyxins (mainly colistin) were the 5th most sold group of antimicrobials based on the total sales of polymyxins in the 26 EU/EEA countries reporting data. Notably, Italy is the European Member State with the second

Table 1
Features of the *mcr-1*-harboring *E. coli* clinical isolates. MICs were determinated by Vitek2.

Strain	Isolation	Source	MLST[a]	MIC mg/L (S/I/R)[b]												
				AMK	AMX/CLAV	CTX	CFT	FEP	IMI	MEM	PIP/TZB	CIP	GEN	SXT	TGC	COL
1O/RA	Aug 2016	urine	ST617	≤2 S	≤2 S	≤1 S	≤1 S	≤1 S	≤0.25 S	≤0.25 S	≤4 S	≤0.25 S	≤1 S	≤20 S	≤0.5 S	8 R
2I/RN	Aug 2016	urine	ST744	≤2 S	8 S	≤1 S	≤1 S	≤1 S	≤0.25 S	≤0.25 S	8 S	≥4 R	≤1 S	≥320 R	≤0.5 S	8 R
3O/RA *ESβL+*[c]	Sept 2016	urine	ST73	≤2 S	16 R	2 I	16 R	2 I	≤0.25 S	≤0.25 S	≤4 S	≥4 R	≥16 R	≤20 S	≤0.5 S	8 R
4O/RA	Sept 2016	urine	ST410	≤2 S	≥32 R	≤1 S	≤1 S	≤1 S	≤0.25 S	≤0.25 S	≤4 S	≥4 R	≤1 S	≥320 R	≤0.5 S	4 R
5I/RA	Sept 2016	blood	ST624	≤2 S	≥32 R	≤1 S	≤1 S	≤1 S	≤0.25 S	≤0.25 S	64 R	≥4 R	2 S	≥320 R	≤0.5 S	8 R
6I/RN	Sept 2016	urine	ST224	≤2 S	16 R	≤1 S	≤1 S	≤1 S	≤0.25 S	≤0.25 S	≤4 S	≥4 R	≤1 S	≥320 R	1 S	8 R
7I/RN	Sept 2016	urine	ST69	≤2 S	≤2 S	≤1 S	≤1 S	≤1 S	≤0.25 S	≤0.25 S	≤4 S	≤0.25 S	≤1 S	≥320 R	≤0.5 S	8 R
8I/CE	Sept 2016	urine	ST69	4 S	16 R	≤1 S	≤1 S	≤1 S	≤0.25 S	≤0.25 S	≤4 S	≥4 R	≥16 R	≥320 R	≤0.5 S	8 R
9O/RA	Sept 2016	urine	ST457	≤2 S	4 S	≤1 S	≤1 S	≤1 S	≤0.25 S	≤0.25 S	≤4 S	2 R	≤1 S	≥320 R	≤0.5 S	8 R
10O/FO	Sept 2016	urine	ST10	≤2 S	4 S	≤1 S	≤1 S	≤1 S	≤0.25 S	≤0.25 S	≤4 S	≥4 R	≥16 R	≥320 R	≤0.5 S	8 R
11O/RA	Sept 2016	wounde	ST354	≤2 S	8 S	≤1 S	≤1 S	≤1 S	≤0.25 S	≤0.25 S	≤4 S	≥4 R	≥16 R	≥320 R	≤0.5 S	8 R
12O/RA	Sept 2016	urine	ST10	≤2 S	4 S	≤1 S	≤1 S	≤1 S	≤0.25 S	≤0.25 S	≤4 S	≥4 R	≥16 R	≥320 R	≤0.5 S	8 R
13I/RN	Oct 2016	urine	ST224	≤2 S	16 R	≤1 S	≤1 S	≤1 S	≤0.25 S	≤0.25 S	16 I	≥4 R	≤1 S	≥320 R	≤0.5 S	8 R
14O/RN	Oct 2016	urine	ST10	≤2 S	≥32 R	≤1 S	≤1 S	≤1 S	≤0.25 S	≤0.25 S	16 I	≥4 R	≤1 S	≥320 R	≤0.5 S	4 R
15O/FO	Nov 2016	urine	ST216	≤2 S	8 S	≤1 S	≤1 S	≤1 S	≤0.25 S	≤0.25 S	≤4 S	≤0.25 S	≤1 S	≥320 R	≤0.5 S	8 R
16O/RA	Nov 2016	urine	ST95	≤2 S	4 S	≤1 S	≤1 S	≤1 S	≤0.25 S	≤0.25 S	≤4 S	2 R	≥16 R	≥320 R	≤0.5 S	8 R
17O/RA	Oct 2016	urine	ST744	≤2 S	≤2 S	≤1 S	≤1 S	≤1 S	≤0.25 S	≤0.25 S	≤4 S	≤0.25 S	≤1 S	≥320 R	≤0.5 S	4 R
18I/RN	Dec 2016	blood	ST10	≤2 S	16 R	≤1 S	≤1 S	≤1 S	≤0.25 S	≤0.25 S	≤4 S	≥4 R	≤1 S	≥320 R	≤0.5 S	8 R
19O/CE *ESβL+*[c,d]	Dec 2016	urine	ST131	≤2 S	4 S	8 R	≤1 S	2 I	≤0.25 S	≤0.25 S	≤4 S	≤0.25 S	≤1 S	≥320 R	≤0.5 S	8 R
20O/RA	Jan 2017	urine	ST131	≤2 S	4 S	≤1 S	≤1 S	≤1 S	≤0.25 S	≤0.25 S	≤4 S	1 I	≤1 S	≥320 R	≤0.5 S	8 R
21I/RN	Feb 2017	urine	ST10	≤2 S	≤2 S	≤1 S	≤1 S	≤1 S	≤0.25 S	≤0.25 S	≤4 S	≥4 R	≤1 S	≤20 S	≤0.5 S	16 R
22O/FO	Apr 2017	urine	ST131	≤2 S	≤2 S	≤1 S	≤1 S	≤1 S	≤0.25 S	≤0.25 S	≤4 S	≥4 R	≤1 S	≤20 S	≤0.5 S	8 R
23O/RA	Apr 2017	urine	ST224	≤2 S	16 R	≤1 S	≤1 S	≤1 S	≤0.25 S	≤0.25 S	≤4 S	≤0.25 S	≤1 S	≥320 R	≤0.5 S	4 R
24I/RA	May 2017	b.asp.[e]	ST10	≤2 S	8 S	≤1 S	≤1 S	≤1 S	≤0.25 S	≤0.25 S	≤4 S	≤0.25 S	≤1 S	≥320 R	≤0.5 S	4 R
25I/RA	June 2017	wounde	ST10	≤2 S	4 S	≤1 S	≤1 S	≤1 S	≤0.25 S	≤0.25 S	≤4 S	≤0.25 S	≤1 S	≥320 R	≤0.5 S	4 R
26O/RN	June 2017	urine	ST131	≤2 S	8 S	≤1 S	≤1 S	≤1 S	≤0.25 S	≤0.25 S	≤4 S	≤0.25 S	≤1 S	≥320 R	≤0.5 S	6 R

[a] **MLST**: multilocus sequence types.
[b] **Antimicrobial drugs abbreviations**: COL-colistin, AMK-amikacin, AMX/CLAV-amoxicillin/clavulanic acid,CTX-cefotaxime, CFT-ceftazidime, FEP-cefepime, IMI-imipenem, MEM-meropenem, PIP/TZB-piperacillin/tazobactam, CIP-ciprofloxacin, GEN-gentamicin, STX-trimethoprim-sulfamethoxazole, TGC-tgecyline. S-susceptible, I-intermediate, R-resistant.
[c] **ESβL+**: extended spectrum beta-lactamase-producing isolate.
[d] **CTX-M group1**.
[e] **b. asp.:** bronchial aspirate.

largest use of polymyxins in veterinary medicine (European Medicines Agency, 2014). The *mcr-1* carrying plasmids can be maintained stably and disseminate more rapidly under the selective pressure imposed by the use of colistin. Emilia-Romagna is the second Italian region for poultry production, with over 1,100 farms, 36 million heads and a value of production (meat and eggs) of over 600 million euros. The role of Romagna in the national poultry landscape is of paramount relevance. This district covers 20% of Italian poultry production, 11% of eggs, and it aggregates 40% of the products processing and marketing division. A retrospective analysis performed on human and veterinary *Salmonella* spp. isolates in Emilia Romagna demonstrated the presence of *mcr-1 in Salmonella* isolates at least since 2012 in our territory. *mcr*-1-positive isolates of human origin were concentrated in 2015, while the largest number of isolates of animals origin was concentrated over the previous two years (Carnevali et al., 2016). The presence of *Salmonella* spp. and *E. coli* in poultry is considered a risk factor for human consumption of meat and eggs. Antimicrobial resistant zoonotic pathogens present in meat food represent a direct hazard to public health. Moreover antimicrobial resistance genes in commensal or pathogenic zoonotic strains form an indirect risk, as they increase the gene pool from which pathogenic bacteria can pick up resistance determinants. Nowadays it is well known that *Salmonella* spp. and *E. coli* could act as donors of antimicrobial resistance genes for other enteric bacilli (Verraes et al., 2013). Therefore an integrated animal, human and environmental health surveillance system is essential to monitor the *mcr-1* emergence and diffusion in our area.

The distribution of plasmid-borne colistin resistance determinants represents a concern for the infection prevention and public health, especially in the context of high CRE prevalence in hospital wards. *mcr-1* was found to be co-localized with other resistance genes on plasmids, such as extended-spectrum β-lactamase (ESBLs) and carbapenemase genes. This means that the *mcr-1* carrying plasmids can be maintained stably and disseminate more rapidly under the selective pressure imposed by the use of antimicrobial agents other than colistin. *Klebsiella pneumoniae* carbapenemases (KPCs) have spread among Gram-negative bacteria, especially *K. pneumoniae*. KPC-producing *K. pneumoniae* is endemic in Italy, and the prevalence rate is around 85% of the total *K. pneumoniae* isolates in some Romagna hospital wards. In addition to this, the percentage of KPC-producing *E. coli* isolates has doubled in the three-year period 2015-2017, reaching 0,6% in 2017. The spread of the *mcr-1* gene to KPC-producing human pathogenic bacteria could pose a real threat because colistin is considered to be the last-resort antibiotic used for the treatment of infections caused by MDR-gram negative bacteria.

There are very limited data on dissemination of mobile colistin resistance genes in Italy, in particular in humans. To our knowledge, this is the first laboratory surveillance program that screened colistin resistant *Enterobacteriaceae* isolated from human specimens for the *mcr-1/2/3/4/5* determinants. Our study reports on the occurrence of *mcr-1*-harboring strains among clinical isolates and the *mcr-1* prevalence rate in the Romagna area. On the other hand, it is advisable to establish an integrated animal, human and environmental survey system in order to monitor the *mcr-1* diffusion in our area.

The knowledge of the *mcr* prevalence among colonized subjects and infected patients would allow evaluation of the dissemination of colistin resistant genes in our area, with the aim of reducing any related risk.

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