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1 **Detection of plasmid-mediated colistin resistance, *mcr-1* gene, in *Escherichia coli* isolated**
2 **from high-risk patients with acute leukemia in Spain.**

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39 **Abstract**

40 **Background:** Bacterial infections in immunocompromised patients are associated with a high
41 mortality and morbidity rate. In this high-risk group, the presence of multidrug-resistant
42 (MDR) bacteria, particularly bacteria that harbor a transferable antibiotic resistance gene,
43 complicates the management of bacterial infections. In this study, we investigated the
44 presence of the transferable colistin resistance *mcr* genes in patients with leukemia in Spain.

45 **Methods:** 217 fecal samples collected in 2013-2015 from 56 patients with acute leukemia and
46 colonized with MDR Enterobacteriaceae strains, were screened on September 2017 for the
47 presence of the colistin resistance *mcr* genes (*mcr-1* to -5) by multiplex PCR. *mcr* positive
48 strains selected on LBJMR and MacConkey supplemented with colistin (2 µg/ml) media were
49 phenotypically and molecularly characterized by antimicrobial susceptibility testing,
50 minimum inhibitory concentration, multilocus sequence typing and plasmid characterization.

51 **Results:** Among 217 fecal samples, 5 samples collected from 3 patients were positive for the
52 presence of the *mcr-1* colistin-resistance gene. Four *Escherichia coli* strains were isolated and
53 exhibited resistance to colistin with MIC= 4 µg/ml. Other genes conferring the resistance to β-
54 lactam antibiotics have also been identified in *mcr-1* positive strains, including *bla*_{TEM-206} and
55 *bla*_{TEM-98}. Three different sequence types were identified, including ST1196, ST140 and
56 ST10. Plasmid characterization allowed us to detect the *mcr-1* colistin resistance gene on
57 conjugative IncP plasmid type.

58 **Conclusion:** To the best of our knowledge, we have identified the *mcr-1* gene for the first
59 time in leukemia patients in Spain. In light of these results, strict measures have been
60 implemented to prevent its dissemination.

61 **Keywords:** *Escherichia coli*; colistin resistance; *mcr-1* gene; leukemic patients; Spain.

62 **Introduction**

63 Acute leukemia patients are considered a high-risk group because of their weakened immune
64 system. Infections with multidrug-resistant (MDR) bacteria are highly responsive in this type
65 of patients, mainly due to the combination of several risk factors such as: hematological
66 disease, intensive/or repeated chemotherapy, neutropenia, healthcare-associated infections,
67 gastrointestinal mucositis and prolonged hospitalization, which promote their colonization by
68 this type of bacteria [1, 2].

69 Antimicrobial therapy in hematology patients, such as leukemia patients, is often used for its
70 important contribution to the survival of these patients, but the emergence of MDR bacteria
71 due to selection pressure complicates the management of these bacterial infections [1].

72 Colistin, an antibiotic long abandoned for its neurological and renal toxicity, has been
73 reintroduced for its effectiveness against MDR Gram-negative bacteria, especially against
74 carbapenemase producers [3, 4]. Indeed, colistin used alone or in combination with other
75 antibiotics, has shown its effectiveness in the treatment of certain bacteremia due to MDR
76 bacteria in hematology patients [1]. Unfortunately, since its use, colistin resistance has
77 increased considerably, represented mainly by chromosomal gene mutations involving a
78 variety of lipopolysaccharide (LPS) modifications [4, 5]. In 2016, Liu et al. reported for the
79 first time colistin resistance mediated by mobile genetic elements identified in
80 Enterobacteriaceae, called plasmid-mediated colistin resistance gene *mcr-1* [6]. Since its first
81 detection, *mcr-1* gene was widespread worldwide in both animals and humans, and several
82 variants of this gene were detected in Enterobacteriaceae [7]. Very few data on PubMed are
83 available on the occurrence of *mcr* genes in leukemic patients and no studies have been
84 conducted in Spain. For this reason, we have sought to detect the presence of these genes in
85 fecal samples collected from patients with acute leukemia in a single institution in Spain.

86 **Materials and methods**

87 *Study design*

88 The FloraStopMRE project (2015 Infect-ERA call) is a collaboration of a multidisciplinary
89 consortium of scientists aiming to understand the role of the human gut microbiome in
90 conferring protection against MDR Enterobacteriaceae infections. Between November 2013
91 and April 2015, a total of 802 fecal samples were collected from 133 patients with acute
92 leukemia at the University Hospital La Fe (Valencia, Spain). All subjects gave their informed
93 consent for inclusion before they participated in the study. The study was approved on the 1st
94 of July 2013 by the Ethics Committee of CEIC Dirección General de Salud Pública y Centro
95 Superior de Investigación en Salud Pública (20130515/08). Samples were collected every
96 week during their hospitalization period. These samples were then screened for the presence
97 of MDR Enterobacteriaceae (MRE) by plating them on Brilliance ESBL Agar (Oxoid) in
98 order to quantify MRE levels and to study the impact of clinical factors and commensal
99 bacteria on MRE intestinal colonization levels (results from this study will be published
100 elsewhere). A subset of 56 patients was included in the present study. These patients had at
101 some point been colonized by an MRE strain, and one or more consecutive samples were
102 collected after the first MRE detection. Two hundred and seventeen samples representing the
103 first positive MRE sample collected during a hospital admission period, plus all consecutive
104 samples collected from this patient during this particular admission period until the MRE is
105 no longer detected, were included in this study. Samples from 2 additional patients matching
106 the criteria described above could not be included in this study since all the fecal material was
107 used in a parallel study involving microbiome analysis.

108 *Microbiological tests and molecular characterization*

109 The 217 fecal samples were screened in September 2017 for the presence of the plasmid
110 mediated colistin resistance *mcr* genes (including *mcr-1*, -2, -3, -4 and -5) by multiplex PCR
111 [8]. PCR positive samples (N=5) were cultured to isolate the colistin-resistant strains
112 harboring *mcr* genes by culture on LBJMR agar (containing 4 µg/ml colistin and 50 µg/ml
113 vancomycin) and MacConkey agar supplemented with colistin (2 µg/ml) [9]. The isolated
114 colonies were identified by matrix-assisted laser desorption ionization–time of flight mass
115 spectrometry (MALDI-TOF MS) (Microflex, Bruker Daltonics, Bremen, Germany).
116 Subsequently, the antibiotics susceptibility of the isolates was determined by evaluating the
117 minimum inhibitory concentration (MIC) using the broth microdilution method according to
118 the Clinical and Laboratory Standard Institute (CLSI) guidelines for the colistin antibiotic,
119 and using Etest method on Mueller Hinton agar for the other antibiotic families tested. In
120 addition, the ESBL (*bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M}) encoding genes were screened in the
121 colistin-resistant isolates.

122 ***Molecular epidemiology***

123 The epidemiological relationship between the colistin resistant strains was determined by
124 multilocus sequence typing (MLST). The seven housekeeping genes (*adh*, *fumC*, *gyrB*, *icd*,
125 *mdh*, *purA*, *recA*) were amplified, sequenced and then blasted on the MLST database
126 available on the Warwick web site
127 (http://enterobase.warwick.ac.uk/species/ecoli/allele_st_search).

128 ***Conjugation experiments and plasmid analysis***

129 Conjugative experiments were conducted using azide-resistant *Escherichia coli* J53 as a
130 recipient, as described [10]. The transconjugants were selected on Luria Bertani (LB) agar
131 (Beckton Dickinson, Le Pont de Claix, France) supplemented with sodium azide (120 µg/ml)
132 and colistin (2 µg/ml). Transconjugant strains were screened for the presence of the colistin

133 resistance genes (*mcr* genes) by PCR, and were subjected to antibiotic susceptibility testing as
134 described above. Plasmid typing experiments were conducted on transconjugant strains using
135 standard PCR [11].

136 **Results**

137 *Microbiological, molecular and epidemiological characterization*

138 Five of the 217 fecal samples tested were positive for the presence of the *mcr-1* gene variant.
139 The PCR product sequence showed 100% identity to the published sequence [3]. These
140 samples corresponded to three patients with acute myeloid leukemia (AML) named Patient-1
141 (two positive samples), Patient-2 (one positive sample) and Patient-3 (two positive samples)
142 aged 59, 48 and 63 years respectively (Table 1). All patients received an antimicrobial therapy
143 prior sampling, including ciprofloxacin, piperacillin, meropenem, vancomycin and
144 teicoplanin, but none had received colistin (Table 1). The patients also been subjected to
145 either chemotherapy, transplant or both. Patient-1 received two chemotherapy and one bone
146 marrow transplant prior sampling the two *mcr-1* positive samples, unlike the positive *mcr-1*
147 sample from Patient-2 was collected while the patient showed clinical signs of infection and
148 after undergoing bone marrow transplantation. During chemotherapy treatment of the Patient-
149 3, the two *mcr-1* positive samples were collected. The culture method allowed us to isolate
150 four *E. coli* strains among the five *mcr-1* positive samples; two strains from Patient-1 (*E. coli*-
151 643 and *E. coli*-648), one strain from Patient-2 (*E. coli*-866) and one strain from Patient-3 (*E.*
152 *coli*-913). The *E. coli* strains isolated were resistant to at least five antibiotics among the
153 sixteen tested, including colistin with MIC= 4 µg/ml (Table 1). The four colistin resistant *E.*
154 *coli* carried the *mcr-1* gene also harbored ESBL genes, including *bla*_{TEM-206} and *bla*_{TEM-98}
155 (Table 1).

156 According to the MLST analysis, three different sequence types (STs) were assigned to the
157 four *E. coli* isolates, including ST1196, ST140 and ST10. *E. coli*-643 and *E. coli*-648 strains
158 retrieved from Patient-1 belonged to the same sequence type, ST1196.

159 ***Conjugation experiments and plasmid analysis***

160 Conjugation experiment was conducted on the three *E. coli* harboring *mcr-1*, including *E.*
161 *coli*-643, *E. coli*-866 and *E. coli*-913. The *E. coli*-648, considered a duplicate of Patient-1 *E.*
162 *coli* strain, was not included in this experiment. Conjugative experiment allowed us to isolate
163 3 transconjugants (*E. coli* J53-643 Azide^r, *E. coli* J53-866 Azide^r and *E. coli* J53-913 Azide^r)
164 resistant to colistin with MIC= 4 µg/ml. The antibiotic susceptibility profile of these
165 transconjugant strains is presented in Table 2. All transconjugant strains were positive to the
166 *mcr-1* gene and the plasmid typing showed that this gene was located on IncP plasmid.

167 **Discussion**

168 Antibiotic resistance in immunocompromised patients was mainly represented by the Gram-
169 positive cocci group, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and
170 vancomycin-resistant *Enterococcus* (VRE). In the last decades, the emergence of infections
171 due to Gram-negative bacteria, especially Enterobacteriaceae and *Pseudomonas*, has been
172 noted [12]. The Gram-negative bacilli identified as the predominant infectious agent in
173 hematological patients is *E. coli*. In this group of patients, *E. coli* most often exhibits a high
174 rate of resistance to quinolones, ceftazidime and beta-lactam antibiotics [12]. These results
175 have been observed in our study where the *E. coli* strains, isolated from our patients with
176 acute leukemia, in addition to other antibiotics, also exhibited resistance to beta-lactam and
177 quinolones.

178 To counter the emergence of MDR bacteria in hematological patients, the use of colistin as
179 monotherapy or in combination with other antibiotics, such as beta-lactams, aminoglycosides,

180 tigecycline or fosfomycin, has been suggested by the current American and European
181 guidelines on febrile neutropenia [1]. Unfortunately, since its use in clinical settings, colistin
182 resistance has increased and this is becoming very alarming, especially since the detection of
183 a plasmid containing the *mcr-1* colistin resistance gene [6]. The result that emerged in our
184 study and which worried us was the fact that we isolated from the leukemia patients *E. coli*
185 strains resistant not only to the antibiotics mentioned above but also to the antibiotic of last
186 resort, colistin, due to the presence of the *mcr-1* gene.

187 The colistin resistance *mcr-1* gene has been reported worldwide, mainly in animals [4]. In
188 Spain, the *mcr-1* gene has also been identified in animals (poultry, pigs and swine) [13, 14], in
189 the environment (wastewater and sewage water) [15, 16] and a few studies reported the *mcr-1*
190 gene in clinical isolates (urine, blood, sputum) [4]. In patients with leukemia, the colistin-
191 resistance *mcr-1* variant has been reported in five studies over the world including China,
192 Austria and Italy. This gene was mostly detected in *E. coli* strains followed by *Klebsiella*
193 *pneumoniae* and conferred to these strains a resistance to colistin with MIC ranging from 4 to
194 8 µg/ml [17-21]. This was reported in our study, where the colistin resistance *mcr-1* gene was
195 detected in *E. coli* strains resistant to colistin with MIC= 4 µg/ml in high-risk patients with a
196 weak immune system, who are leukemia patients, in Spain.

197 Concerning the genetic support of colistin resistance, the *mcr-1* gene was generally identified
198 in different plasmid types such as IncI2, IncHI2, IncX4 and IncP [22]. In our strains, the *mcr-*
199 *1* gene was located on IncP transferable plasmid type. In Spain, the IncP plasmid type has
200 been previously reported to be associated with different resistance genes, such as carbapenem
201 resistant genes [23], but has never been associated with the *mcr-1* gene.

202 Worldwide, different sequence types of *E. coli* harboring the *mcr-1* gene have been detected
203 in leukemia patients such as ST10, ST46, ST58, ST156, ST607 and ST3944 [19-21]. ST10
204 has made a significant contribution to the dissemination of the *mcr-1* gene worldwide [22]. In

205 our study, the ST10 was reported in only one strain. ST1196 was the predominant sequence
206 type detected in our strains (2 out of 4). This ST was reported in only one study in which the
207 *mcr-I* positive *E. coli* strain was recovered from a wastewater treatment plant at West China
208 Hospital [24]. In addition, our study reports for the first time the association of *E. coli* ST140
209 with the presence of the *mcr-I* gene. Unfortunately, the lack of information on the history of
210 patients; if they have been hospitalized, if they have travelled to a high-risk country where the *mcr-I*
211 gene is endemic or if the patient has been in contact with animals, does not allow us to determine the
212 epidemiology and origin of the *mcr-I* gene detected in our study.

213 In the present study, we detected the plasmid-mediated colistin resistance *mcr-I* gene in
214 patients not treated with colistin who were hospitalized between 2013 and 2015 at La Fe
215 University Hospital. This suggests that the appearance of the *mcr-I* gene in these patients was
216 not due to selection pressure with this antibiotic. Despite the fact that our *mcr-I* positives *E.*
217 *coli* strains do not exhibit resistance to all the antibiotics tested, the detection of the *mcr-I*
218 gene on transferable plasmid is alarming. Indeed, the worrying scenarios emerging from this
219 study are the acquisition of the plasmid encoding *mcr-I* genes by MDR bacteria in these
220 leukemia patients whose immune systems are already weakened, and the transmission of these
221 resistant bacteria from one patient to another. This situation can lead the clinician into
222 therapeutic impasse. This is exactly what was reported in a study conducted by Di Pilato et al.
223 where the *mcr-I* gene was identified in an MDR KPC-producing *K. pneumoniae* ST512 [21].
224 In these cases, what measures should be taken to prevent the spread of colistin resistance in
225 this high-risk population? In our situation, the isolation of patients with *mcr* genes encoding
226 colistin resistance could be an urgent solution to avoid any risk of transmission of such genes
227 to other immunocompromised patients. In the long-term, the implementation of rapid tests to
228 detect antibiotic resistance genes and the systematic screening of the carrying of antibiotic

229 resistance genes as soon as they arrive at the hospital, would be a great solution for
230 controlling the spread of antibiotic resistance genes and thus avoid any therapeutic impasse.

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234 **Conflict of interests**

235 The authors declare they have no conflicts of interest

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323 *Microbiol* 2017;8:2094. <https://doi.org/10.3389/fmicb.2017.02094>.

324 **Table 1.** Characterization of samples and strains harboring the *mcr-1* gene identified in leukemia patients in Spain.

Patients	Samples	Sampling date	Antimicrobial therapy	Strains <i>mcr-1</i>	MIC colistin (µg/ml)	Resistant AST phenotype	ESBL genes	ST	Conjugation experiment	Plasmid Typing
Patient-1	643	06/11/2014	MEM, TZP, CIP, VAN	<i>E. coli</i> -643	4	AMX, AMC, CEF, CIP, CST, SXT, DOX	<i>bla</i> _{TEM-206}	1196	+	IncP
	648	12/11/2014	MEM, TZP, CIP, VAN	<i>E. coli</i> -648	4	AMX, AMC, CEF, CIP, CST, SXT, DOX	<i>bla</i> _{TEM-206}	1196	Not tested	Not tested
Patient-2	866	11/03/2015	MEM, TEC, TZP	<i>E. coli</i> -866	4	AMX, AMC, CST, SXT, DOX	<i>bla</i> _{TEM-206}	140	+	IncP
Patient-3	913	29/03/2015	CIP, TZP	<i>E. coli</i> -913	4	AMX, AMC, CEF, CIP, CST, DOX	<i>bla</i> _{TEM-98}	10	+	IncP
	923	01/04/2015	CIP, TZP	No bacteria isolated	/	/	/	/	/	/

325 AMX ; Amoxicillin, AMC ; Amoxicillin/clavulanic acid, CEF ; Cephalothin, CIP ; Ciprofloxacin, SXT ; Trimethoprim-sulfamethoxazole,

326 DOX ; Doxycycline, CST ; Colistin, MEM ; Meropenem, TZP ; Piperacillin-tazobactam, VAN ; Vancomycin, TEC ; Teicoplanin.

327 MIC: Minimum Inhibitory Concentration, AST: Antimicrobial Susceptibility Testing, ESBL: Extended Spectrum β-lactamase, ST: Sequence

328 Type.

329 **Table 2.** Antibiotic susceptibility of *E. coli* strains harboring *mcr-1* gene and their transconjugants^a.

Antibiotic(s) tested	Minimum Inhibitory Concentration (µg/ml)							
	<i>E. coli</i> J53	<i>E. coli</i> -643	<i>E. coli</i> J53-643 Azide ^r	<i>E. coli</i> -866	<i>E. coli</i> J53-866 Azide ^r	<i>E. coli</i> -913	<i>E. coli</i> J53-913 Azide ^r	
Amoxicillin	4 (S)	≥256 (R)	≥256 (R)	≥256 (R)	≥256 (R)	≥256 (R)	≥256 (R)	
Amoxicillin-clavulanate	3 (S)	12 (R)	8 (R)	12 (R)	8 (R)	8 (R)	3 (S)	
Piperacillin-tazobactam	1 (S)	4 (S)	1.5 (S)	0.75 (S)	0.75 (S)	2 (S)	1 (S)	
Ceftriaxone	0.047 (S)	0.047 (S)	0.023 (S)	0.032 (S)	0.016 (S)	0.064 (S)	0.047(S)	
Cefepime	0.064 (S)	0.25 (S)	0.047 (S)	0.032 (S)	0.032 (S)	0.064 (S)	0.047 (S)	
Ertapenem	0.004 (S)	0.008 (S)	0.003 (S)	0.002 (S)	0.002 (S)	0.004 (S)	0.004 (S)	
Imipenem	0.19 (S)	0.125 (S)	0.094 (S)	0.125 (S)	0.094 (S)	0.125 (S)	0.125 (S)	
Amikacin	0.5 (S)	2 (S)	1.5 (S)	1.5 (S)	1.5 (S)	2 (S)	0.5 (S)	
Ciprofloxacin	0.016 (S)	≥32 (R)	0.008(S)	0.008 (S)	0.006 (S)	≥32 (R)	0.023 (S)	
Doxycycline	0.5 (S)	24 (R)	6 (S)	8 (R)	8 (R)	12 (R)	6 (S)	
Fosfomycin	0.38 (S)	4 (S)	4 (S)	6 (S)	4 (S)	3 (S)	0.75 (S)	
Trimethoprim-sulfamethoxazole	0.012 (S)	≥32 (R)	≥32 (R)	≥32 (R)	≥32 (R)	0.012 (S)	0.012 (S)	
Colistin	0.125 (S)	4 (R)	4 (R)	4 (R)	4 (R)	4 (R)	4 (R)	

330 ^aAntibiotic susceptibility testing was performed according to EUCAST recommendations.

331 S; susceptible, R; resistant.