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

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

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Abstract

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

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

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

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

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

Introduction

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

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

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

Materials and methods

Study design

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

Microbiological tests and molecular characterization

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

Molecular epidemiology

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

Conjugation experiments and plasmid analysis

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

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

Results

Microbiological, molecular and epidemiological characterization

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

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

Conjugation experiments and plasmid analysis

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

Discussion

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

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

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

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

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

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

our study, the ST10 was reported in only one strain. ST1196 was the predominant sequence type detected in our strains (2 out of 4). This ST was reported in only one study in which the *mcr-1* positive *E. coli* strain was recovered from a wastewater treatment plant at West China Hospital [24]. In addition, our study reports for the first time the association of *E. coli* ST140 with the presence of the *mcr-1* gene. Unfortunately, the lack of information on the history of patients; if they have been hospitalized, if they have travelled to a high-risk country where the *mcr-1* gene is endemic or if the patient has been in contact with animals, does not allow us to determine the epidemiology and origin of the *mcr-1* gene detected in our study.

In the present study, we detected the plasmid-mediated colistin resistance *mcr-1* gene in patients not treated with colistin who were hospitalized between 2013 and 2015 at La Fe University Hospital. This suggests that the appearance of the *mcr-1* gene in these patients was not due to selection pressure with this antibiotic. Despite the fact that our *mcr-1* positives *E. coli* strains do not exhibit resistance to all the antibiotics tested, the detection of the *mcr-1* gene on transferable plasmid is alarming. Indeed, the worrying scenarios emerging from this study are the acquisition of the plasmid encoding *mcr-1* genes by MDR bacteria in these leukemia patients whose immune systems are already weakened, and the transmission of these resistant bacteria from one patient to another. This situation can lead the clinician into therapeutic impasse. This is exactly what was reported in a study conducted by Di Pilato et al. where the *mcr-1* gene was identified in an MDR KPC-producing *K. pneumoniae* ST512 [21]. In these cases, what measures should be taken to prevent the spread of colistin resistance in this high-risk population? In our situation, the isolation of patients with *mcr* genes encoding colistin resistance could be an urgent solution to avoid any risk of transmission of such genes to other immunocompromised patients. In the long-term, the implementation of rapid tests to 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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Conflict of interests

The authors declare they have no conflicts of interest

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

AMX ; Amoxicillin, AMC ; Amoxicillin/clavulanic acid, CEF ; Cephalothin, CIP ; Ciprofloxacin, SXT ; Trimethoprim-sulfamethoxazole, DOX ; Doxycycline, CST ; Colistin, MEM ; Meropenem, TZP ; Piperacillin-tazobactam, VAN ; Vancomycin, TEC ; Teicoplanin.

MIC: Minimum Inhibitory Concentration, AST: Antimicrobial Susceptibility Testing, ESBL: Extended Spectrum β-lactamase, ST: Sequence 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)						
	E. coli J53	*E. coli*-643	*E. coli* J53-643 Azide^r	*E. coli*-866	*E. coli* J53-866 Azide^r	*E. coli*-913	*E. coli* 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 [a]Antibiotic susceptibility testing was performed according to EUCAST recommendations.

331 S; susceptible, R; resistant.