Colistin-resistant microorganisms and cystic fibrosis: microbiological surveillance in an Italian Children's Hospital

Anna Lisa Montemari,[1] Vanessa Tuccio Guarna Assanti,[1] Giulia Linardos,[1] Giovanni Di Bonaventura,[2] Gabriella Ricciotti,[1] Ersilia Vita Fiscarelli[1]

[1]Cystic Fibrosis Microbiology Laboratory, Department of Laboratories, Bambino Gesù Children's Hospital, IRCCS, Rome; [2]Department of Medical, Oral and Biotechnological Sciences; Centre of Excellence on Ageing and Translational Medicine, "G. d'Annunzio" University of Chieti-Pescara, Chieti, Italy

Summary

Several advances in the medical field are often dependent on the ability to fight infections with the use of antibiotics, including joint replacements, organ transplants, and cancer therapy. The capacity of the bacteria to adapt to and escape from the mechanisms of action of antibiotics makes the antimicrobial resistance a serious public health problem worldwide. Polymyxin E colistin has rarely been used because of its nephrotoxicity and neurotoxicity. More recently, the emergence of multi-drug resistant bacteria as carbapenem-resistant *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* and the re-evaluation of its pharmacokinetic properties have led to a resurgence of colistin as a treatment option, contributing to select resistant strains. Investigating the phenomenon of colistin-resistance in gram-negative bacteria, especially *P. aeruginosa*, is now mandatory, particularly after identification of a plasmid-mediated mechanism for the resistance to colistin (*mcr*) in *Enterobacteriaceae* strains, a mechanism transferable to other species. In this study, we investigated colistin-resistance in gram-negative bacteria isolated from respiratory secretions of cystic fibrosis patients in follow-up at Children's Hospital Bambino Gesù of Rome.

Correspondence: Ersilia Vita Fiscarelli, Children's Hospital Bambino Gesù IRCCS, Piazza Sant'Onofrio, 4 00165, Rome, Italy.
Tel.: +39.06.68592844.
E-mail: evita.fiscarelli@opbg.net

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Introduction

Colistin, also known as polymyxin E, belongs to the group of antibiotic polypeptides derived from *Bacillus polymyxa*. Cationic colistin polypeptide interacts with lipopolysaccharide (LPS) of gram-negative bacteria by shifting the calcium and magnesium ions destabilizing the membrane with a permeability increase (18). Commercialized in the fifties, colistin has rarely been used because of its nephrotoxicity and neurotoxicity; however, the emergence of the antibiotic-resistance and the re-evaluation of its pharmacokinetic properties have recently encouraged the reintegration into clinical practice. Colistin is effective in treating infections caused by carbapenem-resistant *Escherichia coli* and *Klebsiella spp.* (5,15), and Multi-Drug Resistant (MDR) *Acinetobacter baumanni*. It is also commonly used for therapy, including aerosol, in patients with cystic fibrosis (CF) and chronic infection by *Pseudomonas aeruginosa* (20). However, immediately after its re-use, bacteria have developed resistance.

Classical colistin resistance mechanisms include both biochemical and genetic mechanisms. The most frequent is a modification of LPS for the attachment of the 4-amino-4-deoxy-1-arabinose (Lara4N) or the phosphoethanolamine (PEtN) to a phosphate group in the lipid A; this attachment induce an increased net charge leading to a reduction of colistin affinity for polymyxins. The activation of the sensor kinases PhoQ and PmrB under specific stress conditions triggers a phosphorilation of cytoplasmatic regulators PhoP and PmrA which regulate the expression of *arnBCADTEF* and *pmrCAB* genes modulating LPS modifications by Lara4N and PEtN. Loss of LPS for mutations in the *IpxA*, *IpxC* and *IpxD* genes, single mutation in the PmrB protein or involvement of OprH, a membrane protein which contributes to the stability of the cellular shell, are other known mechanisms of resistance to colistin, as well as the presence of a capsule, efflux pumps

expressions and changes in the regulatory loci *pmrA* and *phoP* (3,28). The heteroresistance, namely the resistance to an antimicrobial expressed by a subpopulation in a susceptible population, selects numerous colistin-resistant bacteria.

In 2016, a new mechanism has been discovered in China, where for the first time was described in *E. coli* a plasmid-mediated mechanism for colistin-resistance, called mcr-1 (22). The *mcr-1* gene encoding for a phosphoethanolamine-transferase is capable of modifying the portion of lipid A of LPS, resulting in the inhibition of the polymyxin's binding to the membrane.

Since the first description, new identifications occurred in many countries, including Malaysia (44), Thailand (12), Vietnam (35), Japan (36), USA (21), as well as in several European countries (8,13). The worldwide spread of *mcr-1* represents a great concern because the plasmid allows horizontal transmission of the resistance gene and, therefore, a transferable resistance among species. Its presence, in fact, has been detected also in *Klebsiella pneumoniae* (33), *Acinetobacter* spp.(40) and *P. aeruginosa* (32).

Colistin is used not only in the human field but also in veterinary medicine and its wide use in some countries facilitated the selection of resistant strains and the transmission from animals and environments to humans (29). A Chinese study, investigating the spreading of antimicrobial resistant isolates in a high-density transit area, highlighted that public transportation is a reservoir of *mcr-1* positive bacteria (31). In another study, also carried out in China in 2017, it was found that 34.1% of *Musca domestica* and 51.1% of *Protophormia terraenovae* homogenates are positive for *mcr-1*. (45), thus suggesting that these insects could play a role in the dissemination of plasmid in various ecological niches (16,26). It has recently been highlighted that *mcr-1* is associated with other resistance determinants, *e.g.* NDM-5 in the United States (25), and that this would make the situation even more critical.

In Italy, the first case of *E. coli mcr-1* positive strain was described in 2016 in a study in which the authors carried out a screening of colistin-resistant isolates showing that eight out of nine *E. coli* strains tested, were *mcr-1* positive (6). In a study presented at the ECCMID in Vienna 2017, performed by the University of Palermo, the authors demonstrated a prevalence of 4.1% of *mcr-1* positive isolates on meat for human consumption (17). In the period August 2016-January 2017, three cases of sepsis by *mcr-1* positive bacteria have been described in the Pavia hospital (11). It should be underlined that the three patients did not present any risk factor or relationship, thus suggesting that the presence of *mcr-1* is probably underestimated in Italy.

CF is an autosomal recessive genetic disorder caused by mutations in the trans-membrane conductance regulator (CFTR) gene. Because of the functionally altered protein, the disease is characterized by the formation of thick and viscous mucus in the respiratory tract with a decreased mucociliary clearance and an environment favourable to bacterial colonization. CF airways host pathogen and non-pathogen bacteria resulting in acute or chronic infections. The vicious circle of infections, inflammatory response, overproduction of cytokines and increased bronchial secretions are at the basis of CF lung damage (9,14).

The spectrum of bacterial pathogens colonizing the respiratory tract of CF patients is quite well known. *Staphylococcus aureus* and *Haemophilus influenzae* are frequent during the first decade of life. *Enterobacteriaceae* like *E. coli*, and *K. pneumoniae* can chronically colonize children's airway; however, their clinical role in the lung disease has still to be defined. *P. aeruginosa* is a key pathogen especially in older patients since more than 70% of CF patients aged ≥18 years are colonized by this bacterium (19,20,24,46). Chronic infection by *P. aeruginosa* is the main cause of the lung function decline and mortality among CF patients (37). The high frequency of infectious events, whose resolution requires increasingly intense treatments with antimicrobials, results in the emergence of bacterial strains resistant to most of the antibiotics used in clinical practice.

Colistin is an invaluable antibiotic in CF and it is mostly used against *P. aeruginosa* infections, both intravenously for pulmonary exacerbations and aerosolized for chronic maintenance (34). Therefore, the emergence of colistin resistance mediated by transferable elements, such as *mcr-1*, is particularly alarming in this setting. In 2016, the European Centre for Disease Prevention and Control (ECDC) strongly encouraged to reduce the risk of colonization and infection by mcr-1 positive bacteria suggesting the "One Health Approach" (an integrated surveillance among hospitals, communities, veterinary, environment and food control). It also prompted the laboratories to develop methods for the *mcr-1* gene detection and monitor possible reservoirs of infection. (See: ECDC rapid risk assessment outlines actions to reduce the spread of the *mcr-1* gene, 2016).

Since CF lung may be a reservoir of colistin-resistant gram-negative bacteria and *mcr-1* positive strains, the aim of the present study was to evaluate the rate of colistin resistance and the diffusion of *mcr-1* gene in CF context. To that end, colistin resistance was assessed by molecular testing in *Enterobacteriaceae* (*E. coli*, *K. pneumoniae*) and *P. aeruginosa* isolated from the respiratory samples of CF patients followed at a pediatric hospital in the period March 2017- February 2018.

Materials and Methods

Bacterial isolates

Colistin-resistant gram-negative bacterial isolates were collected from respiratory samples from CF patients in follow-up at the CF Centre of Children's Hospital Bambino Gesù of Rome that takes care of about 350 patients aged from 0 to 57 years old. Bacterial identification was performed, in accordance with internal procedures, by MALDI-TOF MS (Bruker Daltonics Inc. Billerica, MA USA).

In vitro susceptibility testing

Susceptibility to colistin was evaluated, in accordance with EUCAST recommendations, by broth dilution method (SensiTest Colistin, Liofilchem, Roseto degli Abruzzi, Italy). Results were interpreted according to the new MIC breakpoints recently proposed by EUCAST: MIC values ≤2 µg/mL and >2 µg/mL categorized the isolates as sensitive or resistant, respectively. An isolate was also classified as (23): i) multidrug-resistant (MDR), if resistant to at least 1 agent in ≥3 antibiotic classes; ii) extensively drug-resistant (XDR), if non-susceptible to at least 1 agent in all but ≤2 antibiotic classes; iii) pandrug-resistant (PDR), if resistant to all agents in all antimicrobial classes.

Molecular tests

DNA was extracted from bacterial colonies using magnetic particles (EZ1 DNA Extraction Mini Kit, and Biorobot EZ-1 workstation, Qiagen, Germany), according to the manufacturer's instructions, and then stored at -80°C until analysis. MICROSEQ 500 16 S rDNA bact. Seq. (Life Technologies, Italy) was used to check the outcome of the DNA extraction. PCR was performed to detect *mcr-1* gene using primers (forward: 5'CGGTCAGTCCGTTTGTTC'3; reverse: 5'CTTGGTCGGTCTGTAGGG'3) and conditions previously described (22). PCR products were run using Flash gels (Lonza, USA) considering *mcr-1* positive samples those

showing a band at 309 bps. *P. aeruginosa* ATCC27853 and *E. coli* ATCC13846 were used as negative control, whereas a *mcr-1* positive *E. coli* strain, kindly donated by Istituto Zooprofilattico Sperimentale Lazio e Toscana, was used as positive control.

Results

A total of 47, consecutive, non-duplicated colistin-resistant gram-negative bacterial isolates from respiratory samples of 39 CF patients were screened: 42 (89.4%) *P. aeruginosa*, 4 (8.5%) *K. pneumoniae* and one (2.1%) *E. coli.*

Results from antibiotic susceptibility testing are summarized in Table 1. Among 47 colistin-resistant isolates, 13 (27.6%) were classified as MDR (10 *P. aeruginosa*, 2 *K. pneumoniae*, one *E. coli*), 9 (19.1%) as XDR (7 *P. aeruginosa*; 2 *K. pneumoniae*), and 9 (19.1%) were PDR (all *P. aeruginosa*).

P. aeruginosa isolates showed MIC values ranging from 4 to ≥16 µg/mL; we found MIC values equal to 4 µg/mL in 13 isolates, and MIC values equal to or greater than 8 µg/mL (low- and high-level colistin resistance, respectively) in 29 isolates. *E. coli* and *K. pneumoniae* isolates showed a colistin MIC equal to 8 µg/mL and ≥16 µg/mL, respectively.

No correlation was observed between MIC values and resistance profiles (MDR, XDR, PDR) (Table 2).

None of the 47 isolates resulted to be positive for the *mcr-1* gene (Figure 1).

Discussion

The antibiotic colistin, rarely used in the past, today represents one of the last resort agents to combat multi- or even extensively-drug-resistant bacteria, as carbapenem-resistant *Enterobacteriaceae*, *A. baumannii* and *P. aeruginosa*. Unfortunately, colistin-resistant gram-negative bacterial strains have been already detected in several clinical contexts, especially those with a high intensity of care – namely Intensive Care and Complex Chronic Diseases Units - complicating the treatment of multiple life-threating conditions.

Colistin is used in CF patients chronically colonized by *P. aeruginosa* both intravenously to treat pulmonary exacerbations and nebulized for maintenance therapies.

In our CF Centre, out of 163 patients colonized by *P. aeruginosa* (Report 2017), 68 (41.7%), receive colistimethate dry powder for inhalation twice daily. The aerosol administration of antibiotics confers the advantage of reaching high concentrations of the drug in the lung, reducing systemic exposure and toxicity. Among patients treated by inhaled colistin in our CF Centre, 39 subjects (57.3%) developed resistance toward this agent. Even if some studies have demonstrated that the risk of resistance occurrences by inhalatory therapies are less common (10,30), we cannot exclude that the high prevalence of colistin-resistant *P. aeruginosa* isolates we observed could be due to the chronic and intensive usage of colistin.

Recently, EUCAST has coined the term "Area of Technical Uncertainty" (ATU) to warn microbiologists about uncertain interpretation of antimicrobial susceptibility testing results. The ATU is defined by one MIC-value in an area where evaluation and reproducibility are difficult to achieve. For *P. aeruginosa* and colistin the ATU is 4 µg/mL (www.eucast.org). Among the bacterial isolates belonging to our collection, 13 *P. aeruginosa* isolates showed a MIC equal to 4 µg/mL. EUCAST didn't set mandatory actions to perform in this scenario. In our practice, we report the results as resistant ("R") and take the opportunity to discuss the case with clinical colleagues during the daily meeting.

It should be noted that MIC reflects a serum achievable measurement of resistance that may not be applicable to the inhaled route of administration. Pharmacokinetics studies have shown high concentrations of colistin in the lung of mechanically ventilated critically ill patients undergoing inhaled colistin therapy (2). Particularly, colistin concentrations in the epithelial lining fluid were always higher than the MIC value and five-fold higher than those in serum. Therefore, we are reassured that, especially for the maintenance therapies, a MIC value of 4 µg/mL should not be a big concern in our context. In fact, the colistin concentration in the airways during inhaled therapies should be higher than 4 µg/mL leading to the consideration that ATU values should not have a strong impact in clinical outcomes. Anyway, we still maintain close communication with the clinicians in these cases.

Finally, the evidence that, even presenting a high percentage of MDR, XDR or PDR strains, none of our patients has not yet been affected by a gram-negative isolate carrier of *mcr-1* gene confirms

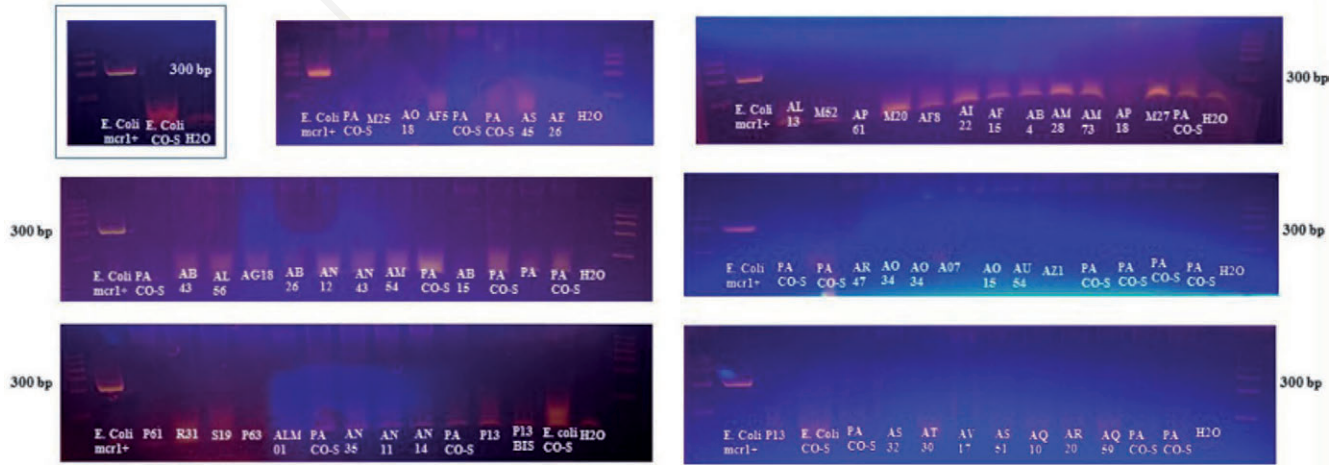

Figure 1. PCR amplification products for mcr-1 gene of colistin resistant Gram-negatives strains isolated from respiratory secretions of CF patients. PA CO-S=colistin-susceptible *Pseudomonas aeruginosa*; *E. coli* CO-S=colistin-susceptible *Escherichia coli*.

Table 1. Phenotypic and genotypic characteristics of the bacterial isolates studied.

Isolates ID	Bacterial species	Sample	Patient age (yrs)	Colony and resistance phenotypes	Colistin MIC (µg/mL)	Resistance phenotype
AZ1	*P. aeruginosa*	Tracheal aspirate	8	Small Colony Variant	4	Carbapenem
AL13	*P. aeruginosa*	Tracheal aspirate	14	Round	4	Fluoroquinolone; Aminoglycoside
M25	*P. aeruginosa*	Sputum	16	Mucoid	4	Carbapenem
AB15	*P. aeruginosa*	Tracheal aspirate	19		4	PDR
AG18	*P. aeruginosa*	Tracheal aspirate	17		4	Carbapenem
M20	*P. aeruginosa*	Tracheal aspirate	7		4	
AP61	*P. aeruginosa*	Tracheal aspirate	13		4	
AN11	*P. aeruginosa*	Tracheal aspirate	1		4	Carbapenem
AN43	*P. aeruginosa*	Sputum	12		4	
AL56	*P. aeruginosa*	Sputum	20		4	PDR
AP18	*P. aeruginosa*	Sputum	17		4	Carbapenem
AB4	*P. aeruginosa*	Sputum	29	Small Colony Variant	4	Fluoroquinolone; Aminoglycoside
M52	*P. aeruginosa*	Sputum	28		4	PDR
AM73	*P. aeruginosa*	Sputum	27		8	MDR
AM54	*P. aeruginosa*	Sputum	15		8	PDR
AM28	*P. aeruginosa*	Sputum	31	Fringed	8	Carbapenem
AO15	*P. aeruginosa*	Tracheal aspirate	15	Small Colony Variant	>8	PDR
AB43	*P. aeruginosa*	Sputum	12		>8	Carbapenem
AN35	*P. aeruginosa*	Sputum	27	Small Colony Variant	>8	Fluoroquinolone
M27	*P. aeruginosa*	Sputum	30	Fringed	>8	MDR
AN14	*P. aeruginosa*	Sputum	25	Smooth	>8	PDR
AF8	*P. aeruginosa*	Sputum	26	Round	>8	XDR
AB26	*P. aeruginosa*	Sputum	29		>8	XDR
AN12	*P. aeruginosa*	Sputum	12	Smooth	>8	MDR
AI22	*P. aeruginosa*	Sputum	16	Small Colony Variant	>8	
AF15	*P. aeruginosa*	Sputum	39		>8	MDR
AF5	*P. aeruginosa*	Sputum	34		>8	
AE26	*P. aeruginosa*	Sputum	37	Small Colony Variant	>8	XDR
A018	*P. aeruginosa*	Sputum	28	Mucoid	≥8	MDR
AQ59	*P. aeruginosa*	Sputum	29		≥16	PDR
AR47	*P. aeruginosa*	Sputum	20	Round	≥16	MDR
AO34	*P. aeruginosa*	Sputum	15		≥16	PDR
AO7	*P. aeruginosa*	Sputum	13		≥16	MDR
AS51	*P. aeruginosa*	Sputum	16		≥16	PDR
AQ10	*P. aeruginosa*	Sputum	32		≥16	XDR
ALM01	*P. aeruginosa*	Sputum	35		≥16	MDR
AR20	*P. aeruginosa*	Sputum	28		≥16	MDR
AS45	*P. aeruginosa*	Sputum	27		≥16	XDR
AU54	*P. aeruginosa*	Sputum	21	Small Colony Variant	≥16	XDR
AT30	*P. aeruginosa*	Sputum	16	Small Colony Variant	≥16	XDR
AV17	*P. aeruginosa*	Sputum	14	Small Colony Variant	≥16	MDR
AS32	*P. aeruginosa*	Sputum	16	Mucoid	≥16	Carbapenem
P13	*E. coli*	Tracheal aspirate	16	ESBL+	8	XDR
P61	*K. pneumoniae*	Tracheal aspirate	1	ESBL+	≥16	MDR
S19	*K. pneumoniae*	Tracheal aspirate	1	ESBL- HODGE– MBL+	≥16	XDR
R31	*K. pneumoniae*	Tracheal aspirate	1	ESBL+	≥16	XDR
P63	*K. pneumoniae*	Pharyngeal swab	1	KPC+	≥16	MDR

MIC, Minimum inhibitory concentration.

Table 2. Minimum inhibitory concentration (MIC) value and category of resistance for *P. aeruginosa* isolates.

MIC	MDR	XDR	PDR
4 µg/mL	0	0	3
≥8 µg/mL	5	3	3
≥16 µg/mL	5	4	3

the effectiveness of prevention and control measures for cross-infection implemented in our CF Centre. The segregation policy adopted by most of CF centres, as already known, is a winning strategy for reducing the incidence of hospital-acquired infections.

Subsequently to the first report on *mcr-1* gene in 2015 in China, in July 2016 a new plasmid-mediated gene was identified in Belgium in a colistin-resistant *E. coli* strain porcine that was negative for *mcr-1*. The gene, named *mcr-2*, is a phosphoethanolamine-transferase found in the IncX4 plasmid, which seems to have a common acquisition pattern with the *mcr-1*. It shows 76.7% homology with *mcr-1* and in Belgium its prevalence in colistin-resistant *E. coli* resulted to be higher than that of *mcr-1* (41).

After the identification of further determinants of resistance to colistin, named *mcr-3* and *mcr-4*, in September 2017 a German study identified an additional phosphoethanolamine-transferase associated transposone, named *mcr-5*, which confers resistance to colistin in *Salmonella paratyphi* B d Ta+ strains (4,7,43). Additionally, *mcr-6/7/8* have been recently identified (1,38,42).

Conclusions

In the light of these new reports, although our results provided evidence about *mcr-1* gene absence we cannot have information about other genetic determinants *mcr-2/3/4/5/6/7/8* (27).

In conclusion, if from one hand results arose from this survey reassure us, on the other they strengthen the need to follow the same path for the other determinants of resistance.

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