Polish Journal of Microbiology
2021, Vol. 70, No 2, 283–288
https://doi.org/10.33073/pjm-2021-021

SHORT COMMUNICATION

Occurrence of Beta-Lactamases in Colistin-Resistant Enterobacterales Strains in Poland – a Pilot Study

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Submitted 19 February 2021, revised 6 May 2021, accepted 10 May 2021

Abstract

Sixty-five colistin-resistant Enterobacterales isolates recovered from different clinical specimens were analyzed. The strains were collected in 12 hospitals all over Poland within a period of nine months. Strains were analyzed for eight genes from the *mcr* family. The presence of *mcr*-1 gene was detected in three *Escherichia coli* strains. The 45/65 isolates were identified as ESBL producers. CTX-M-1-like enzymes were the most common ESBLs (n = 40). One *E. coli* and seven *Klebsiella pneumoniae* strains produced carbapenemases, with the NDM being produced by five isolates. Among all the strains tested, four and five were resistant to new drugs meropenem/vaborbactam and ceftazidime/avibactam, respectively.

K e y w o r d s: Enterobacterales, colistin-resistance, ESBLs, carbapenemases

The number of bacterial isolates extremely resistant to previously effective drugs is growing dynamically. Infections are increasingly being caused by pathogens that are not susceptible to all available antibiotics. This issue is particularly acute for Gram-negative bacilli, both the Enterobacterales strains and non-fermenting rods. Colistin is used as one of the last available treatment options for patients with severe infections caused by carbapenem-resistant Gram-negative rods. Due to the increasing role of colistin in the treatment of human infections caused by multidrug-resistant (MDR) bacteria, the resistance to this antibiotic ought to be monitored (Prim et al. 2017; Petrosillo et al. 2019; Stefaniuk and Tyski 2019).

Until recently, colistin resistance was thought to be dependent only on mutations in the genes regulating LPS synthesis. In 2015, the plasmid-coded colistin resistance associated with the presence of *mcr* genes was first described (Liu et al. 2016). Since then, there have been many reports about plasmid resistance to colistin among strains isolated from human infections (Kluytmans 2017; Elbediwi et al. 2019). In Poland, the first *Escherichia coli* strain with the *mcr*-1 gene was described in 2016 (Izdebski et al. 2016). However, we do not have more information about the presence of *mcr* genes in Poland. As β-lactam antibiotics are "first-line" drugs in the treatment of infections caused by Enterobacterales, the susceptibility of strains to this group of antimicrobial agents was tested; the most important resistance mechanism to this group of drugs is the production of β-lactamases. This study aimed to determine the occurrence of β-lactamases, including carbapenemases, in colistin-resistant Enterobacterales strains in Poland. Such strains are extremely dangerous because of treatment difficulties. Recently, new β-lactam/β-lactamase inhibitor combinations have been introduced into therapy, especially for ESBLs and carbapenemase-producing strains. We have also tested all collected strains against these new drugs as a possible alternative treatment.

The twelve hospitals located all over Poland, in the following voivodeships: Lesser Poland (n = 2), Lublin

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(n = 1), Masovian (n = 2), Pomeranian (n = 1), Silesian (n = 2), Warmia-Masurian (n = 2), and West Pomeranian (n = 2), involved in this study were of similar sizes and had similar profiles, as regional, secondary-care medical centers, with all major types of wards. A total of 65 non-duplicate clinical isolates of Enterobacterales were recovered from inpatients with various infections between April 2019 and December 2019 and were included in this study. The strains were of the following species: *Klebsiella pneumoniae* (n = 45; 69.2%), *E. coli* (n = 15; 23.1%), *Enterobacter cloacae* (n = 3; 4.0%), and *Klebsiella oxytoca* (n = 2; 3.1%). All bacterial strains were identified to the species level in local hospital laboratories, and their susceptibility to antibiotics was determined using available methods. The strains were sent to the Department of Microbiology and Antibiotics of the National Medicines Institute (NMI) in Warsaw, Poland, together with basic clinical information (the date of isolation, the species, the specimen type, the patient's age and sex, and the hospitalization ward). The detailed analysis of patient's demographic data and local antibiotics susceptibility data was performed in the NMI. The strains used in the study were stored at –80°C. Before the investigation, strains were transferred onto the non-selective blood-containing agar (BAP; Columbia Agar with 5% Sheep Blood; Becton Dickinson, USA). All strains were re-identified by using ID GN cards in VITEK 2 Compact (bioMérieux, Marcy l'Etoile, France).

Based on the information provided by the laboratories, the results of antibiotic susceptibility of the studied bacterial strains were pre-analyzed. In the NMI, the colistin MIC value (mg/l) was determined by a reference broth microdilution method according to ISO 20776 (ISO 2019). Susceptibility to colistin was performed in triplicate for each strain, using the same culture to establish a pool of strains with MIC > 2 mg/l of colistin. E-tests with concentration gradients of ceftazidime, ceftazidime/avibactam, imipenem, meropenem, and meropenem/vaborbactam (MIC Strep; Liofilchem, Italy) were used for determination of their MICs (mg/l) in colistin-resistant Enterobacterales strains. Susceptibility results were interpreted according to the guidelines of the EUCAST (EUCAST 2020a). The following strains: *E. coli* ATCC 25922, *E. coli* ATCC 35218, *E. coli* NCTC 13846 (*mcr*-1), and *K. pneumoniae* ATCC 700603 were used as controls (EUCAST 2020b).

All Enterobacterales isolates were tested for ESBLs and carbapenemases production by phenotypic and genotypic methods. ESBLs were detected by the double-disk synergy (DDS) test with disks containing amoxicillin with clavulanate (20 μg and 10 μg, respectively), cefotaxime (30 μg), and ceftazidime (30 μg) (EUCAST 2017). The detection of carbapenemases were assessed by the disk test with phenylboronic acid for KPCs, the synergy test with EDTA for MBLs, and disc with temocillin for OXA-48-like carbapenemases (Żabicka et al. 2015).

Total bacterial DNA was purified with a Genomic DNA Prep Plus kit (A&A Biotechnology, Gdańsk, Poland).

The $bla_{CTX\text{-}M\text{-}1}$-, $bla_{CTX\text{-}M\text{-}2}$-, $bla_{CTX\text{-}M\text{-}8}$- $bla_{CTX\text{-}M\text{-}9}$-, $bla_{CTX\text{-}M\text{-}25}$-, bla_{SHV}-, bla_{TEM}- bla_{KPC}-, bla_{NDM}-, bla_{IMP}-, bla_{VIM}-, $bla_{OXA48\text{-}like}$ genes were identified by PCR as described previously (Woodford et al. 2006; Empel et al. 2008).

All isolates were screened by PCR for the presence of plasmid-mediated *mcr* genes, including *mcr*-1 (Liu et al. 2016), *mcr*-2 (Xavier et al. 2016), *mcr*-3 (Yin et al. 2017), *mcr*-4 (Rebelo et al. 2018), *mcr*-5 (Borowiak et al. 2017), *mcr*-7 (Wang et al. 2018), *mcr*-8 (Yuan et al. 2019) and *mcr*-9 (Carroll et al. 2019), as previously described.

The isolates came from patients of various ages from 1 to 89 years; the most numerous group comprised of patients aged 61–80 (n = 32; 49.2%) and 31–60 years of age (n = 16; 24.6%). The remaining patients were 16–30 years of age (n = 4), ≥ 81 years of age (n = 11), and < 3.1 years (n = 2). The most frequently represented hospital wards were: Intensive Care Unit (n = 20, 30.8%), internal medicine (n = 14, 21.5%), pulmonary (n = 12; 18.5%), and burn wards (n = 8; 12.3%). The remaining patients were hospitalized in the following order: surgery (n = 3), rehabilitation (n = 3), urology (n = 1), and oncology (n = 1). Three patients from whom the tested strains were isolated were patients of the surgical outpatient clinics (n = 2) and one resident of the Long Term Care Facility with documented hospital history.

Just over 40% of all patients' clinical specimens (n = 28; 43.1%) for microbiological testing came from the lower respiratory tract, including: bronchial lavage (n = 16; 24.6%), and sputum (n = 11; 16%), pleural fluid (n = 1), specimens from skin and soft tissue infections (n = 8, 12.3%), and urine (n = 13, 20%). Only 16.9% (n = 11) of the Enterobacterales isolates tested were collected from blood; single isolates came from peritoneal fluid (n = 1), bile (n = 1), and rectal swabs (n = 3). *K. pneumoniae* was the dominant organism in lower respiratory tract infections, followed by *E. coli*. *K. pneumoniae* caused nearly half of the cases of urinary tract infections (UTIs). In seven cases, *K. pneumoniae* (10.8%) was the pathogen isolated from blood.

Resistance to colistin was demonstrated in all 65 isolates. The MIC values of colistin in resistant strains ranged from 4 mg/l to > 64 mg/l. For PCR, positive results were achieved only with primers specific to the *mcr*-1 gene variant in three *E. coli* strains. One was simultaneously resistant to imipenem (MIC = 12 mg/l) and intermediate to meropenem (MIC = 4 mg/l). It was also resistant to ceftazidime/avibactam with an MIC of 32 mg/l, but sensitive to meropenem/avibactam

Table I
Susceptibility of colistin-resistant Enterobacterales strains (n = 65) to ceftazidime, ceftazidime/avibactam, imipenem, meropenem, and meropenem/vaborbactam.

Strains (n; %)	Colistin		*mcr*-1	CAZ		CAZ/AVB		IPM			MEM			MEM/VB	
	MIC (mg/l)			S	R	S	R	S	I	R	S	I	R	S	R
	Value	Numer of isolates													
K. pneumoniae (n = 45; 69.2%)	4	11	0	0	11	9	2	9	0	2	9	1	1	10	1
	8	4	0	4	0	4	0	4	0	0	4	0	0	4	0
	16	2	0	0	2	1	0	2	0	0	2	0	0	2	0
	32	1	0	1	0	1	1	1	0	0	1	0	0	1	0
	64	1	0	1	0	1	0	1	0	0	1	0	0	1	0
	> 64	26	0	14	12	24	2	19	1	6	19	2	5	23	3
E. coli (n = 15; 23.1%)	4	4	0	2	2	4	0	4	0	0	4	0	0	4	0
	8	5	0	3	2	5	0	5	0	0	5	0	0	5	0
	16	3	1	0	3	3	0	3	0	0	3	0	0	3	0
	32	1	1	0	1	0	1	0	0	1	0	1	0	1	0
	64	1	1	0	1	1	0	1	0	0	1	0	0	1	0
	> 64	1	0	0	1	1	0	0	1	0	0	1	0	1	0
E. cloacae complex (n = 3; 4.6%)	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	8	1	0	0	1	1	0	1	0	0	1	0	0	1	0
	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	32	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	64	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	> 64	2	0	1	1	2	0	0	1	1	1	1	1	2	0
K. oxytoca (n = 2; 3.1%)	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	16	1	0	1	0	1	0	1	0	0	1	0	0	1	0
	32	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	64	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	> 64	1	0	1	0	1	0	1	0	0	1	0	0	1	0
Total		65	3	28	37	59	6	52	3	10	53	5	7	61	4

CAZ – ceftazidime, CAZ/AVB – ceftazidime/avibactam, IPM – imipenem, MEM – meropenem, MEM/VB – meropenem/vaborbactam, S – sensitive, I – intermediate, R –resistant

(MIC = 2 mg/l). Twelve isolates from all 65 strains showed elevated MIC values of imipenem and/or meropenem from 2 mg/l to ≥ 256 mg/l: *E. coli* (n = 2), *E. cloacae* (n = 1), and *K. pneumoniae* (n = 9). Four of these strains were resistant to meropenem/vaborbactam, and five to ceftazidime/avibactam. Detailed results of susceptibility testing are presented in Table I.

In Kazmierczak and co-researcher's study (2018) the most common ESBL genes in Polish isolates was CTX-M-15 (80% of 185 ESBL-positive isolates). Authors also observed high percentages of MDR Polish strains (21%); 29.2% of them were ceftazidime-resistant and 0.8% meropenem non-susceptible, but only one isolate produced carbapenemase and it belonged to carbapenemase subtype VIM-1. A higher percentage of Enterobacterales strains resistant to ceftazidime (56.9%) and non-susceptible to meropenem (16.9%) was observed in our study.

Forty-five of the colistin-resistant isolates (69.2%) were identified as ESBL producers by the DDS test. The ESBL-positive strains belonged to three species including *E. cloacae* complex (n = 2, 4.4%), *E. coli* (n = 6, 13.3%), and *K. pneumoniae* (n = 37, 82.2%). Thirty-eight ESBL-positive isolates (84.4%) carried only one β-lactamase gene. The remaining seven strains possessed 2–4 *bla* genes. Forty-one ESBL-positive isolates (91.1% from 45 isolates) carried $bla_{\text{CTX-M-1-like}}$ genes; the most frequent organism was *K. pneumoniae* (n = 34), from which 64.7% of isolates demonstrated a colistin MIC > 64 mg/l. The $bla_{\text{CTX-M-9-like}}$ genes were detected only in two *K. pneumoniae.* Five isolates carried $bla_{\text{SHV-like}}$, and 10 carried $bla_{\text{TEM-like}}$ genes.

One *E. coli* and seven *K. pneumoniae* colistin-resistant isolates produced carbapenemases. Carbapenemase-encoding genes were detected as follows: bla_{KPC} in one *K. pneumoniae*, bla_{NDM} in five *K. pneumoniae,*

Table II
Presence of selected ESBLs and carbapenemases among colistin-resistant Enterobacterales strains (n = 65*).

Strains (n; %)	Colistin			Types of ESBLs			Types of carbapenemases		
	MIC (mg/l)	*mcr*-1	CTX-M-1	CTX-M-9	TEM	SHV	KPC	NDM	OXA-48
	Value	Number of isolates							
K. pneumoniae (n = 45; 69.2%)	4	11	0	6	1	2	0	0	2	0
	8	4	0	4	0	0	0	0	0	0
	16	2	0	0	0	1	0	0	0	0
	32	1	0	1	0	0	0	0	0	0
	64	1	0	1	0	0	0	0	0	0
	>64	26	0	22	1	3	4	1	3	1
E. coli (n = 15; 23.1%)	4	4	0	1	0	1	0	0	0	0
	8	5	0	3	0	0	0	0	0	0
	16	3	1	1	0	1	1	0	0	1
	64	1	1	0	0	1	0	0	0	0
E. cloacae complex (n = 3; 4.6%)	8	1	0	1	0	0	0	0	0	0
	>64	2	0	1	0	1	0	0	0	0
Total		61	3	41	2	10	5	1	5	2

* – in two *E. coli* and two *K. oxytoca* strains (3.1%) resistant to colistin, the β-lactamases were not detected

and $bla_{\text{OXA-48-like}}$ in one *E. coli* (the carbapenem-resistant isolate with *mcr*-1 gene) and one *K. pneumoniae*. Five strains carrying *bla* genes and producing carbapenemases showed MIC > 64 mg/l of colistin, which indicated their clinical significance.

The results of the detection of selected β-lactamases are shown in Table II.

The growing resistance of bacteria to antibiotics is a challenge for 21st-century medicine. Carbapenems were considered so-called "last resort" agents in the treatment of serious infections, especially in hospitalized patients. The spread of carbapenem-resistant Gram-negative rods isolated from outpatients turned out to be a challenge for treating infections (Grundmann et al. 2010; Parisi et al. 2015). The expansion of strains producing carbapenemases has been observed for several years worldwide, including in Poland (Baraniak et al. 2016). Numerous reports have indicated the disturbing phenomenon of large-scale spreading of Enterobacterales strains producing New Delhi metallo-β-carbapenemase, and to a lesser extent producing *Klebsiella pneumoniae* carbapenemase, or OXA-48-carbapenemases and VIM-carbapenemases. Most of the carbapenemases producing strains are multi-drug-resistant (MDR) strains, which significantly limit the therapeutic possibilities of life-threatening infections. Due to the frequent lack of therapeutic options for carbapenem-resistant strains infections, colistin is considered one of the few or sometimes only therapeutic options (Li et al. 2006; Nation and Li 2009; Lim et al. 2010; Sandri et al. 2013; Vicari et al. 2013). The coexistence of colistin resistance along with the production of carbapenemases in multi-drug resistant isolates poses a real threat in the use of carbapenems and colistin to fight infections (Lomonaco et al. 2018; Lee et al. 2019).

Colistin is characterized by high activity against Gram-negative rods, despite numerous reports of increasing bacterial resistance to this drug (Petrosillo et al. 2019), most of which are chromosomally coded. The spread of plasmid-encoded resistance to colistin, related to the presence of *mcr* genes, is alarming, especially since it concerns to a large extent strains with "a low level of resistance to colistin" (with a colistin minimum inhibitory concentration (MIC) range of 2–8 mg/l). The repeatedly described diagnostic problems encountered in determining the MIC values of colistin are largely responsible for the lack of knowledge about the presence of such isolates (Stefaniuk and Tyski 2019). However, numerous reports indicate the universality of such strains (Bardet and Rolain 2018; Jayol et al. 2018), including in Poland, where for the first time *E. coli* strain was identified as possessing the *mcr*-1 gene in 2016 (Izdebski et al. 2016). In Poland, little is known about the scale of the resistance of Gram-negative rods to colistin. Thus, an attempt was made to assess the degree of resistance to other antimicrobial agents of colistin-resistant strains isolated from serious life-threatening infections in patients treated in hospitals throughout Poland.

The project achieved the collection of colistin-resistant Enterobacterales rods over three quarters of 2019. Within the total number of collected strains, isolates with the *mcr*-1 gene constituted only 4.2%. Prim and co-researchers (2017) showed that the *mcr*-1 gene in clinical isolates is still rare in Europe. Our study may indicate that the colistin resistance of Polish Enterobacterales isolates is mainly chromosomally encoded.

Further research is required to confirm this assumption. It is noteworthy that the colistin-resistant *Klebsiella* strains constitute as much as 47/65 (69.2%) of isolates studied in this project, while *E. coli* represented only 15/65 (23.1%). In our study the *mcr*-1 genes were detected only in three *E. coli* strains; two of these strains produced ESBL and were susceptible to the new drugs meropenem/vaborbactam and ceftazidime/avibactam, while the third was resistant to carbapenems (produced OXA-48-like carbapenemase) and resistant to ceftazidime with avibactam. Kazmierczak and co-researchers (2018) showed the activity of ceftazidime/avibactam and other agents against Enterobacteriaceae collected in 18 European countries from 2012 to 2015. The tested isolates also came from Poland; colistin-resistant Enterobacterales isolates accounted for 1.8% of all Polish isolates (Kazmierczak et al. 2018). Ceftazidime/avibactam was the most active agent from all tested antimicrobial agents. From all colistin-resistant isolates in this study, 98.2% were susceptible to ceftazidime with avibactam.

Globally, the *mcr*-gene family is widely disseminated among Enterobacterales, mainly in *E. coli* and *K. pneumoniae* isolated from human infections (Jeannot et al. 2017). Our study suggests that *mcr*-1 is currently more common in *E. coli* strains than in *K. pneumoniae* in Poland. Some authors also report that the MICs of colistin for *E. coli* carrying the *mcr*-1 gene are lower than the MICs of colistin for *K. pneumoniae* (MICs 4–16 mg/l vs. 4–64 mg/l) (Walkty et al. 2016). In our study, MICs of colistin for *E. coli* with the *mcr*-1 gene were higher than indicated by Walkty et al. (2016), ranging from 16 to > 64 mg/l.

This is the first report on the occurrence of β-lactamases in colistin-resistant Enterobacterales strains in Poland. These data broaden the knowledge of the mechanism of resistance to colistin among Enterobacterales causing human infections in Poland. Demographic data of patients, from whom the strains resistant to colistin were isolated, indicate that the problem of this resistance cannot be limited to a selected group of patients. The small number of colistin-resistant isolates (n = 65) obtained from hospitals that participated in the pilot study may indicate that the problem of colistin resistance among Enterobacterales strains is low. However, due to the described issues of the infection therapy, this problem requires further research and analysis. In the future, the authors plan to compare the antibiotics susceptibility of Enterobacterales isolates resistant to colistin and other multidrug-resistant Enterobacterales species susceptible to colistin.

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Acknowledgments
The National Medicines Institute's internal funding financed this work. The authors are thankful to members of the cooperating hospital microbiology laboratories for their excellent technical assistance.

Conflict of interest
The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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