Acta Scientiae Veterinariae, 2024. 52: 1973.

RESEARCH ARTICLE
Pub. 1973

ISSN 1679-9216

Colistin Resistance in Pigs from Serbia: Bioinformatics Insight into *mcr-1* Gene Sequencing

Danijela Petrovic[1], Vesna Lalošević[2], Gordana Kozoderović[1], Vuk Vračar[2] & Dušan Lalošević[3]

ABSTRACT

***Background*:** The increase of antimicrobial resistance is considered a major global threat for human and veterinary medicine. Misuse/overuse of antibiotics contributes to the rapid growth of resistance, also observed for colistin, a drug of choice in the therapy of human infections caused by multidrug resistant (MDR) bacteria. Due to wide usage of colistin as growth promoter in farm animals, the resistance of bacteria to this drug is of high concern in One Health perspective. In our previous study 10 *mcr-1* positive PCR products from pigs' faecal samples were identified and reported. The purpose of this study was to sequence these products and delve deeper into the colistin resistance via bioinformatics method.

***Materials, Methods & Results*:** Using a BLAST database, this study has identified *mcr-1* gene variant(s) responsible for the spreading of resistance. A sequence-led approach identified these ten sequences and 100% alignment score was achieved by all tested (query) sequences. The query coverage for all sequences was between 98-100%, indicating that sequence in the database spans the most of the query sequence or the whole length of the query sequence, respectively. Eight sequences achieved 100%, while one reached 99% and one 98% query coverage. The most of alignments originated in the *Escherichia coli* sequences (97%), followed by *Klebsiella pneumoniae* (2%) and *Salmonella enterica* (1%). All these pathogens are known as MDR bacteria, and in infections they caused, colistin is being used as salvage therapy. Based on the obtained BLAST results, we assumed that DNA isolated from pigs' faecal samples originated from *E. coli*, although our samples were not cultured. The analysis revealed that these sequences were distributed predominantly in Asia, but also in South America and Europe, isolated from different farming animal species and humans, confirming global distribution across different species. Further comprehensive analysis yielded 37 functional genetic alleles of *mcr-1* gene (*mcr-1.1 – mcr-1.37*) registered in NCBI database so far, and in order to identify possible *mcr-1* gene alleles among tested sequences, additional BLAST alignment examinations were carried out. Five *mcr-1* gene alleles were excluded as possible variants, but exact polymorphism in each of the sequences could not be confirmed, most likely due to the lack of full length of the tested sequences. These 10 sequences represent *mcr-1* gene variant(s), registered in Serbia for the 1st time in pigs, and were submitted to the GeneBank and made publicly available, through the Accession numbers.

***Discussion*:** Plasmid-borne *mcr-1* gene has become a significant cause for the dissemination of polymixin resistance in humans and animals worldwide. A closer inquiry in *mcr-1* gene sequences helped to address *mcr-1* gene contribution to the resistance spreading. It was revealed that identified *mcr-1* gene sequences in pigs on Serbian farms have been already disseminated worldwide, on 3 continents, and in different species including farming animals (pigs, chicken, turkey) and their meat samples, companion animals (dogs) and humans. Our results indicated that *mcr-1* gene sequencing contributes to the better understanding of colistin resistance molecular epidemiology. Necessity of colistin resistance monitoring in food producing animals as well as its prudent and responsible use on farms, since it is a last resort drug for combating infections in humans caused by multidrug resistant bacteria, is of crucial importance in One Health approach.

Keywords: BLAST alignments, MDR, molecular epidemiology, swine, colistin resistance, *mcr-1* gene variants, Serbia.

DOI: 10.22456/1679-9216.142295

Received: 3 September Accepted: 29 November 2024 Published: 6 December 2024

[1]Department of Natural Sciences and Management in Education, Faculty of Education in Sombor, University of Novi Sad, Sombor, Serbia. [2]Department of Veterinary Medicine, Faculty of Agriculture & [3]Department of Histology and Embryology, Faculty of Medicine, University of Novi Sad, Novi Sad, Serbia. CORRESPONDENCE: D. Petrovic [petrovid@tcd.ie]. Department of Natural Sciences and Management in Education, Faculty of Education in Sombor, University of Novi Sad. Sombor, Vojvodina, Serbia.

INTRODUCTION

Antibiotic resistance, as one of the main global threats, rapidly increases compromising our capacity to treat and cure infectious disease. Main reasons for such unfavourable outcomes are misuse and/or overuse of antibiotics in human healthcare as well as agricultural sectors [37]. In order to mitigate the devastating consequences of this worldwide burden and to preserve the efficacy of antimicrobials, World Health Organization (WHO) categorized and authorised these drugs for the use in either both or only in human or veterinary medicine [39]. Colistin, as a drug of choice in the therapy of human infections caused by multidrug-resistant (MDR) gram-negative bacteria [21], is classified as "Highest Priority Critically Important Antimicrobials (HPCIA)" [39]. Although being used for the last-hope treatment, colistin is still widely and recklessly used as growth promoter in farm animals, confirming why the rising prevalence of MDR gram-negative bacteria is of high concern in One Health perspective [13,31,34].

To date, 10 *mcr* gene families (*mcr-1* to *mcr-10*) were reported in different bacteria sourced from animals, humans, farms, food and the environment [7,11]. Our recent study confirmed only the presence of *mcr-1* gene (from total DNA) in 350 healthy pigs' faecal samples, collected from 7 different farms. *Mcr-1* gene was detected in 4.85% animals, predominantly in piglets, and all of them were from 3 farms within 100 km apart [15]. The aim of this study was to, starting from obtained sequencing data, examine whether the previously confirmed *mcr-1* gene, represents a novel or worldwide distributed variant(s), knowing that *mcr-1* gene, besides *mcr-3*, has the largest number of reported polymorphisms [18].

MATERIALS AND METHODS

Sample and study design

Ten PCR products positive for the presence of *mcr-1* gene were used in this study. These products were obtained after total DNA samples, extracted from healthy pigs' faeces, were subjected to the multiplex PCR as described in details in our previous study [15]. In order to determine the *mcr-1* gene variant(s), a sequence-led approach was performed and sequencing data obtained for these 10 PCR products were analysed utilising bioinformatics tools. Before being sent for sequencing, these PCR products were checked by gel electrophoresis.

Gel electrophoresis and sequencing

PCR products were electrophoresed in 2% agarose gel, with a molecular weight marker (GeneRuler 100bp Plus DNA Ladder[1]) included in each run. The gels were stained with ethidium bromide (1 µg/mL final concentration in ddH_2O for 30 min) and bands were visualized using UV gel documentation system SERVA Blue-Cube 300[2]. *E. coli* NCTC 13846 strain for *mcr-1* gene was used as a positive control. PCR products were sent for sequencing and the results were obtained using standard sequencing automation system, based on the Capillary Electrophoresis Sequencing technology - Sanger method (ABI 3730xl System)[3].

Bioinformatics approach

The Basic Local Alignment Search Tool (BLAST), within the NCBI (National Center for Biotechnology Information), [https://blast.ncbi.nlm.nih.gov/Blast.cgi] was used to compare obtained *mcr-1* nucleotide DNA sequences (query) to sequences deposited in the BLAST enclosed databases [GenBank, EMBL, DDBJ, PDB, RefSeq sequences - (subject sequences)]. BLAST reads the query sequence, examines parameters, and searches databases [20]. Before query sequences, represented in the standard IUB/IUPAC nucleic acid codes, were entered in FASTA format, they were trimmed using Bioinformatics Software platform for sequence data analysis - Geneious Prime (Annotate and Predict - Trim Ends). Forward query sequence of a particular sample was run as line of sequence query data, without the FASTA definition line. The search set was optimised for standard databases - nucleotid collections (nr/nt), while programme selection was optimised for highly similar sequences (megablast). Algorithm parameters were kept on the default settings. The BLASTn generated alignments and calculated statistical significance of the 100 displayed matches. All of searches yielded small Expect (E) values, indicating database matches of very high quality. The E value threshold was kept at the default value of 0.05.

Statistical analysis

The data were analysed using Microsoft Excel statistical software employing the Student *t* test and $P < 0.05$ was considered statistically significant.

RESULTS

Visualisation of mcr-1 *PCR products and sequencing*

As described previously [20], 17 out of 350 tested samples were positive for *mcr-1* gene only, as confirmed by multiplex PCR. Ten of these *mcr-1* positive PCR products of 320 bp in length (as positive control for *mcr-1*, Ec+), were visualised on 2% agarose gel (Figure 1). Subsequently, these PCR products were sequenced using Sanger sequencing method. The output of the sequencing was presented as chromatogram (ab1 file) and as text-based files (notepad, php1). Insights into chromatograms confirmed that peaks were sharp, well-spaced and with assigned bases for all sequences for all samples. Text-based sequence files for all samples yielded BLAST alignments. All query sequences were submitted to the NIH genetic sequence database, GeneBank, and have received Accession numbers, so all are publicly available (Table 1).

BLAST complete alignment: taxonomy and geographic distribution

In order to determine whether the *mcr-1* gene detected from 10 samples obtained from the 3 Serbian farms, represent certain variants of the very polymorphic *mcr-1* gene family, a sequence-led approach was adopted. The obtained *mcr-1* nucleotide sequences were compared with sequences deposited in BLAST enclosed databases. The program algorithms compared the sequences and calculated the statistical significance of the matches. Performed alignments disclosed a score of 100% identity achieved by all 10 tested sequences D09, D12, D14, D15, VJ04, VJ05, VJ06, VJ09, VJ10 and VJ11. Description tab (default BLAST result) displayed a list of subject sequences in database that completely matched the query sequence. The results are represented by an alignment given as a definition line (a row) with identifiers and descriptions of the hits, and the small E-value, as statistical measures of the significance, indicated great uniqueness of the match for all query sequences (100%). In addition, query coverage for 8 sequences was 100%, indicating that sequence in the database spans the whole query sequence length, with an exception for VJ06 (99%) and VJ09 (98%). Maximum (max) and total scores for each particular query sequence was identical, demonstrating perfect match without deletion/insertion across the alignment. The closer insight has confirmed the same subject sequences displayed for the majority of query sequences (n=8), and besides these identical counterparts, only 1 new subject sequence was reported in a BLAST search for the rest of 2 query sequences (D14/VJ06).

Taxonomic BLAST results summarised the distribution of matched subject sequences in the database regarding the bacterial species. Total numbers of hits were 101 and 102 (in 8 and 2 query sequences respectively). The most of alignments originated in *Escherichia coli* (*E. coli*) subject sequences: D09/D12/D15/VJ04/VJ05/VJ09/VJ10/VJ11 - 98, D14/VJ06 - 99; followed by *Klebsiella pneumoniae*: 2 and *Salmonella enterica*: 1 for all sequences (Figure 2). Incidence of *E. coli* sequences across all matched subject sequences was significantly higher in comparison to 2 other pathogens ($P = 0.000$). *E. coli* was by far the most dominant species for all query sequences: 97%, followed by *Klebsiella pneumoniae* and *Salmonella enterica* present to the smallest extent (2% and 1% respectively). Although our samples were not cultured, according to the obtained BLAST results, we hypothesised that isolated DNA originated from *E. coli*.

Further comprehensive searches of all BLAST hits, revealed that these sequences were distributed in Asia, Europe, and South America. Detailed analysis of these BLAST hits, obtained through the accession link for each hit, yielded information on countries that reported particular sequence, as well as the host and the isolate (Table 2). Table is based on thorough analysis of each BLAST hits.

Mcr-1 *gene alleles identification in completely aligned sequences*

Up to date, 37 functional genetic alleles of *mcr-1* gene (*mcr-1.1 – mcr-1.37*) encoding phosphoethanolamine-lipid A transferase, have been registered in NCBI database i.e. National Database of Antibiotic Resistant Organisms (NDARO), as in August 2024 (Table 3). In order to identify *mcr-1* gene polymorphism(s), further investigation included BLAST search based on the particular programme option - Align 2 or more sequences. Comparison of query sequences with all previously reported *mcr-1* gene alleles (both in FASTA format) revealed no conclusion on particular polymorphisms. However, it facilitated to exclude the presence of certain alleles, and they were as follow: *mcr-1.3* (NG_052861.1), *mcr-1.10* (NG_055583.1), *mcr-1.14* (NG_057460.1), *mcr-1.20* (NG_065450.1), and *mcr-1.23* (NG_067235.1).

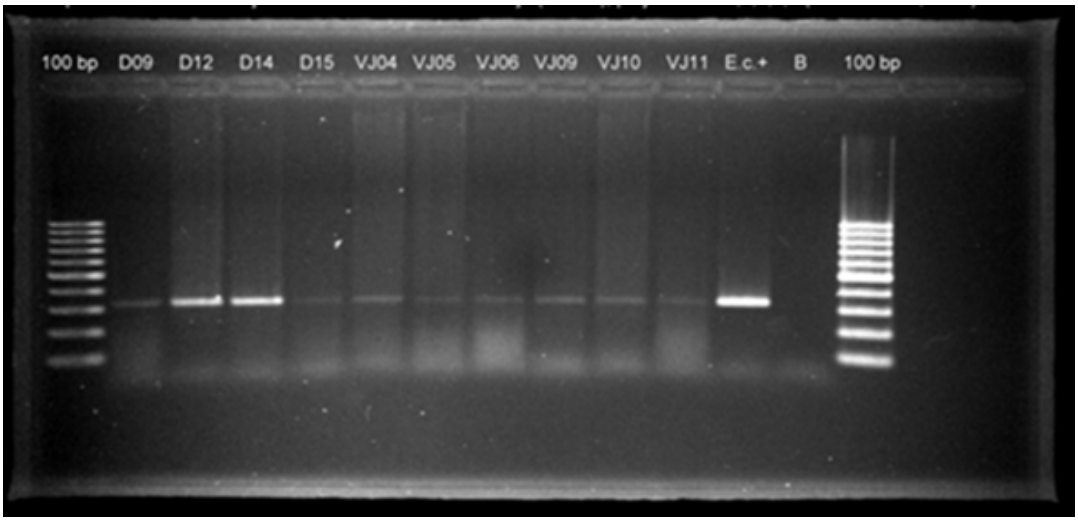

Figure 1. Electrophoresed PCR products (D09/12/14/15, sampled from the North-Backa region and VJ04/05/06/09/10/11 obtained from North-Banat region). [2% agarose gel; Ec+ - positive control; B - blanc; Ladder - 100 bp. Product size 320 bp].

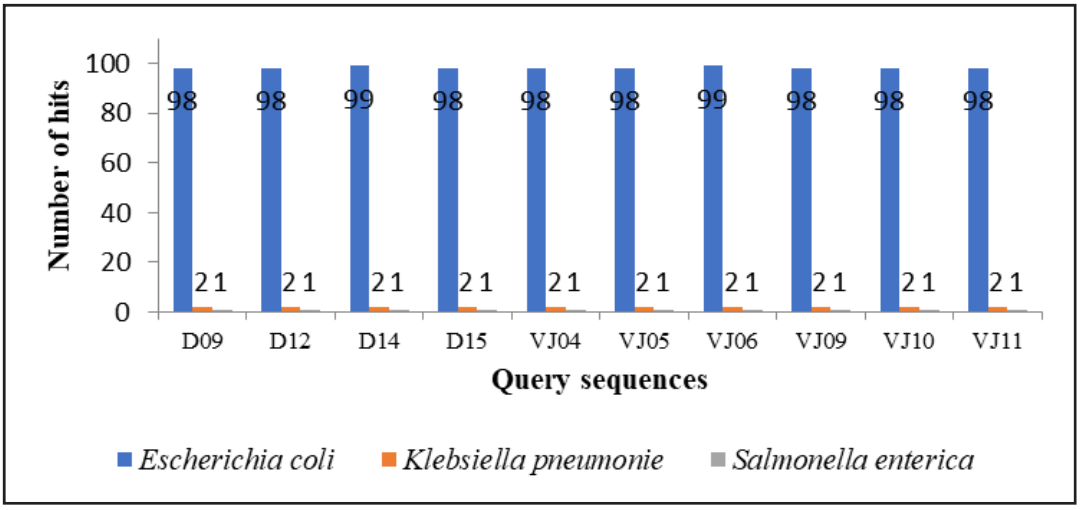

Figure 2. Total number of hits scattered among different bacterial species for each query sequence. Data are based on the Taxonomy BLAST reports that included the organisms found in the BLAST hitlist that have the strongest scores.

Table 1. GenBank accession numbers and main characteristics associated with query sequences.

Sequence ID	Organism	Host	Source	Isolate	Accession N.
D09	Uncultured bacterium	Swine	Farm	Faeces	PP334135
D12	Uncultured bacterium	Swine	Farm	Faeces	PP334136
D14	Uncultured bacterium	Swine	Farm	Faeces	PP315912
D15	Uncultured bacterium	Swine	Farm	Faeces	PP334137
VJ04	Uncultured bacterium	Swine	Farm	Faeces	PP315913
VJ05	Uncultured bacterium	Swine	Farm	Faeces	PP334138
VJ06	Uncultured bacterium	Swine	Farm	Faeces	PP315914
VJ09	Uncultured bacterium	Swine	Farm	Faeces	PP334139
VJ10	Uncultured bacterium	Swine	Farm	Faeces	PP315915
VJ11	Uncultured bacterium	Swine	Farm	Faeces	PP334140

Table 2. The geographical distribution of the 100% aligned subject sequences. Details on the countries of sequence origin, the host and isolates were assembled through the accession link for each BLAST hit.

Sequence ID	*Escherichia coli*	*Klebsiella pneumoniae*	*Salmonella enterica*
D09/D12/D15/VJ04/VJ05/ VJ09/VJ10/VJ11	CHINA - pork, chicken and pig faeces, human urin VIETNAM - human faeces BULGARIA - human urin GERMANY - turkey ITALY - dog urin ECUADOR - human faeces	CHINA - pork, chicken faeces	VIETNAM - human diarrheal stool
D14/VJ06	CHINA - pork, chicken and pig faeces, human urin VIETNAM - human faeces BULGARIA - human urin GERMANY - turkey ITALY - dog urin POLAND - turkey ECUADOR - human faeces	CHINA - pork, chicken faeces	VIETNAM - human diarrheal stool

Table 3. Overview of all *mcr-1* alleles (*mcr-1.1 – mcr-1.37*) available in NCBI (as on August 31th, 2024)

Mcr-1 gene alleles	NCBI Ref.Seq.	Species	Strain	Host (Source)	Authors[1]	Country
mcr-1.1	NG_050417.1	*Escherichia coli*	SHP45	pig	Liu Y., 2016	China
mcr-1.2	NG_051170.1	*Klebsiella pneumoniae*	KP-6884	human	Di Pilato V., 2016	Italy
mcr-1.3	NG_052861.1	*Escherichia coli*	HeN867	chicken	Yang Y., 2016	China
mcr-1.4	NG_052664.1	*Escherichia coli*	WCHEC1606	sewage	Zhao F., 2016	China
mcr-1.5	NG_052663.1	*Escherichia coli*	1256822	human	Estabrook M., 2016	Argentina
mcr-1.6	NG_052893.1	*Salmonella enterica*	YL14P053	human	Lu X., 2016	China
mcr-1.7	NG_054678.1	*Escherichia coli*	WCHEC1604	sewage	Zhao F., 2017	China
mcr-1.8	NG_054697.1	*Escherichia coli*	ST101	poultry	Abdul Momin H.F.,2017	Brunei
mcr-1.9	NG_055582.1	*Escherichia coli*	LV23529	pig	Manageiro V., 2017	Portugal
mcr-1.10	NG_055583.1	*Moraxella sp.*	MSG13-C03	pig	AbuOun M., 2017	UK
mcr-1.11	NG_055784.2	*Escherichia coli*	347-43491A	human	Deshpande L., 2018	Peru
mcr-1.12	NG_056412.1	*Escherichia coli*	EC15-101	pig	Nishino Y., 2017	Japan
mcr-1.13	NG_057466.1	*Escherichia coli*	12-AB00501	turkey	Hammerl J.A., 2018	Germany
mcr-1.14	NG_057460.1	*Klebsiella pneumoniae*	SC24	chicken	Yang Y., 2019	China
mcr-1.15	NG_061610.1	*Klebsiella pneumoniae*	KP138	chicken	Xiang R., 2018	China
mcr-1.16	NG_064787.1	*Escherichia coli*	EC32	chicken	Zhu Y., 2019	China
mcr-1.17	NG_064788.1	*Escherichia coli*	ECK2	cow	Zhu Y., 2019	China
mcr-1.18	NG_064789.1	*Escherichia coli*	sc12-96	chicken	Wu C., 2018	China
mcr-1.19	NG_065449.1	*Salmonella enterica*	CFSA629	egg	Hu Y., 2019	China
mcr-1.20	NG_065450.1	*Escherichia coli*	nd.[2]	pig	Duggett N., 2018	UK
mcr-1.21	NG_065451.1	*Escherichia coli*	QHXN2016-F200	human	Li C., 2019	China
mcr-1.22	NG_065944.1	*Escherichia coli*	NIG-EC49	chicken	Carretto E., 2019	Nigeria
mcr-1.23	NG_067235.1	*Salmonella enterica*	SAUVM	poultry	Uddin M.B., 2020	Bangladesh
mcr-1.24	NG_067236.1	*Escherichia coli*	SAUVM_E3	poultry	Uddin M.B., 2020	Bangladesh
mcr-1.25	NG_067237.1	*Escherichia coli*	SAUVM_E6	poultry	Uddin M.B., 2020	Bangladesh
mcr-1.26	NG_068217.1	*Escherichia coli*	803_18	human	Neumann B., 2020	Germany
mcr-1.27	NG_068218.1	*Escherichia coli*	844_18	human	Neumann B., 2020	Germany
mcr-1.28	NG_070762.1	*Escherichia coli*	nd.[2]	nd.[2]	Ruan Z., 2020	China
mcr-1.29	NG_070763.1	*uncultured bacterium*	nd.[2]	environ.[3]	LV D., 2020	China
mcr-1.30	NG_070764.1	*uncultured bacterium*	nd.[2]	swine	LV D., 2020	China
mcr-1.31	NG_074755.1	*Escherichia coli*	2018-290904-002-01	human	Bolzoni L., 2021	Italy
mcr-1.32	NG_074756.1	*Escherichia coli*	N8114	sputum	Falgenhauer L., 2021	Spain
mcr-1.33	NG_079262.1	*Escherichia coli*	nd.[2]	urine	Bao D., 2021	China
mcr-1.34	NG_079263.1	*Escherichia coli*	EC3769	human	Qian C., 2022	China
mcr-1.35	NG_203419.1	*Escherichia coli*	20ZX79	chicken	Hu Y., 2023	China
mcr-1.36	NG_231577.1	*Escherichia coli*	IHIT44909	pig	Goepel L., 2023	Germany
mcr-1.37	OR879175.1	*Escherichia coli*	IHIT23707	dog	Ewers,C. 2023.	Germany

[1]Only first author listed. [2]Not determined. [3]Environment. *Mcr* alleles in bold were excluded as possible alleles.

DISCUSSION

Our findings provide a glimpse on the possible risky burden that hovers above Serbian farming sector. This report on pigs' faecal samples, harbouring plasmid(s) carrying *mcr-1* gene, points out towards colistin resistance problem, emphasizing transmission risk, since in the neighbouring country (Bulgaria) the same *mcr-1* gene sequences were isolated from human urine sample. Furthermore, our research has revealed that *mcr-1* gene sequences identical to ours, for the 1st time reported in pigs from Serbia, have been already disseminated on 3 continents and among different animal species including farming animals (pigs, chicken, turkey) and their meat samples, companion animals (dogs) and humans. A recent study was the 1st to report a presence of IncX4 replicon type plasmid carrying *mcr-1.1* gene variant in 5 *Escherichia coli* isolates sourced from healthy turkeys in Serbian farms [23], while our previous study was the 1st to state the presence of *mcr-1* gene in farming pigs' faecal samples in Serbia [15]. Therefore, findings from these studies clearly indicate necessity for continuous monitoring for colistin resistance in farming facilities in Serbia to avoid possible dissemination of resistance to humans. Particular monitoring requests significant attention since it was recently revealed that diverse strains of *E. coli,* harboring *mcr-1* on multiple plasmids, were not eradicated in healthy pigs years after the colistin treatment was ceased on the farm [40]. Intensified surveillance of *mcr* genes, besides thoughtful use of important antibiotics for therapeutic purposes, improved vaccination, increased hygiene, development and use of rapid diagnostic tests, etc., contribute the most in successful control of colistin resistant bacteria dissemination from poultry sector across low- and middle-income countries [3]. Extensive usage of colistin as a growth promotor, as well as, in prevention and treatment of infections caused by gram-negative bacteria in food producing animals, contributed enormously to the emergence of the resistance to this antibiotic [5], and we might not wait too long to see the consequences in our hospitals. This is even more important, since colistin has recently re-emerged as one of the last resorts in battling the certain infections in absence of newly created drugs and when widely used antibiotics failed to treat gram-negative bacteria [1,6]. During the COVID-19 pandemic in the case of severe pneumonia and the need for mechanical ventilation, very often patients used to develop ventilator-associated pneumonia (VAP) with the most striking feature being multiple antimicrobial resistance. In these cases, the aerosolized administration of colistin has become very important in the treatment of severe infections [17,29]. However, the recent study in Serbia has revealed that 40% of molecularly confirmed isolates positive to *Acinetobacter baumannii*, were colistin-resistant and were isolated from COVID-19 patients and patients on mechanical ventilation [14].

Several studies have reported that colistin resistance was caused by mutations in chromosomal genes mostly involved in the synthesis of the lipopolysaccharide (LPS), component of the bacterial outer membrane [4,24,28]. Shortly after, Liu *et al*. proposed a different mechanism involving mobilized colistin resistance-1 (*mcr-1*) gene in the emergence of plasmid-mediated colistin resistance in farm animals and human probands in China [19]. *Mcr-1* gene encodes for a phosphoethanolamine transferase, an enzyme that catalyses the transfer of phosphoethanolamine moiety to lipid A, a component of bacterial outer membrane. This modification results in neutralisation of lipid A negative charge, resulting in reduced binding affinity of cationic colistin [28,32]. This diminished colistin affinity increases membrane resistance to the antibiotic, thus protecting the bacterial integrity and its intracellular targets. Moreover, similar mechanism is involved in resistance of numerous gram-negative bacteria to other extracellular material, resulting in fade host immune response [26]. Plasmid-borne nature of *mcr* genes coding colistin resistance indicates their ability of horizontal transmission throughout diverse bacterial species in gut microbiome [19]. Of note, only 3 months after the 1st report of *mcr-1* gene, more than 20 countries have also stated its presence [11]. Therefore, global health concern arises on the ability of plasmids to be quickly transferred between pathogenic bacterial strains, species and genera, accelerating dissemination of resistance. Yet, we cannot ignore the fact that a recent study demonstrated that spontaneous chromosomal modifications, mainly mutations of genes involved in the cell signalling of the LPS synthesis, also contribute to the colistin resistance to a large extent [33]. Very shortly after the 1st ever *mcr-1* report in China [19], the *mcr-1* gene was identified on 5 continents and isolated from human, farm animal, wild animal, vegetable and meat samples [13], showing accelerated worldwide

spreading. *Mcr-1* gene variants are embraced within very diversified plasmids, what contributes to their prompt expansion and, subsequently, rapid evolution (mutation rate) [2]. Recent metagenomic study has revealed that *mcr-1* and *mcr-9* gene variants were the most frequent in comprehensive research data regarding metagenomics samples' alignments, hosts, countries, timescale and pathogenic bacteria genomes (51.08% and 40.38% respectively) [22]. Three years ago 27 *mcr-1* subvariants in form of single amino acid substitutions were scientifically known (25 registered in NCBI) [27]. Our research has yielded data on 37 *mcr-1* gene alleles, suggesting successful evolutionary trend, yet under unknown selective factors, as it was noted by Ling *et al*. [18]. Unfortunately, using bioinformatics tools we were not able to conclude the particular *mcr-1* gene alleles for query sequences, probably due to the lack of full length of the sequences. However, we managed to exclude 5 alleles.

This study presented the 1st sequencing data on *mcr-1* gene obtained from farming pigs' faecal samples in Serbia. *Mcr-1* gene sequences identified in our study previously were reported within the genome of Enterobacteriaceae (*Escherichia coli*, *Klebsiella pneumoniae* & *Salmonella enterica*). All these pathogens are known as MDR bacteria, and in infections they caused, colistin is being used as salvage therapy. The global threat is recognized and it resulted in the edition of "International Consensus Guidelines for the Optimal Use of the Polymyxins" [34], with numerous recommendations how to deal with this issue in healthcare settings. As reported by numerous studies, *mcr-1* gene is, with different frequencies, abundant mainly in *Escherichia coli* all across the world [7,8,12,25,30,35,41]. Besides, other gram-negative bacteria, such as *Klebsiella* sp. and *Salmonella* sp., have been reported to harbour *mcr-1* gene, but with significantly lower prevalence [36]. Knowing that outbreaks or high prevalence of nosocomial gram negative bacteria harbouring *mcr-1* gene could be reality in Serbia very soon, our results contribute to the surveillance of colistin resistance in our country, although it was proven to be quite demanding and challenging to screen colistin resistance, as confirmed by experts [38]. In contrast to plasmid mediated colistin resistance, a recent study reported that the main mechanism of colistin resistance in *Acinetobacter baumannii* isolates tested in Serbia, was accumulation of chromosomal mutations within the *pmrB* and *pmrC* genes [14].

Therefore, finding of this study contributes to an incredibly complex puzzle of colistin resistance phenomenon, although the explanation on mechanisms such as colistin binding, its antimicrobial activity, or emergence and evolution of plasmid-mediated resistance based on LPS modifications, still remains elusive [10]. Colistin abuse in farming practice, especially pig and poultry production, represents a strong selective pressure resulting in food safety fears and public health threats [9]. Interestingly, *mcr-1* colistin resistance, on the other hand, facilitates functional impairment and decreased permeability of the bacterial outer membrane towards hydrophobic antibiotics, such as gentamycin, kanamycin and rifampicin. This situation challenges bacterial populations and their fitness cost, forcing them to balance between *mcr-1* expression and its own survival [16], indicating a very complex interplay between selective pressure created by both antibiotics as well as the host immunity and bacterial ability to escape. Who will be victorious in this interactions' maze, depends mostly on wise human moves in the future.

CONCLUSION

Plasmid-borne *mcr-1* gene has become a significant cause for the dissemination of colistin resistance in humans and animals worldwide. Our results demonstrated that sequencing of *mcr-1* gene may contribute to the better understanding of molecular epidemiology of colistin resistance. The results also contribute to the increasing body of evidence for necessity of colistin resistance monitoring in food producing animals as well as restricting colistin abuse on farms, since it is among rare agents, i.e. last resort drug, effective in treatment of infections in humans caused by multidrug-resistant gram-negative bacteria. Thus, colistin antimicrobial effectiveness must not be compromised via its further misuse in food producing livestock.

MANUFACTURERS

[1]Thermo Scientific, Thermo Fisher Scientific Inc. Waltham, MA, USA.

[2]SERVA Electrophoresis GmbH. Heidelberg, Germany.

[3]Macrogen Inc. Seoul, Republic of South Korea.

***Funding*.** This project was supported by Ministry of Science, Technological Development and Innovations, Rep. of Serbia (grant number: 451-03-47/2023-01/ 200117) and by the Provincial Secretariat for Higher Education and Scientific Research of the Autonomous Province of Vojvodina, Rep. of Serbia (grant number 142-451-2626/2021-01/2).

Ethical approval. This research employed bioinformatics approach only (sequencing, BLAST alignment, etc.) and, for all work performed in this study, no ethical approval was required. DNA samples that were sent for sequencing were obtained from faecal samples of the pigs in our previous study. For these animals, no ethical approval of the Institutional Committee was needed, because that study did not involve a prospective evaluation, did not involve laboratory animals and only involved non-invasive procedures (faecal samples). All procedures, performed in that study involving human participants, were in accordance with the ethical standards of the Institutional Committee and with the 1964 Helsinki declaration and its later amendments.

Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing in the paper.

REFERENCES

1 **Andrade F.F., Silva D., Rodrigues A. & Pina-Vaz C. 2020.** Colistin Update on Its Mechanism of Action and Resistance, Present and Future Challenges. *Microorganisms*. 8(11): 1716. DOI: 10.3390/microorganisms8111716

2 **Anyanwu M.U., Jaja I.F., Okpala C.O.R., Jaja C.J.I., Oguttu J.W., Chah K.F. & Shoyinka V.S. 2021.** Potential sources and characteristic occurrence of mobile colistin resistance (*mcr*) gene-harbouring bacteria recovered from the poultry sector: a literature synthesis specific to high-income countries. *PeerJ*. 9: e11606. DOI: 10.7717/peerj.11606

3 **Anyanwu M.U., Jaja I.F., Okpala C.O.R., Njoga E.O., Okafor N.A. & Oguttu J.W. 2023.** Mobile Colistin Resistance (*mcr*) Gene-Containing Organisms in Poultry Sector in Low- and Middle-Income Countries: Epidemiology, Characteristics, and One Health Control Strategies. *Antibiotics*. 12(7): 1117. DOI: 10.3390/antibiotics12071117.

4 **Cannatelli A., D'Andrea M.M., Giani T., Di Pilato V., Arena F., Ambretti S., Gaibani P. & Rossolini G.M. 2013.** *In vivo* emergence of colistin resistance in *Klebsiella pneumoniae* producing KPC-type carbapenemases mediated by insertional inactivation of the PhoQ/PhoP mgrB regulator. *Antimicrobial Agents and Chemotherapy*. 57(11): 5521-5526.

5 **Catry B., Cavaleri M., Baptiste K., Grave K., Grein K., Holm A., Jukes H., Liebana E., Lopez Navas A., Mackay D., Magiorakos A.-P., Moreno Romo M.A., Moulin G., Muñoz Madero C., Matias Ferreira Pomba M.C., Powell M., Pyörälä S., Rantala M., Ružauskas M., Sanders P., Teale C., Threlfall E.J., Törneke K., van Duijkeren E. & Torren Edo J. 2015.** Use of colistin-containing products within the European Union and European Economic Area (EU/EEA): development of resistance in animals and possible impact on human and animal health. *International Journal of Antimicrobial Agents*. 46(3): 297-306.

6 **El-Sayed Ahmed M.A.E.G., Zhong L.L., Shen C., Yang Y., Doi Y. & Tian G.B. 2020.** Colistin and its role in the Era of antibiotic resistance: an extended review (2000–2019). *Emerging Microbes & Infections*. 9(1): 868-885.

7 **Ewers C., Göpel L., Prenger-Berninghoff E., Semmler T., Kerner K. & Bauerfeind R. 2022.** Occurrence of *mcr-1* and *mcr-2* colistin resistance genes in porcine *Escherichia coli* isolates (2010–2020) and genomic characterization of *mcr-2*-positive *E. coli*. *Frontiers in Microbiology*. 13. DOI: 10.3389/fmicb.2022.1076315

8 **Falgenhauer L., Waezsada S.E., Yao Y., Imirzalioglu C., Käsbohrer A., Roesler U., Michael G.B., Schwarz S., Werner G., Kreienbrock L., Chakraborty T. & RESET consortium. 2016.** Colistin resistance gene *mcr-1* in extended-spectrum β-lactamase-producing and carbapenemase-producing Gram-negative bacteria in Germany. *The Lancet. Infectious Diseases*. 16(3): 282-283.

9 **Gao R., Hu Y., Li Z., Sun J., Wang Q., Lin J., Ye H., Liu F., Srinivas S., Li D., Zhu B., Liu Y.-H., Tian G.-B. & Feng Y. 2016.** Dissemination and Mechanism for the *MCR-1* Colistin Resistance. *PLoS Pathogens*. 12(11): e1005957.

10 **Gogry F.A., Siddiqui M.T., Sultan I. & Haq Q.M.R. 2021.** Current Update on Intrinsic and Acquired Colistin Resistance Mechanisms in Bacteria. *Frontiers in Medicine*. 8. DOI: 10.3389/fmed.2021.677720

11 **Hussein N.H., AL-Kadmy I.M.S., Taha B.M. & Hussein J.D. 2021.** Mobilized colistin resistance (*mcr*) genes from 1 to 10: a comprehensive review. *Molecular Biology Reports*. 48(3): 2897-2907.

12 **Irrgang A., Roschanski N., Tenhagen B.-A., Grobbel M., Skladnikiewicz-Ziemer T., Thomas K., Roesler U. & Käsbohrer A. 2016.** Prevalence of *mcr-1* in *E. coli* from Livestock and Food in Germany, 2010-2015. *PloS One*. 11(7): e0159863.

13 **Jeannot K., Bolard A. & Plésiat P. 2017.** Resistance to polymyxins in Gram-negative organisms. *International Journal of Antimicrobial Agents*. 49(5): 526-535.

14 **Kabic J., Novovic K., Kekic D., Trudic A., Opavski N., Dimkic I., Jovcic B. & Gajic I. 2023.** Comparative genomics and molecular epidemiology of colistin-resistant *Acinetobacter baumannii*. *Computational and Structural Biotechnology Journal*. 21: 574-585.

15 **Kozoderović G., Lalošević V., Süli T. & Vračar V. 2022.** The first record of *mcr-1* gene for colistin resistance in pigs from Serbia: should we be worried? *Polish Journal of Veterinary Sciences*. 25(3): 463-467.

16 **Li B., Yin F., Zhao X., Guo Y., Wang W., Wang P., Zhu H., Yin Y. & Wang X. 2020.** Colistin Resistance Gene *mcr-1* Mediates Cell Permeability and Resistance to Hydrophobic Antibiotics. *Frontiers in Microbiology*. 10. DOI: 10.3389/fmicb.2019.03015

17 **Lima W.G., Brito J.C.M. & Nizer W.S.C. 2020.** Ventilator-associated pneumonia (VAP) caused by carbapenem-resistant *Acinetobacter baumannii* in patients with COVID-19: Two problems, one solution? *Medical Hypotheses*. 144: 110139. DOI: 10.1016/j.mehy.2020.110139

18 **Ling Z., Yin W., Shen Z., Wang Y., Shen J. & Walsh T.R. 2020.** Epidemiology of mobile colistin resistance genes *mcr-1* to *mcr-9*. *Journal of Antimicrobial Chemotherapy*. 75(11): 3087-3095.

19 **Liu Y.Y., Wang Y., Walsh T.R., Yi L.X., Zhang R., Spencer J., Doi Y., Tian G., Dong B., Huang X., Yu L.F., Gu D., Ren H., Chen X., Lv L., He D., Zhou H., Liang Z., Liu J.H. & Shen J. 2016.** Emergence of plasmid-mediated colistin resistance mechanism *MCR-1* in animals and human beings in China: a microbiological and molecular biological study. *The Lancet Infectious Diseases*. 16(2): 161-168.

20 **Madden T. 2013.** The BLAST Sequence Analysis Tool. *The NCBI Handbook [Internet]*. 2nd edn. National Center for Biotechnology Information (US). Corpus ID: 7454137

21 **Magiorakos A.P., Srinivasan A., Carey R.B., Carmeli Y., Falagas M.E., Giske C.G., Harbarth S., Hindler J.F., Kahlmeter G., Olsson-Liljequist B., Paterson D.L., Rice L.B., Stelling J., Struelens M.J., Vatopoulos A., Weber J.T. & Monnet D.L. 2012.** Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infection*. 18(3): 268-281.

22 **Martiny H.-M., Munk P., Brinch C., Szarvas J., Aarestrup F.M. & Petersen T.N. 2022.** Global Distribution of mcr Gene Variants in 214K Metagenomic Samples. *mSystems*. 7(2): e00105-22.

23 **Mišić D., Kiskaroly F., Szostak M.P., Cabal A., Ruppitsch W., Bernreiter-Hofer T., Milovanovic V., Feßler A.T., Allerberger F., Spergser J., Müller E., Schwarz S., Braun S.D., Monecke S., Ehricht R., Korus M., Benković D., Korzeniowska M. & Loncaric I. 2021.** The First Report of *mcr-1*-Carrying *Escherichia coli* Originating from Animals in Serbia. *Antibiotics*. 10(9): 1063. DOI: 10.3390/antibiotics10091063

24 **Moffatt J.H., Harper M., Harrison P., Hale J.D.F., Vinogradov E., Seemann T., Henry R., Crane B., St Michael F., Cox A.D., Adler B., Nation R.L., Li J. & Boyce J.D. 2010.** Colistin resistance in *Acinetobacter baumannii* is mediated by complete loss of lipopolysaccharide production. *Antimicrobial Agents and Chemotherapy*. 54(12): 4971-4977.

25 **Nang S.C., Li J. & Velkov T. 2019.** The rise and spread of *mcr* plasmid-mediated polymyxin resistance. *Critical Reviews in Microbiology*. 45(2): 131-161.

26 **Needham B.D. & Trent M.S. 2013.** Fortifying the barrier: the impact of lipid A remodelling on bacterial pathogenesis. Nature Reviews. *Microbiology*. 11(7): 467-481.

27 **Neumann B., Rackwitz W., Hunfeld K.P., Fuchs S., Werner G. & Pfeifer Y. 2020.** Genome sequences of two clinical *Escherichia coli* isolates harboring the novel colistin-resistance gene variants *mcr-1.26* and *mcr-1.27*. *Gut Pathogens*. 12(1): 40. DOI: 10.1186/s13099-020-00375-4.

28 **Olaitan A.O., Morand S. & Rolain J.M. 2014.** Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Frontiers in Microbiology*. 5. DOI: 10.3389/fmicb.2014.00643

29 **Russo A., Bruni A., Gullì S., Borrazzo C., Quirino A., Lionello R., Serapide F., Garofalo E., Serraino R., Romeo F., Marascio N., Matera G., Longhini F., Trecarichi E.M. & Torti C. 2023.** Efficacy of cefiderocol- vs colistin-containing regimen for treatment of bacteraemic ventilator-associated pneumonia caused by carbapenem-resistant Acinetobacter baumannii in patients with COVID-19. *International Journal of Antimicrobial Agents*. 62(1): 106825. DOI: 10.1016/j.ijantimicag.2023.106825.

30 **Schwarz S. & Johnson A.P. 2016.** Transferable resistance to colistin: a new but old threat. Journal of Antimicrobial Chemotherapy. 71(8): 2066-2070.

31 **Srinivas P. & Rivard K. 2017.** Polymyxin Resistance in Gram-negative Pathogens. *Current Infectious Disease Reports*. 19(11): 38. DOI: 10.1007/s11908-017-0596-3

32 **Stojanoski V., Sankaran B., Prasad B.V.V., Poirel L., Nordmann P. & Palzkill T. 2016.** Structure of the catalytic domain of the colistin resistance enzyme MCR-1. *BMC Biology*. 14(1): 81. DOI: 10.1186/s12915-016-0303-0.

33 Torres D.A., Seth-Smith H.M.B., Joosse N., Lang C., Dubuis O., Nüesch-Inderbinen M., Hinic V. & Egli A. 2021. Colistin resistance in Gram-negative bacteria analysed by five phenotypic assays and inference of the underlying genomic mechanisms. *BMC Microbiology*. 21(1): 321. DOI: 10.1186/s12866-021-02388-8

34 Tsuji B.T., Pogue J.M., Zavascki A.P., Paul M., Daikos G.L., Forrest A., Giacobbe D.R., Viscoli C., Giamarellou H., Karaiskos I., Kaye D., Mouton J.W., Tam V.H., Thamlikitkul V., Wunderink R.G., Li J., Nation R.L. & Kaye K.S. 2019. International Consensus Guidelines for the Optimal Use of the Polymyxins: Endorsed by the American College of Clinical Pharmacy (ACCP), European Society of Clinical Microbiology and Infectious Diseases (ESCMID), Infectious Diseases Society of America (IDSA), International Society for Anti-infective Pharmacology (ISAP), Society of Critical Care Medicine (SCCM), and Society of Infectious Diseases Pharmacists (SIDP). *Pharmacotherapy*. 39(1): 10-39.

35 Unger F., Eisenberg T., Prenger-Berninghoff E., Leidner U., Ludwig M.-L., Rothe M., Semmler T. & Ewers C. 2017. Imported reptiles as a risk factor for the global distribution of *Escherichia coli* harbouring the colistin resistance gene *mcr-1*. *International Journal of Antimicrobial Agents*. 49(1): 122-123.

36 Valiakos G. & Kapna I. 2021. Colistin Resistant mcr Genes Prevalence in Livestock Animals (Swine, Bovine, Poultry) from a Multinational Perspective. A Systematic Review. *Veterinary Sciences*. 8(11): 265. DOI: 10.3390/vetsci8110265.

37 WHO. Antibiotic resistance. n.d. Available at: <https://www.who.int/news-room/fact-sheets/detail/antibiotic-resistance>.

38 WHO. GLASS: the detection and reporting of colistin resistance, 2nd ed. n.d. Available at: <https://www.who.int/publications-detail-redirect/glass-the-detection-and-reporting-of-colistin-resistance-2nd-ed>.

39 WHO. Public discussion on the WHO Medically Important Antimicrobial List, 7th Revision 2023 (CLOSED). n.d. Available at: <https://www.who.int/news-room/articles-detail/public-discussion-on-the-who-medically-important-antimicrobial-list--7th-revision>.

40 Yoshizawa N., Hikoda-Kogiku Y., Tamamura-Andoh Y. & Kusumoto M. 2023. *mcr-1* remains detectable in various *Escherichia coli* lineages isolated from healthy swine after withdrawal of colistin use on the farm. *The Journal of Veterinary Medical Science*. 85(5): 536-540.

41 Zhang R., Huang Y., Chan E.W., Zhou H. & Chen S. 2016. Dissemination of the *mcr-1* colistin resistance gene. *The Lancet Infectious Diseases*. 16(3): 291-292.