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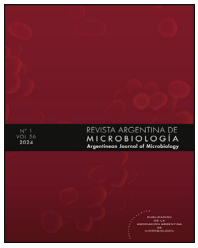

ORIGINAL ARTICLE

Colistin-resistant *Escherichia coli* mediated by the *mcr-1* gene from pigs in northeastern Argentina

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Abstract The emergence and spread of multidrug-resistant *Escherichia coli* carrying *mcr-1* is recognized as a threat to public health. The aim of this study was to determine the prevalence of the *mcr-1* gene in colistin-resistant *E. coli* isolates from commercial pig farms in Chaco, Argentina from 2020 to 2021. A total of 140 rectal swab samples were collected from pigs in six different pig production farms. Antimicrobial susceptibility was determined by broth microdilution. *mcr-1* to *mcr-5* genes were identified by multiplex PCR and clonality was assessed by ERIC and REP-PCR. The prevalence of *mcr-1* was 16.4% and *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5* genes were not detected. Colistin MIC values showed a bimodal distribution with a MIC50, MIC90 and a range of 4, 8 and 4–8 μg/ml, respectively. The resistance profile to other antimicrobials was: ampicillin, 87% (20); ampicillin–sulbactam, 47.8% (11); amoxicillin–clavulanic, 13% (3); chloramphenicol, 82.6% (19); ciprofloxacin, 60.9% (14); minocycline, 26.1% (5) and trimethoprim/sulfamethoxazole, 43.5% (10). Eighty-seven percent (87%) of the strains were categorized as MDR and 12 phenotypic resistance patterns with different clonality profiles were observed. A high prevalence of *mcr-1* is demonstrated in colistin-free pig farms from Chaco, Argentina. The *mcr-1* positive *E. coli* isolates showed an alarming level of multidrug resistance and high clonal diversity. It is necessary to continuously monitor the presence of the *mcr-1* gene not only in pig production, but also in humans and the environment.

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PALABRAS CLAVE
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Cerdos

Resistencia a colistina mediada por *mcr-1* en *Escherichia coli* aislada de cerdos del nordeste argentino

Resumen La aparición y propagación de *Escherichia coli* resistente a múltiples fármacos, portadora de *mcr-1*, se reconoce como una amenaza para la salud pública. El objetivo de este estudio fue determinar la prevalencia del gen *mcr-1* en aislamientos de *E. coli* resistentes a colistina en granjas porcinas de Chaco, Argentina. Se recolectaron 140 muestras de hisopados rectales de cerdos en seis granjas de producción entre marzo de 2020 y julio de 2021. La sensibilidad antimicrobiana se evaluó mediante el método de microdilución en caldo. Los genes *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* y *mcr-5* se identificaron por PCR multiplex; la clonalidad se evaluó mediante ERIC y REP-PCR. La prevalencia de *mcr-1* fue del 16,4% y no se detectaron los genes *mcr-2*, *mcr-3*, *mcr-4* y *mcr-5*. Los valores de CIM de colistina presentaron una distribución bimodal, con unas CIM_{50} y CIM_{90} de 4 y 8 μg/ml, respectivamente, y un rango de 4-8 μg/ml. El perfil de resistencia a otros antimicrobianos dentro de los aislamientos *mcr-1* positivos fue el siguiente: ampicilina, 87% (20); ampicilina-sulbactam, 47,8% (11); amoxicilina-clavulánico, 13% (3); cloranfenicol, 82,6% (19); ciprofloxacina, 60,9% (14); minociclina, 26,1% (5) y trimetoprima/sulfametoxazol, 43,5% (10). El 87% de las cepas se categorizaron como multidrogorresistentes (MDR) y se observaron 12 patrones fenotípicos de resistencia con diferentes perfiles de clonalidad. Se corroboró una elevada prevalencia de *mcr-1* en granjas porcinas de Chaco libres de colistina. Los aislamientos de *E. coli* positivos para *mcr-1* mostraron un nivel alarmante de multirresistencia y una alta diversidad clonal. Es necesario monitorear continuamente la presencia del gen *mcr-1*, no solo en la producción porcina, sino también en humanos y en el ambiente.

Introduction

Antimicrobial resistance (AMR) is recognized as a global threat to human, animal and environmental health. One of the main causes of AMR is the overuse of antimicrobials in both humans and animals. This practice selects for multidrug-resistant (MDR) bacteria that can be transmitted from animals to humans and vice versa. Transmission occurs through direct contact between animals and humans or through the food chain and the environment[33]. The emergence of MDR gram negative bacterial infections in humans has been the cause of the resurgence of colistin as a last-line antibiotic therapy[25].

Colistin is a polycationic polypeptide belonging to the polymyxin family. It has been widely used in veterinary medicine for prophylaxis, metaphylaxis and treatment of enteric diarrhea in pigs[2]. It has also been used extensively as an animal growth promoter (AGP) in food-producing animals. In this way, animals achieve increased weight gain, improved growth performance and good feed efficiency[1].

Until 2015, colistin resistance was only associated with mutations and regulatory changes mediated by chromosomal genes[21,30]. More recently, the first plasmid-associated colistin resistance gene (*mcr-1*) was reported in food, humans and pigs from China[18]. Soon after this finding, the presence of the *mcr-1* gene and other variants (*mcr-2* to *mcr-10*) were described worldwide[13]. This gene encodes an enzyme from the phosphoethanolamine (pEtN) transferase group, which introduces a pEtN group into lipid A. As a result, there is an increase in the positive charge of lipopolysaccharide and a decrease in the binding of polymyxins[29].

Recently, worldwide reports have been published on the detection and characterization of the *mcr-1* gene in *Escherichia coli* strains[12]. It has been reported mainly in animal and environmental samples and, to a lesser extent, in human clinical samples[15]. In Argentina, *mcr-1*-mediated colistin resistance was first documented in human infections caused by *E. coli* in 2016[24]. Since then, the *mcr-1* gene has been found in a variety of sources, including gulls[17], poultry[6], pigs[4] and companion animals[27].

The aim of this study was to investigate the prevalence of the *mcr-1* gene in colistin-resistant *E. coli* isolates collected from intensive pig farms in Chaco, Argentina. In addition, we sought to characterize the susceptibility profiles of these isolates to different antimicrobial agents and to assess the clonal diversity among strains harboring the *mcr-1* gene.

Materials and methods

Study design and sample collection

A prospective, observational and cross-sectional study was conducted. A total of 140 rectal swabs were collected from pigs in six different farms (A, B, C, D, E and F). Sampling was carried out from March 2020 to July 2021 in different geographical areas of the province of Chaco, Argentina. For each farm, asymptomatic pigs were randomly selected on the basis of their age and production stage. Therefore, 35 weaned piglets (4–8 weeks old), 35 growing pigs (10–18 weeks old) and 70 fattening pigs (20–26 weeks old) were studied. All samples were immediately transported at 4 °C

to the laboratory for microbiological examination and processed within 4 h. An epidemiological survey was conducted in each of the pig farms studied.

Isolation of colistin-resistant *E. coli*

Rectal swab samples were incubated overnight at 37 °C in 10 ml buffered peptone water (pH ≥7.2 ± 0.2, Britania, Argentina). Subsequently, 50 μl of each enrichment broth was inoculated on colistin-supplemented (3 μg/ml, Sigma-Aldrich, US) Mac Conkey agar (Britania, Argentina). Plates were incubated at 37 ± 1 °C for 22 ± 2 h. Colistin-resistant *E. coli* isolates were selected by colony morphology and identified by biochemical methods[3].

Antimicrobial susceptibility testing

Susceptibility to colistin was evaluated based on growth or no growth on Mueller-Hinton agar plates containing 3 μg/ml colistin (COLTEST, Britania, Argentina). The minimum inhibitory concentration (MIC) was determined by the broth microdilution method (Sensititre, Thermo Fisher, USA) according to CLSI[5] and EUCAST[7] guidelines. The antibiotics tested were ampicillin (8–16 μg/ml), ampicillin/sulbactam (8/4 to 16/8 μg/ml), amoxicillin/clavulanic (8/4 to 16/8 μg/ml), cefuroxime (4–16 μg/ml), cefotaxime (1–32 μg/mL), ceftazidime (2–32 μg/ml), cefepime (2–16 μg/ml), piperacillin/tazobactam (8/4 to 64/4 μg/ml), ciprofloxacin (0.06–2 μg/ml), levofloxacin (0.12–4 μg/ml), gentamicin (4–8 μg/ml), amikacin (8–32 μg/ml), chloramphenicol (8–16 μg/ml), trimethoprim/sulfamethoxazole (2/38 μg/ml), imipenem (0.5–8 μg/ml), meropenem (1–16 μg/ml), nitrofurantoin (32–64 μg/ml), colistin (1–4 μg/ml) and tigecycline (0.5–2 μg/ml). *E. coli* ATCC 25922 was used for quality control in antimicrobial susceptibility testing. MIC_{50} and MIC_{90} were calculated for all antimicrobials based on the MIC of each strain. In this study, we define MDR as non-susceptibility to one or more antimicrobial agents in at least three different categories, as previously defined[20].

Detection of *mcr* genes and molecular typing of colistin-resistant *E. coli* isolates

All *E. coli* isolates with colistin MICs ≥4 μg/ml were screened by multiplex PCR for the detection of *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5* genes, as previously described[16]. DNA was extracted using the boiling bacterial lysis method. Clonal relatedness between isolates was determined by enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) and repetitive element palindromic-polymerase chain reaction (REP-PCR), as the previously established[32].

Statistical analysis

The Statistical Packages of Social Sciences (SPSS) software, version 22.0, was used for the statistical analysis. Pearson's chi-squared test was used to determine the relationship between strains based on the presence of the *mcr-1* gene. Statistical significance was defined as $p < 0.05$.

Results

Presence of colistin-resistant *E. coli* and the *mcr-1* gene

Of 140 rectal swab samples analyzed, 23 (16.4%) showed growth on colistin-supplemented McConkey. All these isolates were confirmed to be *E. coli* and the presence of the *mcr-1* gene was detected in all of them. Prevalence of *mcr-1*-positive *E. coli* for weaning piglets was 27.7% (95% CI; [11%;49%]), followed by 14.3% (95% CI; [5%;36%]) for growing pigs, and 12.9% (95% CI; [3%;34%]) for fattening pigs. The analysis of the remaining colistin resistance genes showed the absence of the *mcr-2*, *mcr-3*, *mcr-4*, and *mcr-5* genes.

Of the total number of farms analyzed, three were located in the northwestern part of the province, two in the eastern central region and one in the southwestern zone. At least one *mcr-1*-positive isolate was detected in 66.7% of the pig farms studied (4/6), three of which engaged in another type of activity, such as bovine production. Farms with *mcr-1* positive isolates were located in two of three agricultural regions of Chaco, Argentina. At the same time, only three farms reported the use of at least one antibiotic during pig fattening and, in particular, two farms had used colistin as a growth promoter in the past (Table 1).

Antimicrobial susceptibility profile

Colistin MIC values showed a bimodal distribution with a range of 4–8 μg/ml and a MIC value of 4 was observed in 82.6% (19/23) of the isolates (Table 2). The observed resistance profile was as follows: ampicillin, 87% (95% CI; [66%;97%]); ampicillin–sulbactam, 47.8% (95% CI; [27%;69%]); amoxicillin–clavulanic, 13% (95% CI; [3%;34%]); chloramphenicol, 82.6% (95% CI; [61%;95%]); ciprofloxacin, 60.9% (95% CI; [38%;80%]); levofloxacin, 56.5% (95% CI; [34%;77%]); minocycline, 26.1% (95% CI; [10%;48%]) and trimethoprim/sulfamethoxazole, 43.5% (95% CI; [23%;65%]). In contrast, 100% of the isolates were susceptible to cefotaxime, ceftazidime, meropenem, imipenem, gentamicin, amikacin, nitrofurantoin, fosfomycin and tigecycline (Fig. 1).

On the other hand, 12 phenotypic resistance patterns were observed in 23 *mcr-1*-positive *E. coli* isolates analyzed. Eighty-seven percent (87%) (95% CI; [66%;97%]) of the strains were classified as MDR based on previously defined criteria (Table 2). However, molecular typing of the isolates studied revealed a wide diversity of ERIC and REP profiles and no similarity in these patterns was observed.

Discussion

In recent years, many studies have highlighted an increase in colistin-resistant *Enterobacteriaceae* isolates in both humans and animals[14,31]. Recently, the *mcr-1* gene has been reported in different types of food-producing animals such as pigs and poultry in Argentina[6]. Therefore, in the present

Table 1 Epidemiologic characteristics of the intensive pig farms studied.

Farms	Region of Chaco	Pig breed	Breeding system	Lockdown	Other activities	ATB used during study	Past COL use	*mcr-1* gene
A	Northwestern	Landrace	Rearing	Mixed	Poultry	No use	No	–
B	Southwestern	Yorkshire	Complete cycle	Permanent	No	No use	No	–
C	Northwestern	Yorkshire	Growth-finishing	Permanent	Bovine	AMX	Yes	+
D	Northwestern	Yorkshire	Complete cycle	Permanent	No	No use	No	+
E	Eastern Center	Yorkshire	Growth-finishing	Permanent	Bovine	TML	No	+
F	Eastern Center	Landrace	Complete cycle	Permanent	Bovine	FFC, TML, CTC	Yes	+

ATB: antibiotic; COL: colistin; AMX: amoxicillin; TML: tiamulin; FFC: florfenicol; CTC: chlortetracycline.

Table 2 Characterization of colistin-resistant *E. coli* isolates (n = 23).

Isolate	Pig category	Weight (kg)	CIM COL (μg/ml)	COLTEST	*mcr* gene	MDR
MFC5	Fattening pigs	70	8	+	*mcr-1*	–
CBC1	Weaned piglets	6	4	+	*mcr-1*	+
CBC2	Weaned piglets	6	4	+	*mcr-1*	+
CBC3	Weaned piglets	6	4	+	*mcr-1*	+
CBC4	Weaned piglets	6	4	+	*mcr-1*	+
CBC5	Weaned piglets	6	4	+	*mcr-1*	+
CBC7	Growing pigs	10	4	+	*mcr-1*	+
CBC8	Growing pigs	10	4	+	*mcr-1*	+
CBC9	Growing pigs	10	4	+	*mcr-1*	+
CBC10	Growing pigs	10	4	+	*mcr-1*	+
CBC16	Fattening pigs	14	4	+	*mcr-1*	–
CBC18	Fattening pigs	14	4	+	*mcr-1*	+
CBC19	Fattening pigs	14	4	+	*mcr-1*	+
CBC20	Fattening pigs	14	4	+	*mcr-1*	+
MC6	Growing pigs	90	4	+	*mcr-1*	+
DMC1	Weaned piglets	25	4	+	*mcr-1*	+
DMC4	Weaned piglets	25	8	+	*mcr-1*	+
DMC5	Weaned piglets	25	4	+	*mcr-1*	+
DMC10	Weaned piglets	25	8	+	*mcr-1*	+
DMC21	Fattening pigs	80	8	+	*mcr-1*	–
DMC27	Fattening pigs	80	4	+	*mcr-1*	+
DMC29	Fattening pigs	80	4	+	*mcr-1*	+
DMC30	Fattening pigs	80	4	+	*mcr-1*	+

MDR: multidrug-resistant.

study, we analyzed the prevalence of *mcr-1*-positive *E. coli* isolates in pig farms in Chaco, Argentina. To the best of our knowledge, our manuscript highlights the first report of the *mcr-1* gene within the pig production chain in northeastern Argentina, despite the fact that the use of colistin as a growth promoter has been suspended.

Previous studies in pigs have shown that the frequency of *mcr-1* was 0.5–9.9% in Europe[23,26] and 20.6% in China[18]. In Latin America, the circulation of *mcr-1*-harboring *E. coli* strains in swine showed a prevalence of 47% in Ecuador[34], while in Peru and Brazil it was 12% and 3.1%, respectively[4,10]. In January 2019, the Ministry of Agriculture of Argentina published Resolution No. 22/2019, which prohibits the use of colistin and its derivatives in food-producing animals[28]. Prior to this resolution, the prevalence of *mcr-1*-positive *E. coli* isolates in swine from Argentina was 38.7%[8]. However, in our study we found the *mcr-1* gene in 16% of pigs tested between 2020 and 2021. These results showed a significant decrease in the prevalence of *mcr-1* carrying *E. coli* in pig production, which could be due to the prohibition of colistin for veterinary use in our country.

In Argentina, the most frequently utilized antimicrobial classes in pigs include beta-lactams (e.g., amoxicillin, ceftiofur), macrolides (e.g., tylosin), tetracyclines (e.g., oxytetracycline, chlortetracycline), and fluoroquinolones (e.g., enrofloxacin and norfloxacin)[11]. When comparing the three stages of pig production, it was found that weaning piglets had a significantly higher resistance rate to colistin than other stages. This may be explained by the fact that more than 70% of the antimicrobial agents used in pigs are administered during the first 10 weeks of life[22]. Therefore, our results suggest that weaning piglets could act as an important reservoir for the *mcr-1* gene. Furthermore, this study showed that three pig farms presented *mcr-1* positive isolates and were simultaneously engaged in another type of activity such as bovine production. This situation

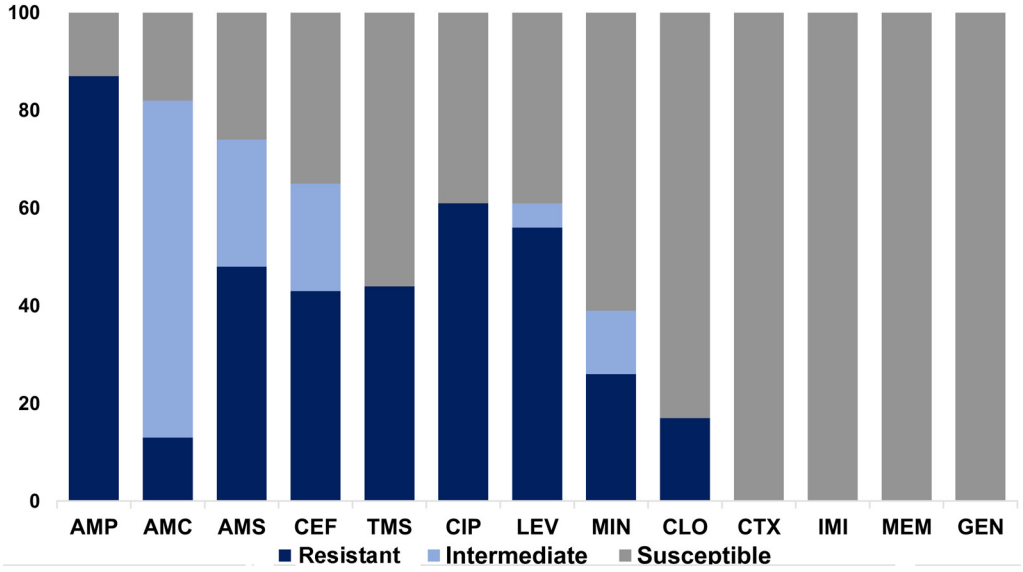

Figure 1 Antimicrobial susceptibility profile of *mcr-1*-positive *E. coli* isolates to other antibiotics. AMP: ampicillin; AMC: amoxicillin/clavulanic; AMS: ampicillin–sulbactam; CEF: cefalotin; TMS: trimethoprim–sulfamethoxazole; CIP: ciprofloxacin; LEV: levofloxacin; MIN: minocycline; CHL: chloramphenicol; CTX: cefotaxime; IMI: imipenem; MER: meropenem; GEN: gentamicin.

is worrying because it could contribute to the spread of multidrug-resistant microorganisms carrying *mcr-1* from pigs to cattle, thereby affecting a new production chain in the region.

The analysis of the *mcr* genes by PCR in colistin-resistant *E. coli* isolates showed that they all carried solely *mcr-1*. In concordance with our results, *mcr-1* is the only gene reported so far in pig farms in Argentina[8] and, as far as we know, in other farm animals[6] and even in pets[27]. Molecular typing of *mcr-1*-positive *E. coli* strains revealed the presence of a wide diversity of ERIC and REP profiles, indicating that these microorganisms are probably not clonally related. This finding could be attributed to the fact that the establishments under investigation are situated in geographically distinct locations, with no connection between the production sites and animals, resulting in the emergence of distinct clones. In addition, antimicrobial susceptibility to other antibiotics showed that 87% of *mcr-1*-positive *E. coli* isolates were classified as MDR, in agreement with a previous study describing a high prevalence of MDR (71%)[8].

Current data indicate a higher prevalence of *mcr*-carrying bacteria in animals than in humans. This supports the hypothesis that colistin resistance encoded by *mcr-1* is most commonly transferred from animals to humans[19]. It is important to clarify that fewer studies have been conducted in humans than in animals. In Argentina, only one retrospective multicenter study of human samples from 2012 to 2018 has been reported. A total of 192 human isolates of *mcr-1*-positive *E. coli* were analyzed, only one (0.5%) of which was from the province of Chaco[9]. Therefore, it would be of great interest to conduct further studies on *mcr-1*-mediated colistin resistance in humans and the environment in our region. This would allow a better understanding of the transmission cycle of the *mcr-1* gene from a One Health perspective.

Finally, our study showed the presence of the *mcr-1* gene from pig farms in the province of Chaco. This is of great concern because this province is the fourth largest producer of pork in Argentina. Accordingly, this situation could contribute to the spread of multiresistant microorganisms carrying *mcr-1* in the pig production chain from northeastern Argentina. Some limitations of the present study should be mentioned. First, circulating allelic variants of the *mcr-1* gene were not identified. Secondly, we did not investigate the *mcr-6*, *mcr-7*, *mcr-8*, *mcr-9* and *mcr-10* genes, which are also involved in colistin resistance.

Conclusion

The results of this study show a high prevalence (16%) of *mcr-1*-positive *E. coli* from COL-free intensive pig farms in the province of Chaco, Argentina. The *mcr-1*-positive *E. coli* isolates exhibited diverse resistance profiles with 87% MDR and high clonal diversity. Our study suggests that healthy pigs may act as *mcr-1* carrying *E. coli* reservoirs. Therefore, active surveillance of antimicrobial resistance and strict monitoring of biosecurity measures are necessary in the pig farms studied, which will prevent the spread and dissemination of the *mcr-1* gene in the pig production chain.

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Conflict of interest

The authors declared no potential conflicts of interest.

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