Original Article

Antibiotic resistance profile of Enterobacteriaceae isolated from healthy pigs in the Republic of Armenia

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Abstract

The rising prevalence of antimicrobial resistance (AMR) among Enterobacteriaceae in livestock presents a significant threat to both animal and human health. This study investigates the antibiotic resistance profiles of Enterobacteriaceae isolated from 160 samples collected from the lymph nodes of healthy pigs in Armenia. Fifty isolates were identified, including *Escherichia coli* (24), *Klebsiella* spp. (10), *Salmonella* spp. (6), *Raoultella ornithinolytica* (4), *Pseudomonas aeruginosa* (4), and *Proteus mirabilis* (2). Resistance was evaluated using the disk diffusion method against 22 antibiotics from nine classes. Alarmingly, 94.5% of isolates were resistant to clarithromycin and 90.3% to erythromycin, while resistance to cephalothin and amoxicillin averaged 71.7% and 56.4%, respectively. All *E. coli* isolates displayed multidrug resistance (MDR), with resistance to 3–5 antibiotic classes, and other Enterobacteriaceae exhibited MDR spanning up to seven classes. Notably, *Proteus mirabilis* showed resistance to eight antibiotic classes, underscoring its potential as a reservoir of resistance genes. The findings highlight the risk of zoonotic transmission of AMR pathogens within the food chain. This study underscores the urgent need for antimicrobial stewardship, robust surveillance systems, and improved farming practices to mitigate the AMR crisis in livestock. The results serve as a call to action for policymakers and researchers to address the public health implications of antibiotic resistance in food production systems.

Keywords: disk diffusion, identification, microorganism, pigs, resistance

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Received January 14, 2025
Accepted March 4, 2025

Introduction

The indiscriminate and sometimes excessive use of antimicrobials in diverse livestock farming practices fosters the emergence of various mechanisms of resistance among microorganisms (Baudoin *et al.*, 2021). Overuse and misuse of these drugs in animal agriculture can lead to the development of Antimicrobial Resistance (AMR) bacteria, which can then be transmitted to humans through contaminated food or direct contact (Almansour *et al.*, 2023). Antimicrobial agents are employed in livestock farming for therapeutic, prophylactic, and growth-promoting purposes. Their utilization is notably elevated in intensively reared species, such as pigs and poultry, compared to extensively farmed cattle and sheep (Woolhouse *et al.*, 2015). From a geographical standpoint, pigs are raised across diverse regions globally, representing one of the primary sources of sustenance for humans. In fact, pigs rank among the foremost contributors to the global food supply, with more than 40% of the total meat consumption worldwide derived from this species (Peng *et al.*, 2022). The stressful conditions under which farmed pigs are managed contribute significantly to their heightened susceptibility to infections (Papatsiros *et al.*, 2024). This susceptibility necessitates increased utilization of antibiotics within farming operations. Particularly pronounced in low- and middle-income countries, where regulatory oversight may be lacking, inappropriate Antimicrobial Use (AMU) practices are prevalent due to the unrestricted availability of antibiotics (Albernaz-Gonçalves *et al.*, 2021). Consequently, this confluence of factors not only drives up AMU rates but also fosters the emergence and spread of antibiotic resistance among pig populations, presenting a significant challenge to infection control efforts within these agricultural settings. The emergence and dissemination of antibiotic-resistant bacteria (ARB) and antibiotic-resistant genes (ARGs) throughout the farm-to-plate continuum pose significant health and socioeconomic implications worldwide. This direct interaction accelerates the dissemination of these resistant entities, exacerbating the global challenge of combating antibiotic resistance (Founou *et al.*, 2021; Chinemerem Nwobodo *et al.*, 2022). Within pig populations, ARB and ARGs are primarily associated with Enterobacteriaceae and Staphylococci. These microorganisms, notably *Escherichia coli (E. coli)* (Shivley *et al.*, 2023; Songsaeng *et al.*, 2024), serve as significant reservoirs for AMR in pig farming. They possess the ability to acquire ARGs through various mechanisms, contributing to the spread of resistance within the pig population and potentially to humans through direct or indirect contact (Monger *et al.*, 2021).

Pig breeding is a key sector of the national economy in the Republic of Armenia (RA). It is most developed in regions near the capital, Yerevan, where the highest concentration of pigs is found, including commercial pig farms. In the northern part of the country, backyard and free-range breeding is more common. According to data from the Statistical Committee of the Republic of Armenia (*armstat.am*), as of January 1, 2024, the pig population was 186,951, including 30,077 sows. Of these, 49,543 pigs were raised on commercial farms, while 137,408 were kept in rural and urban households. The country produces approximately 15.7 thousand tons of pork (slaughter weight) annually. In many regions, particularly in small-scale backyard farms, pigs are maintained for both personal consumption and occasional sale. Unfortunately, these environments often lack adequate biosecurity measures, leading to suboptimal hygiene standards and increased susceptibility to a spectrum of diseases, both infectious and non-infectious, among the animals. A concerning aspect within this context is the emergence of AMR. This phenomenon is exacerbated by the unregulated availability and unrestricted use of antibiotics within the veterinary domain in all countries. The lack of stringent oversight means that farmers and veterinarians have unhindered access to antibiotics, facilitating their inappropriate and excessive usage.

The objective of this study is to isolate Enterobacteriaceae from the lymph nodes of healthy pigs, identify them at the genus and species level, and evaluate the antimicrobial resistance patterns of each isolate to the same antibiotics. Lymph nodes serve as key immune sites and reservoirs for systemic bacteria, including Enterobacteriaceae, providing insights into persistent colonization and AMR transmission risks. They are crucial for food safety, easily accessible during slaughter, and minimize contamination. Though isolating bacteria from lymph nodes is challenging, viable commensal and pathogenic bacteria can still be detected (Mann *et al.*, 2015).

Materials and Methods

Ethical approval for this research was obtained from the scientific committee of the Scientific Centre for Risk Assessment and Analysis in Food Safety Area CJSC (protocol N6 from 17.07.2024).

Sample collection: All pigs sampled in this study were from various backyard farms located in the Armavir, Ararat, and Kotayk regions near the capital, Yerevan. Samples were collected from three abattoirs located in Yerevan, Ararat, and Armavir regions. Sampling was conducted on randomly selected pigs on various days without a strict seasonal or monthly pattern. The pigs ranged in age from 6 months to 1.5 years, with 80% of them being between 6 and 10 months old.

The Enterobacteriaceae family of bacteria was isolated from the lymph nodes of healthy pigs after slaughtering in abattoirs. The lymph nodes sampled included the mandibular, mesenteric, and iliac nodes. During 2022-2023, samples were collected from 80 pigs in sterile containers, each labeled with all necessary information, including the region, community, age of the pigs, date of sampling, and sample identification number. A total of 160 lymph nodes were collected, including 80 mesenteric lymph nodes, 44 iliac, and 36 mandibular lymph nodes. Each pig contributed a mesenteric lymph node, along with either an iliac or a mandibular lymph node.

Sample preparation: The surface of each lymph node was disinfected with 70% ethanol before the tissue was

minced with sterile scalpels. Minced tissues were then transferred into sterile bottles containing 9 mL of buffered peptone water. The samples were homogenized using a stomacher for 1-2 minutes to ensure thorough mixing.

Bacterial culture: The homogenized samples were incubated at 37°C for 20 hours to promote bacterial growth, then inoculated onto selective and differential agar media (MacConkey agar, Bismuth sulfite agar, and Simmons Citrate Agar) and incubated again at 37°C for 24-48 hours. Colony morphology typical of Enterobacteriaceae was assessed, and Gram staining was performed following standard protocols to confirm isolate identity (Quinn *et al.*, 2011; Tripathi and Sapra, 2024). Well-isolated colonies were picked and streaked onto fresh agar plates to obtain pure cultures, followed by another incubation at 37°C for 18-24 hours.

Identification of Enterobacteriaceae: Identification of Enterobacteriaceae species was performed using the API 20E Test identification system (bioMérieux, France). This system comprises 20 miniature biochemical tests in a strip format, which assess metabolic activities such as carbohydrate fermentation, enzymatic reactions, and amino acid metabolism. Suspensions of bacterial isolates were inoculated into the API 20E strips and incubated at 37°C for 18–24 hours. Results were interpreted using the API database to determine the biochemical profile, enabling accurate identification of Enterobacteriaceae species (Aryal, 2019).

Antimicrobial susceptibility test: The resistance of isolated strains of staphylococci and enterobacteria to antibiotics was assessed using the disk diffusion method, following the *Guidelines set forth standard methods for determining the sensitivity of microorganisms to antibacterial drugs* (МУК 4.2.1890-04; Krut, 2020). Mueller-Hinton agar was used as the medium for the test, prepared in accordance with the Clinical and Laboratory Standards Institute (CLSI, 2022) or The European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2022) standards. Antibiotic disks were carefully placed on the agar surface, with each disk labeled with the antibiotic name, content in micrograms, and expiration date to ensure reliability. After incubation at 35°C for 18–24 hours, zones of inhibition were measured, and results were interpreted based on standard breakpoints provided by EUCAST or CLSI. The clear zone formed is then grouped into sensitive (S), intermediate (I), or resistant (R) groups.

As a main panel, the following 22 antibiotics discs from 9 antibiotic groups have been used for each isolated and identified Enterobacteriaceae: Penicillins (Amoxicillin 10 µg - AMX; Ampicillin sulb. 20 µg - AMP/SUL), Cephalosporines (Ceftazidime 30 µg - CAZ; Cefotaxime 30 µg - CTX; Cephalothin 30 µg - CLO), Carbapenems (Imipenem 10 µg - IPM), Polypeptides (Polymyxin B 30 µg - PMB), Macrolides (Azithromycin 15 µg - AZM; Clarithromycin 15 µg - CLR; Erythromycin 15 µg - ERY), Tetracyclines (Tetracycline 30 µg - TET; Minocycline 30 µg - MIN), Aminoglycosides (Gentamicin 10 µg - GEN; Streptomycin 10 µg - STM; Neomycin 30 µg - NEO; Amikacin AK 30 µg - AMK; Netilmicin 30 µg - NET), Fluoroquinolones (Levofloxacin 5 µg - LVX; Ofloxacin 5 µg - OFL; Norfloxacin 10 µg - NOR; Nalidixic acid 30 µg - NAL), Amphenicols (Chloramphenicol 30 µg - CHL).

The following ATCC control strains were used for quality control in antibiotic susceptibility testing: *Salmonella Typhimurium* ATCC 14028 (Quanti-CultiControl, Liofilchem) and *Escherichia coli* ATCC 8739 (Quanti-CultiControl, Liofilchem).

The Multidrug-resistance (MDR) and Multiple Antimicrobial Resistance (MAR) index: Multidrug-resistant (MDR) strains were identified, with MDR defined as resistance to at least one agent in three or more antimicrobial categories, as outlined by international guidelines such as those from the Centers for Disease Control and Prevention (CDC) and European Centre for Disease Prevention and Control (ECDC). (Magiorakos *et al.*, 2012). The Multiple Antibiotic Resistance (MAR) Index has been calculated. MAR is a quantitative measure used to assess the antibiotic resistance patterns of bacterial isolates. It is a valuable tool for evaluating the extent of resistance and identifying high-risk sources of contamination. The formula for the MAR Index:

MAR Index = number of antibiotics to which the isolate is resistant/total number of antibiotics tested in the panel.

Interpretation: MAR Index ≤ 0.2: Indicates that the bacterial isolate originates from environments where antibiotics are rarely used, posing a lower risk. MAR Index > 0.2 suggests the isolate comes from environments with high antibiotic usage, such as hospitals, farms, or wastewater, representing a higher public health risk (Krumperman, 1983).

Result

From 160 samples from 80 pigs in general 50 Enterobacteriaceae family of bacteria (31.2%; n=160) have been isolated from which: *E. coli* 24 isolates (15.0%), *Klebsiella* spp. 10 isolates (6.2%), *Salmonella* spp. 6 isolates (3.7%), *Raoultella ornithinolytica* 4 isolates (2.5%), *Pseudomonas aeruginosa* 4 isolates (2.5%), *Proteus mirabilis* 2 isolates (1.2%).

Among the 22 antibiotics tested (belonging to 9 groups), the isolated Enterobacteriaceae showed high resistance to several of them. Specifically, on average, 94.5% of the isolated and identified Enterobacteriaceae exhibited resistance to CLR; 90.3% to ERY; 71.7% to CLO; and 56.4% to AMX.

E. coli exhibited varying resistance across different antibiotics, with a notable resistance to ERY (91.7%), STM-CLO (83.3%), and CLR (66.7%). The majority of antibiotics, such as CAZ-IPM-MIN-NET, showed relatively lower resistance levels, indicating a degree of susceptibility. *Klebsiella* spp. generally demonstrated higher resistance, especially to CLR-ERY (100.0%) and AMX-CLO (80%). *Salmonella* spp. displayed higher resistance, particularly to CLR; ERY (100.0%), and AMX-CLO-TET (66.6%). *Raoultella ornithinolytica*, while fewer isolates (n=4), showed resistance patterns,

particularly high resistance to AMX-CLO-CLR (100.0%). *Proteus mirabilis* showed a higher resistance to IPM-PMB-AZM-CLR- ERY-MIN-STM-CHL (100.0%) (Table 1).

In general, we observed zones ranging from 0 to 48 mm (Fig. 1). Among the 50 Enterobacteriaceae tested, only one *E. coli* isolated showed a mean value of 13.1 mm for all 22 antibiotics. In the remaining cases (n=23), the results ranged from 17.4 mm to 23.4 mm for *E. coli*. In the case of other Enterobacteriaceae (n=26), the results ranged from 14.9 mm (*Klebsiella aerogenes*) to 24.5 mm (*Salmonella enterica).*

MDR profiles of Enterobacteriaceae isolates demonstrated notable differences between *E. coli* (n=24) and other Enterobacteriaceae species (n=26). Among the *E. coli* isolates, 50% exhibited resistance to five antibiotic groups, while 33.3% displayed resistance to three groups. In contrast, other Enterobacteriaceae exhibited a more varied resistance profile. Resistance to five antibiotic groups was observed in 38.5% of isolates, followed by 23.0% with resistance to six groups (Table 2).

The MAR index further delineates the resistance characteristics of the isolates. For *E. coli*, 33.3% of isolates had MAR indices of 0.2 and 0.3, while 16.7% exhibited a MAR index of 0.1. Notably, no *E. coli* isolates were recorded with MAR indices of 0.6 or higher. Other Enterobacteriaceae displayed a more varied MAR index distribution. The most frequent MAR index values were 0.3 (30.8%) and 0.4 (23.0%), followed by 0.1 and 0.5, each accounting for 15.3% of isolates (Table 3).

Table 1 Antibiotic resistance profile (% of resistance) of various Enterobacteriaceae species.

Antibiotic discs	*E. coli*	*Klebsiella* spp.	*Salmonella* spp.	*Raoultella ornithinolytica*	*Pseudomonas* spp.	*Proteus mirabilis*	*Total Enterobacteria*
	n=24	*n=10*	*n=6*	*n=4*	*n=4*	*n=2*	*n=50*
AMX 10	41.7	80.0	66.6	100.0	50.0	0.0	56.4
AMP/SUL 20	8.3	0.0	33.3	0.0	50.0	0.0	15.3
CAZ 30	0.0	20.0	0.0	0.0	50.0	0.0	11.7
CTX 30	8.3	20.0	0.0	0.0	50.0	0.0	13.1
CLO 30	83.3	80.0	66.6	100.0	100.0	0.0	71.7
IPM 10	0.0	20.0	0.0	50.0	50.0	100.0	36.7
PMB 30	8.3	0.0	0.0	0.0	0.0	100.0	18.1
AZM 15	25.0	40.0	0.0	0.0	50.0	100.0	35.8
CLR 15	66.7	100.0	100.0	100.0	100.0	100.0	94.5
ERY 15	91.7	100.0	100.0	50.0	100.0	100.0	90.3
TET 30	25.0	40.0	66.6	50.0	50.0	50.0	46.9
MIN 30	0.0	0.0	0.0	0.0	0.0	100.0	16.7
GEN 10	8.3	0.0	0.0	0.0	0.0	50.0	9.7
STM 10	83.3	0.0	33.3	50.0	0.0	100.0	44.4
NEO 30	41.7	0.0	0.0	50.0	0.0	0.0	15.3
AMK 30	16.7	0.0	0.0	0.0	0.0	0.0	2.8
NET 30	0.0	0.0	0.0	0.0	0.0	0.0	0.0
LVX 5	8.3	40.0	0.0	50.0	50.0	0.0	24.7
OFL 5	8.3	0.0	0.0	50.0	50.0	0.0	18.1
NOR 10	8.3	0.0	0.0	50.0	50.0	0.0	18.1
NAL 30	25.0	0.0	33.3	50.0	100.0	0.0	34.7
CHL 30	25.0	40.0	33.3	0.0	100.0	100.0	49.7

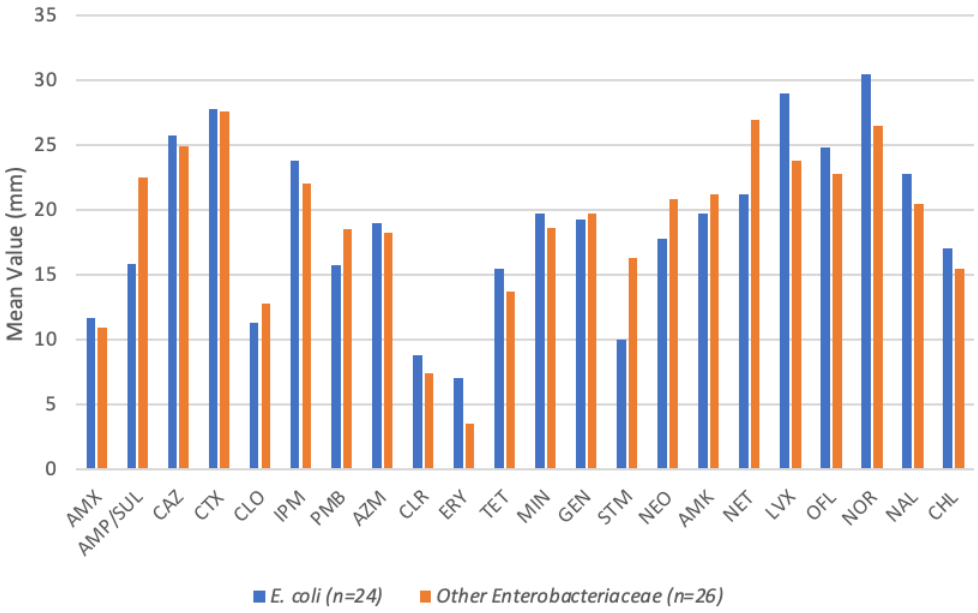

Figure 1 Average zone diameters of antibiotic discs for tested 50 Enterobacteriaceae.

Table 2 MDR profile of isolated Enterobacteriaceae.

N of Antibiotic group	*E. coli* *n=24*	*E. coli* *(%)*	*Other Enterobacteriaceae* *n=26*	*Other Enterobacteriaceae (%)*
1	0	0.0	4	15.4
2	2	8.3	0	0.0
3	8	33.3	2	7.7
4	2	8.3	2	7.7
5	12	50.0	10	38.5
6	0	0.0	6	23.0
7	0	0.0	2	7.7

Table 3 MAR index of isolated Enterobacteriaceae.

MAR Index	*E. coli* *n=24*	*E. coli* *(%)*	*Other Enterobacteriaceae* *n=26*	*Other Enterobacteriaceae (%)*
0.0	0	0.0	2	7.8
0.1	4	16.7	4	15.3
0.2	8	33.3	2	7.8
0.3	8	33.3	8	30.8
0.4	2	8.3	6	23.0
0.5	2	8.3	4	15.3
0.6	0	0.0	0	0.0
0.7	0	0.0	0	0.0
0.8	0	0.0	0	0.0
0.9	0	0.0	0	0.0
1.0	0	0.0	0	0.0

Discussion

The findings of this study underscore the significant public health challenge posed by MDR Enterobacteriaceae isolated from healthy pigs. A considerable proportion of the isolates exhibited resistance to multiple antibiotic classes. These results align with previous studies documenting high levels of resistance among Enterobacteriaceae isolated from livestock, driven largely by inappropriate antibiotic use in animal farming (Uzeh *et al*., 2021; Kamel *et al*., 2024).

The high resistance levels observed in *Klebsiella* spp. and *Pseudomonas* spp. highlight their well-documented intrinsic resistance mechanisms, such as efflux pumps and low outer membrane permeability (Pang *et al*., 2019; Calland *et al*., 2023; Ajayi *et al*., 2023). However, in addition to intrinsic resistance, these bacteria are also known to acquire resistance through horizontal gene transfer, including plasmids, transposons, and integrons carrying resistance genes (Scott *et al*., 2019). The identification of resistance to critically important antibiotics, such as cephalosporins (e.g., CLO) and macrolides (e.g., ERY), raises concerns about the potential spread of resistance genes from animals to humans. This transfer can occur through direct contact with animals or indirectly via contaminated food products, exacerbating the global burden of antimicrobial resistance (Lechner *et al*., 2020).

In our research *Pseudomonas* spp. exhibited significant resistance, particularly to CLO, CLR, ERY, NAL, and CHL (100.0%), which aligns with the well-documented natural resistance of *Pseudomonas* species to multiple antibiotic classes (Adejobi *et al*., 2021; Elfadadny *et al.* 2024).

The observed variation in resistance profiles across different bacterial genera reflects their unique genetic and physiological adaptations. For instance, in our research, *Proteus mirabilis* displayed notable resistance to eight antibiotics from four antibiotic classes. Similarly, *E. coli*, identified as MDR across all isolates, serves as a critical indicator of AMR dynamics in pig farming due to its role as a commensal organism and reservoir for resistance genes (Brisola *et al*., 2019).

The analysis reveals striking differences in the MDR profiles of *E. coli* and other *Enterobacteriaceae*. While half of the *E. coli* isolates showed resistance to five antibiotic groups, this level of resistance was less common in other *Enterobacteriaceae*. The absence of *E. coli* isolates with resistance to six or more antibiotic groups contrasts with the significant proportion of other *Enterobacteriaceae* displaying resistance to six or seven groups. These results suggest that non-*E. coli Enterobacteriaceae* may harbor a broader range of resistance mechanisms, potentially reflecting their ability to thrive in diverse environments or acquire resistance genes through horizontal gene transfer. Previous studies (Partridge, 2015) have shown that Enterobacteriaceae are highly adapted to exchanging genetic material, with much of their antimicrobial resistance attributed to mobile resistance genes. The presence of multiple resistance genes on a single plasmid allows bacterial cells to acquire multidrug resistance in a single genetic event. This co-localization of resistance genes also means that the use of one antibiotic can co-select for the spread of resistance to other, unrelated antibiotics, further exacerbating the challenge of controlling antimicrobial resistance.

The MAR index values highlight a notable level of antimicrobial resistance among both *E. coli* and other Enterobacteriaceae species. The predominance of moderate MAR index values (0.2–0.4) in both groups suggests that the bacteria have been exposed to subtherapeutic levels of antibiotics, which is commonly associated with agricultural use. The absence of isolates with MAR indices ≥0.6 suggests that extreme multidrug resistance remains rare in this population. Interestingly, the presence of two fully susceptible isolates among other Enterobacteriaceae and the absence of such isolates among *E. coli* highlight potential differences in selective pressure or intrinsic

resistance mechanisms between these groups. The suggestion of differing selective pressures and intrinsic resistance mechanisms aligns with existing literature (Holmes *et al.*, 2016).

The broader distribution of MAR was observed in non-*E. coli* Enterobacteriaceae underscores the considerable heterogeneity in resistance mechanisms within this family. These findings suggest that different species of Enterobacteriaceae harbor distinct resistance profiles, which may be influenced by factors such as genetic diversity, the presence of resistance genes, and environmental pressures (Munita and Arias, 2016; Davin-Regli *et al.*, 2019). Such variability in resistance mechanisms complicates efforts to control these pathogens and underscores the need for targeted surveillance and treatment strategies (Singh and Kim, 2025).

Interestingly, antibiotics such as polymyxin B and amikacin showed relatively lower resistance rates, suggesting their potential as alternative therapeutic options for severe human infections caused by resistant Enterobacteriaceae. However, their judicious use is imperative to prevent the development of further resistance. The risk posed by polymyxin-resistant nosocomial pathogens, including members of the Enterobacteriaceae family, is extremely alarming (Mohapatra *et al.*, 2021) because these pathogens exhibit resistance to nearly all available antibacterial agents, severely limiting treatment options and posing a significant threat to patient outcomes and public health. The effectiveness of amikacin against Enterobacteriaceae was established long ago and has been well-documented in clinical and microbiological studies (Jones and Packer, 1982; Moody *et al.*, 1987). In addition, as we reference the emergence of polymyxin-resistant nosocomial pathogens, it is important to emphasize the necessity of adhering to clinical guidelines for antibiotic selection, which are based on the principles of antimicrobial susceptibility testing. These guidelines help ensure appropriate use and contribute to the reduction of contradictions between therapeutic options and the rise of resistant pathogens.

Comparing these findings with existing literature highlights the need for stringent regulatory policies and surveillance programs to mitigate AMR in animal agriculture, particularly in regions with limited oversight, such as low- and middle-income countries (Schar *et al.*, 2018; Ya *et al.*, 2023).

In conclusion, this study underscores the serious challenge of MDR Enterobacteriaceae in pig farming, which has high resistance, particularly to some cephalosporins and macrolides. In Armenia, AMU-related resistance in healthy pigs, despite the absence of active infection, raises concerns about the potential transfer of these bacteria to the human food chain. The variation in resistance profiles between *E. coli* and other Enterobacteriaceae highlights the complexity of AMR transmission, suggesting that non-*E. coli* strains may possess broader resistance mechanisms due to horizontal gene transfer or environmental adaptability. The moderate MAR index values emphasize the role of subtherapeutic antibiotic use in Armenia's backyard pig farming. Given these findings, immediate action is necessary, including stricter regulations to control antibiotic overuse, especially in regions like Armenia with limited oversight. Enhanced surveillance, improved farming practices, and stronger biosecurity measures are crucial to mitigate AMR and its potential public health impact.

Acknowledgment

This work was funded by the Science Committee of the Ministry of Education, Science, Culture and Sports of the Republic of Armenia (research project number 21T-4A187).

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