

Prevalence of Multidrug-resistant *Escherichia coli* in the Swine Production Chain: Implications for Food Safety in Brazilian Slaughterhouses

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Abstract

Multidrug resistance is a significant threat to global public health. This study aimed to detect and characterize antimicrobial-resistant microorganisms in swine feces and carcasses obtained from slaughterhouses. A total of 214 samples were collected, comprising 111 swine feces and 103 from carcasses, across two different slaughterhouses. Polymerase chain reaction was used to detect antimicrobial resistance genes, identifying 22 resistant isolates that were subsequently confirmed to be *Escherichia coli*. Pulsed-field gel electrophoresis was performed on these 22 isolates to investigate their genetic similarity. The Kirby–Bauer disk diffusion method characterized phenotypic resistance to different antimicrobials. The *bla*_{CTX-M-1} gene was detected in all 22 *E. coli* isolates. Additionally, 90.9% of the strains were considered multidrug-resistant, exhibiting resistance to at least three different antimicrobial classes. The isolates exhibited high genetic diversity. The presence of these multidrug-resistant bacteria in swine and animal-derived foods emphasizes the importance of sanitation measures during production to protect public health.

Keywords: antimicrobial resistance; *bla*_{CTX-M}; carcasses; ESBL; pig; public health.

Practical Application: Resistant microorganisms contaminating swine carcasses in Brazil's food chain production.

1 INTRODUCTION

Escherichia coli is one of the most important pathogenic microorganisms in animal production, responsible for causing significant gastrointestinal infections, known as colibacillosis, in animals. In swine, this microorganism can cause both neonatal and post-weaning diarrhea. Additionally, it is one of the microorganisms responsible for Metritis–Mastitis–Agalactia (MMA) syndrome in sows. MMA affects colostrum and milk production, consequently harming piglet nutrition and leading to increased morbidity and mortality at this stage (Hirsch et al., 2003). Although outbreaks caused by *E. coli* strains are not common in pig farms, when they occur, they cause significant economic losses due to high rates of mortality and morbidity, as well as a significant impact on zootechnical performance (Tran et al., 2018).

Due to these challenges in pig farming, in low- to middle-income countries and some regions of America, producers often resort to treatment and prevention strategies, including antimicrobial therapy and growth promoters, to mitigate damage and

minimize negative impacts on production (Holman & Chénier, 2015; Van Boeckel et al., 2019). However, indiscriminate use of antimicrobials in these countries poses significant risks to human, animal, and environmental health. These risks include residues in food, microbial resistance, and contamination of water and soil (Cheng et al., 2019).

The risks can also arise from the presence of microorganisms in meat and meat products, including toxin-producing species like *E. coli* O157 hemorrhagic responsible for producing vero/shiga toxin, which can contaminate food during slaughter and processing. This contamination compromises food safety and can lead to outbreaks in humans (Conedera et al., 2007; Guragain et al., 2024).

Concerns about the contamination of foods by multidrug-resistant (MDR) bacteria have increased over the years (Albernaz-Gonçalves et al., 2021). This issue is attributed to the selection pressure favoring microorganisms that adapt through the dissemination and expression of resistance genes and other mechanisms (Holmes et al., 2016). Several genes related to

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antimicrobial resistance have been detected in *E. coli* strains isolated from animal production. The *bla*_{CTX-M} gene is one of the most prevalent genes conferring resistance to beta-lactam antibiotics, specifically to third- and fourth-generation cephalosporins, which are widely used in both human and veterinary medicine (Cantón et al., 2012; Cormier et al., 2019). This gene encodes extended-spectrum beta-lactamase (ESBL) enzymes, which can hydrolyze the beta-lactam ring in these antimicrobials, rendering them ineffective (Lima et al., 2020). Over the years, extensive research has been conducted on ESBL enterobacteria in livestock animals (Benavides et al., 2021; Tseng et al., 2023).

Extensive research on multidrug resistance has shown a growing resistance in opportunistic bacteria to various antimicrobial agents, including streptomycin, ampicillin, tetracycline, and sulfamethoxazole. This resistance is particularly predominant in *E. coli* strains from farm animals, as reported in countries like China (Zhou et al., 2022), the United States (Sodagari & Varga, 2023), Tanzania (Kimera et al., 2021), the United Kingdom (Yang et al., 2020), Estonia (Aasmäe et al., 2019), and Ghana (Larbi et al., 2021). The spread of resistance between strains is facilitated by the presence of genes on mobile genetic elements, such as plasmids, integrons, and insertion sequences, which enable gene transfer between different bacteria. This promotes the dissemination of resistance genes (Shafiq et al., 2023). Due to the significant spread and increasing prevalence of MDR bacteria, there is a growing emphasis on the importance of constant monitoring of antimicrobial resistance in production animals. Such monitoring is crucial for producing safe food and providing solutions to antimicrobial resistance (Hesp et al., 2021).

Given the critical need to monitor antimicrobial resistance, this study focused on the identification of antimicrobial resistance genes, the isolation of microorganisms carrying these genes, phenotypic analyses of resistance to various antimicrobial agents, and the assessment of genetic similarity between isolates obtained from fecal samples and swine carcasses.

1.1 Relevance of the work

Escherichia coli is a major pathogen in animal production, impacting swine health through neonatal and post-weaning diarrhea, as well as in the final fattening and slaughter phase. This study investigated antimicrobial resistance genes in *E. coli* isolated from swine feces and carcasses, revealing a high prevalence of multidrug-resistant strains. The *bla*_{CTX-M-1} was detected in all isolates, indicating resistance to beta-lactam antibiotics. The findings highlight the risks of indiscriminate antimicrobial use in animal production, emphasizing the need for better monitoring and regulatory measures in the food production chain to ensure food safety and protecting both animal and human health.

2 MATERIALS AND METHODS

2.1 Sampling

Samples were previously collected in 2012 from two distinct slaughterhouses in Jaboticabal region of Sao Paulo State and

were stored in a freezer at -80°C (Borges et al., 2012). These samples were later recovered from the Bacterial Cell Bank of the Biotechnology and Bacterial Resistance laboratory (BIOBAC) for analysis in the present study. The samples included 51 fecal samples and 45 carcass samples from Slaughterhouse A, and 60 fecal samples and 58 carcass samples from Slaughterhouse B, totaling 214 samples from different pigs (111 fecal and 103 carcass samples). Each sample was streaked onto MacConkey agar plates and was incubated at 37°C for 24 h. Five colonies with distinct morphologies from each sample were then selected, streaked onto brain–heart infusion (BHI) agar, and subsequently transferred to BHI broth for DNA extraction and screening for resistance-related genes (*bla*_{CTX-M-1}, *bla*_{CTX-M-2}, *bla*_{CMY-1}, *bla*_{CMY-2}, *bla*_{NDM}, *bla*_{KPC}, and *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*) by polymerase chain reaction (PCR). Colonies positive for at least one resistance gene were further analyzed to confirm species via PCR. Finally, these isolates were subjected to antibiogram testing by disk diffusion to assess phenotypic resistance and genetic similarity analysis using the PFGE technique (Figure 1).

2.2 Polymerase chain reaction for the identification of resistance genes and species

Bacterial DNA was extracted using the boiling method for 10 min (Ewers et al., 2004). PCR was then performed with a mixture containing 0.4 μL of 10 mM dNTPs (ThermoFisher Scientific®), 2 μL of 10X Taq Polymerase Buffer (ThermoFisher Scientific®), 0.8 μL of 50 mM MgCl_2 (ThermoFisher Scientific®), 1 μL of 5 pmole primer (Sigma Aldrich®), 0.2 μL of Taq Polymerase, 1 μL of DNA template, and ultrapure water to reach a total volume of 20 μL . This technique was used to screen beta-lactam resistance genes such as *bla*_{CTX-M-1}, *bla*_{CTX-M-2}, *bla*_{CMY-1}, and *bla*_{CMY-2}; carbapenem resistance genes *bla*_{KPC} and *bla*_{NDM}; and genes *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* (Table 1). Then, PCR was also used to identify the species of the resistant isolates with primers specific to *E. coli*. It is important to note that the colonies of the resistant isolates were lactose-positive. Therefore, we considered confirming other species, but all 22 isolates were identified as *E. coli*.

2.3 Antimicrobial susceptibility testing

The isolates underwent antimicrobial susceptibility testing using the Kirby–Bauer disk diffusion method, following the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2024b). A variety of antimicrobials were tested, including amoxicillin+clavulanic acid (30 μg), ampicillin (10 μg), amikacin (30 μg), ceftiofur (30 μg), ceftazidime (30 μg), cefotaxime (30 μg), cefoxitin (30 μg), ceftriaxone (30 μg), ciprofloxacin (5 μg), fosfomycin (50 μg), norfloxacin (10 μg), tetracycline (30 μg), streptomycin (10 μg), sulfamethoxazole + trimethoprim (25 μg), and imipenem (10 μg). The inhibition zone diameters were measured with a graduated ruler and compared with reference breakpoint tables for humans, provided by the CLSI (2024b) for most antimicrobials. For ceftiofur, the CLSI VET (CLSI, 2024a) breakpoint table for swine was used (CLSI VET 01S-ED7, 2024). Isolates were classified as MDR if they exhibited resistance to at least three different classes of antimicrobials (Magiorakos et al., 2011).

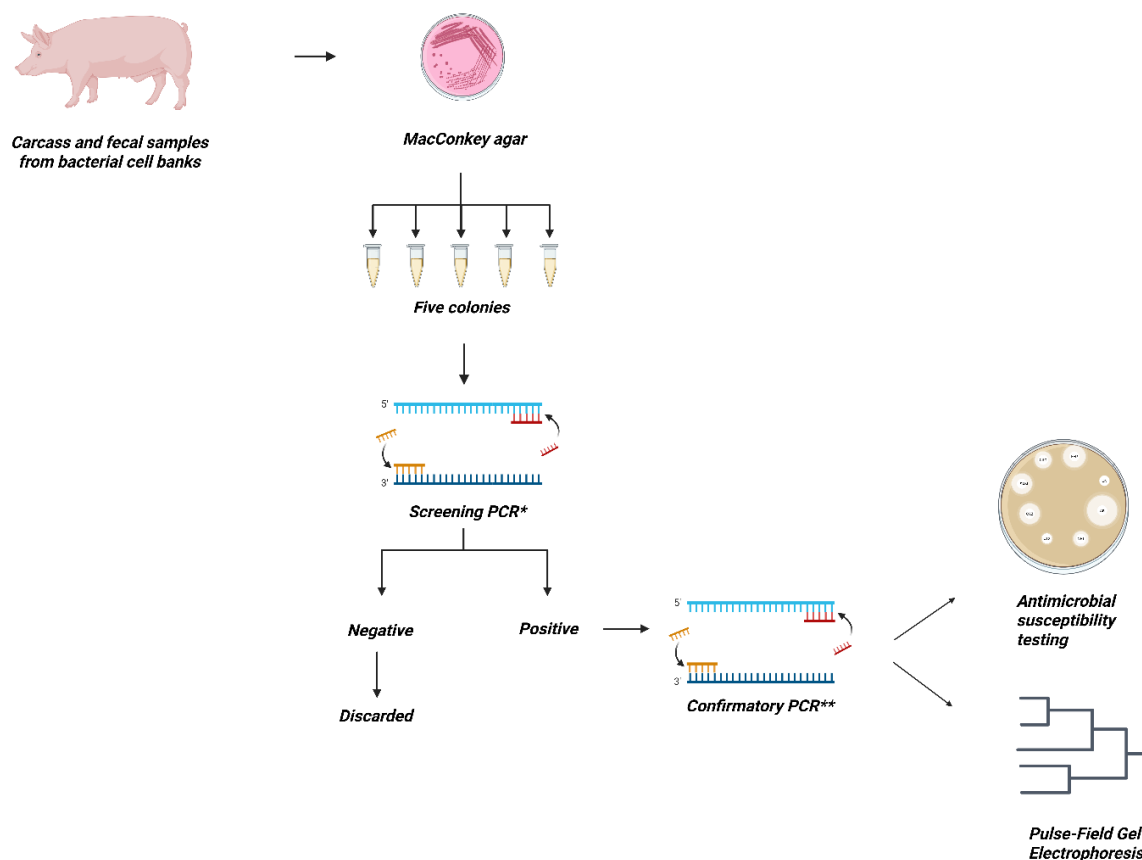


Figure 1. Flowchart for sample processing and antimicrobial resistance analyses and genetic similarity evaluation.

PCR: polymerase chain reaction.

*Screening PCR for antimicrobial resistance genes (*bla*_{CTX-M-1}, *bla*_{CTX-M-2}, *bla*_{CMY-1}, *bla*_{CMY-2}, *bla*_{NDM}, *bla*_{KPC}, and *mcr*-1 to 4)

**PCR for confirmation of positive isolate species.

Table 1. Gene, primer sequence, and the size of the product utilized for polymerase chain reaction.

Groups	Gene	Primer sequence (5' – 3')	Size of the product (pb)	Reference
β-Lactam	<i>bla</i> _{CTX-M-1}	F – TTAGGAARTGTGCCGCTGYA R – CGATATCGTTGGTGGTRCCAT	688	[25]
	<i>bla</i> _{CTX-M-2}	F – CGTTAACGGCACGATGAC R – CGATATCGTTGGTGGTRCCAT	404	[25]
	<i>bla</i> _{CMY-1}	F – ATGCAACAACGACAATCCATCCTG R – TCAACCGGCCAACTGCCGAGGAT	1560	[26]
	<i>bla</i> _{CMY-2}	F – ATGATGAAAAAATCGTTATGCT R – TTATTGCAGCTTTTCAAGAATGCG	3202	[26]
Carbapenem	<i>bla</i> _{NDM}	F – GGTTTGGCGATCTGGTTTTTC R – CGGAATGGCTCATCACGATC	621	[27]
	<i>bla</i> _{KPC}	F – CGTCTAGTTCTGCTGTCTTG R – CTTGTCATCCTTGTTAGGCG	798	[27]
Polymyxins	<i>mcr</i> -1	F – AGTCCGTTTGTCTTGTGGC R – AGATCCTTGGTCTCGGCTTG	320	[28]
	<i>mcr</i> -2	F – CAAGTGTGTTGGTCGCAGTT R – TCTAGCCCGACAAGCATACC	715	[28]
	<i>mcr</i> -3	F – AAATAAAAAATTGTTCCGCTTATG R – AATGGAGATCCCCGTTTTT	929	[28]
	<i>mcr</i> -4	F – TCACCTTCATCACTGCGTTG R – TTGGTCCATGACTACCAATG	1116	[28]
Species	<i>Escherichia coli</i>	F – GGGAGTAAAGTTAATACCTTTGCTC R – TTCCCGAAGGCACATTCT	584	[24]

2.4 Genetic similarity analysis

To assess the genetic similarity between strains isolated from fecal samples and pig carcasses, we employed the pulsed-field gel electrophoresis (PFGE) technique as described by Ribot et al. (2006). Chromosomal DNA and plugs were prepared by digesting with the Xba I enzyme (Invitrogen®, USA), and the *Salmonella* strain Braenderup H9812 was used as a molecular weight reference. Electrophoresis was conducted for 21 h at 14°C using a 1% pulse field-certified agarose gel following the standard protocol. Fragment similarity was analyzed using the Dice coefficient with 1% tolerance and 0.5% optimization. The resulting data were used to generate a dendrogram through unweighted pair group method with arithmetic mean (UPGMA) clustering, performed with BioNumerics software version 7.1 (Applied Maths, Sint-Matens-Latem, Belgium).

3 RESULTS

A total of 22 *E. coli* strains were identified: 13 from fecal samples and 9 from carcasses, all from different animals, using PCR. Among the resistance genes examined (*bla*_{CTX-M-1}, *bla*_{CTX-M-2}, *bla*_{CMY-1}, *bla*_{CMY-2}, *bla*_{NDM}, *bla*_{KPC}, and *mcr-1*, 2, 3, and 4), only *bla*_{CTX-M-1} was detected in all strains.

The strains exhibited the highest resistance rates to the following antimicrobial agents: streptomycin (90.9%), tetracycline (90.9%), sulfamethoxazole + trimethoprim (86.4%), and ampicillin (72.7%).

Imipenem was effective against all isolates, while 95.5% of the isolates were sensitive to amikacin, ceftiofur, ceftazidime, and fosfomycin, and 90.9% were sensitive to ceftiofur and ceftriaxone (Table 2). According to the antimicrobial susceptibility test, 90.9% of the isolates exhibited multidrug resistance

(Magiorakos et al., 2011), with the most common resistance phenotype including streptomycin, sulfamethoxazole + trimethoprim, and tetracycline (Table 2).

In the PFGE technique used for genetic similarity analysis, the isolates displayed a range of genetic diversity (Figure 2). Isolates 4 and 6 from the fecal sample were genetically similar. Isolates 14 and 15 from one carcass sample showed genetic similarity to each other, while isolates 18 and 19 from another carcass sample exhibited 100% genetic similarity among themselves. Isolates 18 and 19 exhibited identical resistance patterns to the same antimicrobial agents. In contrast, isolates 4 and 6 differed in their resistance profiles: isolate 4 was resistant to ciprofloxacin, ceftazidime, and ampicillin, whereas isolate 6 was sensitive to these agents. Furthermore, strains 14 and 15 also differed in their resistance to ceftiofur, with strain 14 being sensitive and strain 15 resistant.

4 DISCUSSION

E. coli predominates in the intestines of both animals and humans due to its strong competitive ability and capacity for colonization. This characteristic led to the isolation of only *E. coli* from the samples analyzed, resulting in the identification of 22 isolates. Additionally, it has been shown that commensal and potentially pathogenic bacteria in the gastrointestinal tract often carry genes that confer resistance to various antimicrobials (Crits-Christoph et al., 2022).

The ESBL enzyme is commonly found in bacteria, especially *E. coli*, isolated from various sources, including humans, poultry, cattle, and even wild animals (Gundran et al., 2019; Guyomard-Rabenirina et al., 2020; Yamanaka et al., 2020). This widespread occurrence is largely due to the *bla*_{CTX-M} gene, which is widely distributed by plasmid-mediated transfer

Table 2. Phenotypic resistance profile of isolates to different antimicrobial agents.

		Slaughterhouse A										Slaughterhouse B											
		Feces										Carcasses											
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Aminoglycosides	AMI	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	EST	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R	R
Cephalosporins (2nd generation)	CFO	S	R	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S
	CFT	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S
Cephalosporins (3rd generation)	CRO	S	S	S	S	S	S	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S
	CTX	S	S	S	S	S	S	S	S	S	R	R	S	S	S	R	S	S	S	S	S	R	S
	CAZ	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Carbapenems	IPM	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Penicillins	AMP	R	S	R	R	R	S	R	S	R	R	R	R	R	R	R	S	S	R	R	R	S	R
	AMC	S	S	S	S	S	S	S	S	R	R	R	R	R	S	S	S	S	R	R	R	S	R
Fluoroquinolones (2nd generation)	CIP	R	S	S	R	S	S	S	R	S	R	S	R	R	R	R	S	R	S	S	S	S	R
	NOR	R	R	R	S	R	S	S	R	R	R	S	S	R	S	S	S	R	S	S	S	S	R
Fosfomycin	FOS	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S
Sulfonamides + trimethoprim	SUT	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	R	R	R	R	R	R
Tetracyclines	TET	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	S	R	R	R	R	R	R

*AMI: Amikacin, EST: Streptomycin, CFO: Cefoxitin, CFT: Ceftiofur, CRO: Ceftriaxone, CTX: Cefotaxime, CAZ: Ceftazidime, IPM: Imipenem, AMP: Ampicillin, AMC: Amoxicillin + clavulanic acid, CIP: Ciprofloxacin, NOR: Norfloxacin, FOS: Fosfomycin, SUT: Sulfamethoxazole + trimethoprim, TET: Tetracycline. **S: Sensible, R: Resistant.

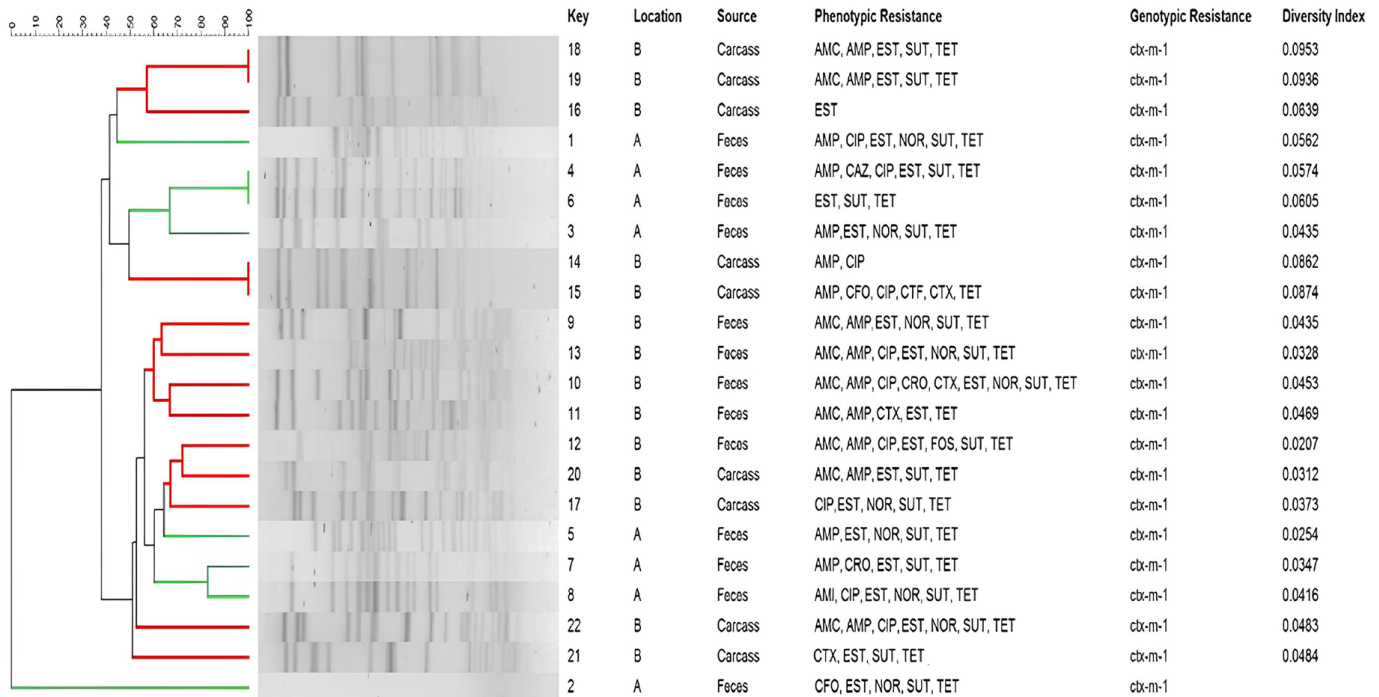


Figure 2. Dendrogram demonstrating the genetic diversity among the 22 strains of *Escherichia coli* isolated from pig carcass and fecal samples and their origin.

and expressed among different bacterial strains (Yu et al., 2024). Enterobacteria carrying the *bla*_{CTX-M-1} gene have been isolated from pig samples in slaughterhouses and breeding facilities in several countries, including the United Kingdom, Switzerland, Argentina, and Thailand (Faccone et al., 2019; Geser et al., 2011; Mitsuan et al., 2023; Randall et al., 2014). In this study, *E. coli* strains carrying the *bla*_{CTX-M-1} gene were detected in samples from swine carcasses and feces. This finding is consistent with a study also conducted in Brazil, which identified *bla*_{CTX-M-1}-positive *E. coli* in healthy pigs (Oliveira, Silva et al., 2024).

Genes from the *bla*_{CTX-M} family have been identified in bacteria isolated from a range of animal species, including dogs, chickens, swine, horses, cattle, as well as in humans. This widespread distribution indicates significant gene dissemination across various environmental niches. Such an extensive spread may be attributed to the presence of these genes in mobile genetic elements that can be transferred between different bacterial species (Cormier et al., 2019).

In this study, we analyzed the *mcr*-1, 2, 3, and 4 genes, which are related with resistance to the polymyxin antimicrobial class (Nakano et al., 2021; Wang et al., 2018). Although none of these four genes were detected, the presence of newer variants such as *mcr*-5, 6, 7, 8, 9, and 10, which are found in various plasmids, cannot be excluded due to ongoing genetic evolution and discovery (Hamel et al., 2021; Hussein et al., 2021).

The highest antimicrobial resistance rates among the isolates in this study were observed for streptomycin (90.9%), tetracycline (90.9%), sulfamethoxazole (86.4%), and ampicillin (72.7%). These findings suggest that MDR *E. coli* isolated from

healthy swine, as described in the literature, show that both sick and healthy pigs can harbor this resistance pattern (Zhou et al., 2022). This resistance can be explained due to the indiscriminate use of these drugs in animal farming (Kieffer et al., 2018; Rabello et al., 2020; Spindola et al., 2018).

In Brazilian swine farming, antimicrobials like penicillin, amoxicillin, gentamicin, quinolones, tetracyclines, and sulfonamides are administered indiscriminately through feed additives to serve as prophylactics and growth promoters, enhancing production efficiency and zootechnical performance (Oliveira, Santa Rosa et al., 2024). However, the indiscriminate use of these drugs contributes to the development of bacterial resistance, which poses risks to animals, humans, and the environment (Viana et al., 2020). In contrast, several European countries, such as Norway, Sweden, Denmark, and the Netherlands, have banned the use of antimicrobials in pig farming. This has resulted in a significant decrease in the use of these drugs in several European countries, through a joint effort among different production sectors, leading to the hope of reversing microbial resistance and providing benefits for public health. Thus, Europe is becoming a model to be followed in the fight against antimicrobial resistance (Dewulf et al., 2022).

MDR bacteria remain a significant challenge in animal production, continuing to be a problem even after nearly a decade. The present study, along with recent literature, highlights the substantial prevalence of antimicrobial resistance. Additionally, MDR *E. coli* strains were isolated from two different swine slaughterhouses in the Federal District of Brazil between 2019 and 2021. The study revealed a high prevalence of strains resistant to amoxicillin, ampicillin, and streptomycin, and some

strains even showed resistance to colistin. These findings underscore the widespread dissemination of resistance genes among bacteria on surfaces, utensils, and equipment that may come into contact with carcasses (Santos et al., 2022).

The only antimicrobial showing 100% sensitivity was imipenem, a beta-lactam antibiotic from the carbapenem subgroup. This high sensitivity is consistent with recent findings (Egbule et al., 2021) and is likely due to the lack of imipenem use in local swine farming, leading to minimal bacterial exposure to this drug. Additionally, over 90% of strains were found to be susceptible to third-generation cephalosporins, such as ceftiofur, ceftazidime, and ceftriaxone. This susceptibility can be attributed to the reduced use of these antimicrobials in animal farming over the past two decades.

Using the PFGE technique (Figure 1), the formation of distinct clusters in the dendrogram was observed, indicating a high degree of genetic diversity among the isolates. This suggests the presence of various enterobacterial strains in animals and their products, such as meat, each with different antimicrobial resistance profiles.

In contrast to the present study, other research has reported high genetic similarity among *E. coli* strains found in swine at slaughterhouses and on their carcasses, suggesting that animal products may be contaminated by the same strains present in production and potentially transmitted to humans (Zelendova et al., 2020). In this study, isolates with 100% genetic similarity were identified, but these came from the same source and exhibited only phenotypic differences (isolates 4 and 6, 14 and 15, 18 and 19). This implies that while these isolates may share a common origin, they could have acquired mobile genes over time, leading to variations in antimicrobial resistance expression. Such a genetic transfer can occur through plasmid-mediated conjugation, where different genes are exchanged between strains (Headd & Bradford, 2020).

5 CONCLUSIONS

In this study, 90.9% of the isolates were multidrug-resistant, highlighting concerns about the improper use of antimicrobials in swine farming. Identifying genes associated with antimicrobial resistance in *E. coli* is crucial for detecting strains from swine that could pose a risk to human health.

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