Vietnam Journal of Food Control
A Scientific Journal of National Institute for Food Control
Journal homepage: https://vjfc.nifc.gov.vn

Research Article

Changes in antibiotic resistance trends (phenotype and genotype) of *Salmonella* isolated from pork at traditional markets in Hanoi

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(Received: 25 Feb 2025; Revised: 21 Apr 2025; Accepted: 13 May 2025)

Abstract

The study aims to analysis the trend of the phenotype and genotype resistance profiles of *Salmonella* isolates from pork collected in Hanoi traditional markets in 2018 and 2023 for commonly used antibiotics. The results showed a significant increase in the resistance rate of two antibiotics including ceftazidime (2.4% and 10.2%) and amoxicillin/clavulanic acid (0% and 35.6%) over five years ($p<0.05$). *Salmonella* spp. isolates in 2018 and 2023 were highly resistant to tetracycline (83.3% and 79.7%), sulfonamide (78.6% and 83.1%), ampicillin (71.4% and 67.8%), while were completely susceptible to imipenem and norfloxacin. However, the change in resistance to these antibiotics between the 2018 and 2023 isolates were not statistically significant. The study identified 2/110 (1.98%) colistin-resistant *Salmonella* isolates, of which the 2018 isolate carried gene *mcr1*, and the 2023 isolate carried gene *mcr3*. In addition, we found 7 *Salmonella* isolates with ESBL-producing phenotypes, of which 6/59 (10.2%) isolates were in 2018 and 1/42 (2.4%) isolate was in 2023. Of these, 5 isolates carried gene *blaCTX*-M, 2 isolates carried both the *blaCTX*-M and *blaTEM*, and no isolates carried the *blaSHV*. The research results provide scientific evidence showing the need to strengthen management and monitoring of antibiotic status to limit resistance rates and the spread of drug-resistant bacteria.

Keywords: *Salmonella, antibiotic resistance, pork, traditional market, Hanoi.*

1. INTRODUCTION

Pig sector is the most important for food supply in Vietnam and that has grown strongly currently. According to the report of the Department of Animal Husbandry, Vietnam is one of the 10 countries with the highest pork consumption in the world, with the average pork consumption per capital increasing continuously over the years. Specifically, in 2021, about 30 kg of pork meat/person/year, in 2022, about 32 kg of pork meat/person/year, and in 2023, about 33.8 kg of pork meat/person/year, and in 2024, it is estimated to reach 37.04 kg of pork meat/person/year [1]. However, the use of antibiotics in livestock farming has not been well monitored, which might be the cause of drug resistance [2]. Many previous studies have pointed out the potential risk of transmitting drug-resistant bacteria between livestock and humans through the food supply chain (food, drinking water), through contact or through the environment [3, 4]. More seriously, these bacteria can be transmitted to humans through food, making it difficult to treat for patients.

To ensure the health of consumers, the Ministry of Agriculture and Rural Development has promptly and strongly directed to control the use of antibiotics in livestock farming. The national surveillance program for drug-resistant bacteria in agriculture is being implemented in a number of provinces and cities according to Decision No. 3609/2021/QD-BNN-TY. However, recent research and monitoring results show that the current

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https://doi.org/10.47866/2615-9252/vjfc.4491

situation of antibiotic use in livestock farming has not been strictly controlled and the rate of resistance to many types of antibiotics in bacteria is still high [5, 6].

Salmonella is a Gram-negative bacillus belonging to the Enterobacteriaceae family that can be transmitted through food supply chance and is the leading cause of many food poisoning cases [7]. More seriously, antibiotic-resistant *Salmonella* strains are becoming more common, and they are resistant to many important antibiotics. The resistance of bacteria carrying resistance genes is not only transmitted vertically to bacteria of the same species but can also be transmitted horizontally to bacteria of different species, leading to the rapid development of drug-resistant microorganisms in the One Health ecosystem (humans-livestock-environment), increasing the challenge in disease control and treatment [8]. In the context that Vietnam does not have a National program to monitor *Salmonella* bacteria and their antibiotic resistance levels in food, this study was carried out to continue obtain scientific evidence on the risk of antibiotic resistance in bacteria and can be the support management units to continue to strongly implement activities to monitor the use of antibiotics in livestock.

2. RESEARCH CONTENT, MATERIALS AND METHODS

2.1. Research contents

To determine the antibiotic resistance profiles of *Salmonella* spp. isolates from pork collected at local markets in Hanoi in 2018 and 2023.

To investigate the the relationship between phenotypic and genotypic antibiotic resistance in *Salmonella* spp. isolates collected at different time points.

2.2. Materials

A total of 101 *Salmonella* isolates obtained from pork samples collected at traditional markets in Hanoi in 2018 (42 isolates) and 2023 (59 isolates) from previous studies conducted by the Department of Veterinary Hygiene, National Institute of Veterinary Research (NIVR). Sampling procedure and *Salmonella* isolation process were performed according to TCVN 4833-1:2002/ISO 3100-1 and TCVN 10780-1:2017/ISO 6579:1-2017, respectively.

Media for biochemical characterization and antibiotic susceptibility testing of *Salmonella* isolates including XLT4 agar (Oxoid), Nutrient agar (Merck), Kligler agar (Oxoid), Urea broth (Merck), L-Lysine Decarboxylase Broth (Himedia), ONPG broth (Himedia), Mueller Hinton agar (Oxoid) and Muller Hinton broth No. 2 (Merck)...

Laboratory facilities at the Department of Veterinary Hygiene, National Institute of Veterinary Research

Research period: From April to September 2024.

2.3. Methods

2.3.1. Bacterial revival, biochemical characterization, and serological confirmation

Salmonella spp. isolates stored at -40°C were revived by streaking on the surface of XLT4 agar plates and incubated overnigh at 37°C. A typical Salmonella colony on XLT4 agar plate was transfered to the nutrient agar before subjected to biochemical tests, including: glucose/lactose fermentation, gas/H_2S production, urease activity, lysine decarboxylation, and β-galactosidase activity. In addition, Salmonella isolates were confirmed by slide agglutination using Salmonella Polyvalent O and H polyvalent antisera according to TCVN 10780-1:2017/ISO 6579:1-2017.

2.3.2. Antibiotic susceptibility testing

All *Salmonella* spp. isolates were tested for susceptibility to 12 commonly used antibiotics by the Kirby–Bauer disk diffusion assay including: ampicillin (AMP, 10 µg); ceftriaxone (CRO, 30 µg); cefotaxime (CTX, 30 µg); ceftazidime (CAZ, 30 µg); amoxicillin/clavulanic acid (AMC, 20/10 µg); imipenem (IMP, 10 µg); sulfonamides (SUL, 300 µg); tetracycline (TET, 30 µg); nalidixic acid (NAL, 10 µg); ciprofloxacin (CIP, 5µg); norfloxacin (NOR, 10 µg); gentamicin (GEN, 10 µg). At the same time, micro-broth dilution assay was used to confirm colistin (COL) resistance, using 2-fold dilutions of colistin sulfate (Sigma Aldrich, St Louis, MO). *E. coli* ATCC 25922 strain was used as the control. Interpretation of antimicrobial susceptibility test results was according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2023).

2.3.3. Detection of ESBL-producing phenotype

Extended-spectrum β-lactamase (ESBL)-producing *Salmonella* spp. were detected using the Double Disc Synergy Test (DDST) as described by Iqbal et al. [9].

2.3.4. Detection of antibiotic resistance genes

** DNA* extraction *by heat shock method*

Selected *Salmonella* isolates was streaked onto the surface of the Nutrient agar plate and incubated at 37°C for 24 h. Three to five colonies were suspended in 1 mL DNase/RNase-free water (Thermo Fisher) before a centrifugation at 5000 rpm for 10 minutes. The supernatant was discarded, and the pellet washed again with 500 µL of free-DNase/RNase water and centrifuged to collect a purified bacterial cell. The pellet was resuspended in 100 µL of free-DNase/RNase water, then heated at 100°C for 10 minutes, cooled rapidly. After centrifuging at 13000 rpm for 1 minute, the supernatant containing the bacterial DNA was collected to use for PCR.

** Detection of antibiotic resistance genes by PCR*

Salmonella isolates showing antibiotic resistance phenotypes were screened for colistin resistance genes (*mcr1–mcr5*) and cephalosporin resistance genes (*blaTEM*, *blaSHV*, *blaCTX-M*). Primers used and PCR conditions show in **Table 1**.

Table 1. *Primers used and PCR conditions for detection colistin and cephalosporin resistance genes*

Primer name	Sequence (5'-3')	Size (bp)	Temperature cycle	Reference
*mcr*1-F	AGTCCGTTTGTTCTTGTGGC	320	94°C: 5min, 35 cycles (94°C: 30s, 58°C: 30s, 72°C: 30s), 72°C: 5min	[10]
mcr1-R	AGATCCTTGGTCTCGGCTTG			
mcr2-F	CAAGTGTGTTGGTCGCAGTT	715		
mcr2-R	TCTAGCCCGACAAGCATACC			
mcr3-F	AAATAAAAATTGTTCCGCTTATG	929		
mcr3-R	AATGGAGATCCCCGTTTTT			
mcr4-F	TCACTTTCATCACTGCGTTG	1,116		
mcr4-R	TTGGTCCATGACTACCAATG			
mcr5-F	ATGCGGTTGTCTGCATTTATC	1,644		
mcr5-R	TCATTGTGGTTGTCCTTTTCTG			
blaTEM-F	GGTCGCCGCATACACTATTCTC	372	95°C: 5min, 25 cycles (95°C: 30s, 60°C: 90s, 72°C: 90s), 68°C: 10min	[11]
blaTEM-R	TTTATCCGCCTCCATCCAGTC			
blaSHV-F	CCAGCAGGATCTGGTGGACTAC	231		
blaSHV-R	CCGGGAAGCGCCTCAT			
blaCTX-F	ATGTGCAGYACCAGTAARGTKATGGC	593	94°C: 3min, 25 cycles (94°C: 60s, 60°C: 60s, 72°C: 60s), 72°C: 10min	[12]
blaCTX-R	TGGGTRAARTARGTSACCAGAAYCAGCGG			

PCR products were electrophoresed on 1.5% agarose gel in 1X TBE buffer, stained, and visualized using an ImageQuant LAS500 system (Sweden).

2.4. Data analysis

Antibiotic resistance levels were analyzed and compared using Fisher's exact test in SPSS version 26.0.

3. RESULTS AND DISCUSSION

3.1. Biochemical characteristics and serological confirmation of Salmonella isolates

The results of biochemical tests (**Table 2**) showed that among the 101 tested isolates, 17 isolates (16.83%) were able to ferment glucose, while 84 isolates (83.17%) gave questionable results due to high H_2S production, which obscured the color change in the medium under the slant. Most of the isolates (94/101; 93.07%) were unable to ferment lactose. All isolates produced H_2S (100%) and 80/101 isolates (79.21%) were capable of gas production. All isolates were positive for urease activity (100%). In addition, 99 isolates (98.02%) were able to decarboxylate lysine, and 92 isolates (91.09%) were negative for β-galactosidase. The serological test showed that all 101 isolates (100%) agglutinated with polyvalent O and H antisera. This allowed us to confirm that all 101 isolates selected for study were Salmonella spp. (according to TCVN 10780-1:2017/ISO 6579-1: 2017).

Table 2. *Biochemical characteristics and serological confirmation of Salmonella isolates*

Test parameter	Requirement for *Salmonella*	Results		
		Positive isolates (%)	Suspected isolates (%)	Negative isolates (%)
Glucose fermentation	+	17 (16.83)	84 (83.17)	0
Lactose fermentation	-	0	7 (6,9)	94 (93.07)
Gas production	+	80 (79.21)	0	21 (20.79)
H_2S production	+	101 (100)	0	0
Urease activity	-	0	0	101 (100)
Lysine decarboxylation	+	99 (98.02)	0	2 (1.98)
β-galactosidase reaction	-	9 (8.91)	0	92 (91.09)
Polyvalent O-H antisera	+	101 (100)	0	0

Note: +: Positive; -: Negative (following TCVN10780-1:2017/ISO 6579:1-2017)

3.2. Antibiotic susceptibility of Salmonella isolates

A total of 101 *Salmonella* spp. isolates were tested for susceptibility to 12 antibiotics using the disk diffusion method, results shown in **Figure 1.**

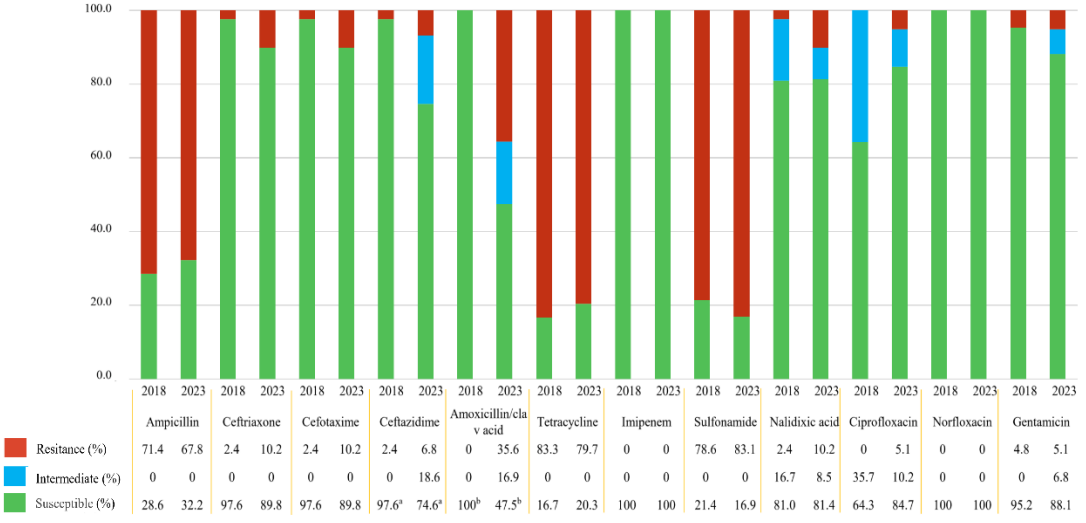

Figure 1. *Susceptibility profiles of Salmonella isolates to 12 tested antibiotics*

The results revealed that the *Salmonella* spp. isolates from both 2018 and 2023 showed the highest resistance rates to tetracycline (83.3% and 79.7%, respectively), sulfonamides (78.6% and 83.1%), and ampicillin (71.4% and 67.8%). Resistance to ceftazidime increased significantly from 2.4% in 2018 to 10.2% in 2023 (p=0.001935), while resistance to amoxicillin/clavulanic acid rose sharply from 0% in 2018 to 35.6% in 2023, the difference was statistically significant with $p=1.87 \times 10^{9}$. Differences in resistance of *Salmonella* spp. to other tested antibiotics were not statistically significant between the two sampling periods. All *Salmonella* isolates remained fully susceptible to imipenem and norfloxacin during both years.

Our study results are quite similar to the research reported by Cam Thi Thu Ha et al. (2023), who found that *Salmonella* isolates from pork in Soc Son had high resistance rates to tetracycline (97.56%) and ampicillin (63.41%), but low resistance rates to ceftazidime (7.32%) and ciprofloxacin (7.32%) [14]. Another study conducted in Hanoi, Bac Ninh and Nghe An showed that *Salmonella* spp. isolates from pork exhibited high resistance rates to sulfonamide (79.2%), ampicillin (68.8%), and tetracycline (67.7%), while 100% of the isolates were susceptible to ceftazidime [15]. Nguyen Thanh Trung (2023) reported that 119 *Salmonella* isolates recovered from chicken, duck, and goose meat samples collected at markets in Hanoi in 2019 showed the highest resistance rates to ampicillin (89.08%) and tetracycline (87.39%) [16]. In contrast, our study results revealed higher resistance rates than those reported by Truong Huynh Anh Vu (2021) in Ho Chi Minh City, who observed the highest resistance to tetracycline (31.68%), followed by ampicillin (22.98%), and the lowest to amoxicillin/clavulanic acid (1.86%) [17].

Multidrug resistance (MDR) is defined as the ability to resist at least three antibiotics belonging to three different antibiotic classes [18]. In this study, we tested the resistance of *Salmonella* isolates to 12 antimicrobial agents representing β-lactams, tetracycline, sulfonamides, and aminoglycosides. The levels of multidrug resistance of *Salmonella* isolates are presented in **Figure 2.**

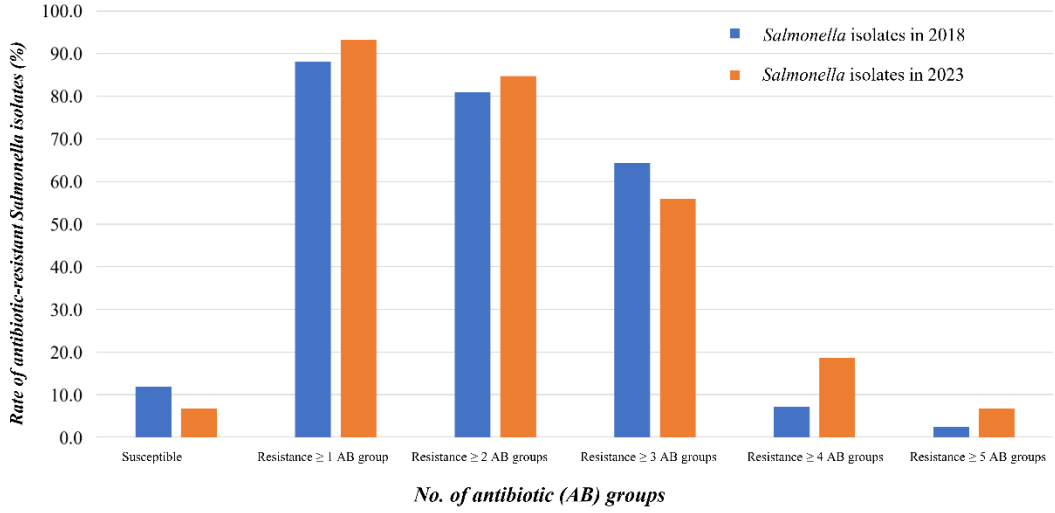

Figure 2. *The levels of multidrug resistance of Salmonella isolates*

The research results show that 64.3% of *Salmonella* isolates recovered in 2018 and 55.9% of isolates recovered in 2023 were multidrug-resistant, with no statistically significant difference (p = 0.4195). Notably, the proportion of isolates resistant to at least four antibiotic classes increased from 7.1% in 2018 to 18.6% in 2023. Similarly, 2.4% of *Salmonella* isolates recovered in 2018 and 6.8% in 2023 were resistant to at least five antibiotic classes. These results provide scientific evidence for stakeholders to strengthen measures to limit the increase in resistance rates and multi-resistance rates of pathogenic bacteria.

Our findings are quite similar to the results of Cam Thi Thu Ha et al. (2023), who reported that 68.29% of *Salmonella* isolates from pork in Soc Son were multidrug-resistant [14]. The study by Tran Thi Nhat et al. (2019) found that 69.8% of *Salmonella* isolates from pork and 76.7% from chicken were multidrug-resistant [15]. However, our results show higher resistance rates than other previous studies. Truong Huynh Anh Vu et al. (2021) reported that 22.22% and 38.46% of *Salmonella* isolates from pork and chicken, respectively, were multidrug-resistant [17]. Tran Thi Thuy Nga et al. (2019) reported that 63.2% of *Salmonella* isolates from

chicken, 47.5% from pork, and 34.6% from beef collected in the Central region had a multidrug-resistant phenotype [19]. Differences in multidrug resistance rates among studies may be due to variations in sampling locations, geographical regions, and local awareness regarding the prudent use of antibiotics. Magiorakos et al. (2012) also noted that multidrug resistance can vary by region and the endemicity of each area or country [18]. The widespread use of antibiotics, incorrect dosages, and improper treatment regimens are considered major causes of antimicrobial resistance and multidrug resistance in Vietnam [20].

3.3. Relationship between phenotypic and genotypic colistin resistance in *Salmonella* isolates

Colistin resistance in *Salmonella* spp. was assessed by determining the minimum inhibitory concentration (MIC). The results showed that the MIC distribution of the tested *Salmonella* isolates was concentrated at three values: 0.25 µg/mL, 0.5 µg/mL, and 1 µg/mL. Among them, 43 out of 59 (71.9%) isolates recovered in 2018 and 34 out of 42 (80.9%) isolates recovered in 2023 had an MIC of 0.5 µg/mL (**Table 3**).

Table 3. *Distribution of MIC values for colistin resistance of Salmonella isolates*

Source	No. of tested isolates	Colistin-MIC value distribution (µg/mL)										Cutoff* value (µg/mL)	Resistant isolates (%)
		0.06	0.125	0.25	0.5	1	2	4	8	16	32		
Isolated in 2018	59	0	0	2	43	11	2	0	0	1	0	≥ 4	1 (1.69%)
Isolated in 2023	42	0	1	3	34	3	0	0	1	0	0		1 (2.38%)

*Note: * Cutoff value following CLSI (2023)*

According to CLSI guidelines (2023), Enterobacteriaceae with MIC values for colistin ≥ 4 µg/mL are considered colistin-resistant, representing isolates with a colistin-resistant phenotype. Accordingly, in this study, 2 out of 110 (1.98%) *Salmonella* isolates exhibited a colistin-resistant phenotype. Previous studies in Vietnam also reported that no *Salmonella* isolates recovered from pork and chicken were resistant to colistin [14, 15]. Our results are comparable to those of Lay et al. (2021), who found that only 2.6% of 463 *Salmonella* isolates recovered from pork carcasses at slaughterhouses were resistant to colistin [21]. Similarly, in several Southeast Asian countries, the prevalence of colistin-resistant *Salmonella* recovered from pork has been reported to be very low compared to *E. coli* and other enteric bacteria. For example, Pungpian et al. (2021) reported that only 1.7% of *Salmonella* isolates were resistant to colistin, while the resistance rate in *E. coli* was 17.8% [22]. This rate was even lower than that in other countries included in the same study, such as Laos and Thailand.

These two colistin-resistant isolates were further tested for the presence of colistin resistance genes by PCR. The results showed that the *Salmonella* isolates recovered in 2018 carried the *mcr-1* gene, whereas the isolates recovered in 2023 carried the *mcr-3* gene (**Figure 3**). These genes are among the most common colistin resistance determinants found in Vietnam as reported in previous studies [23–26]. They have also been reported in *Salmonella* isolates recovered from pork in Cambodia and Thailand [22].

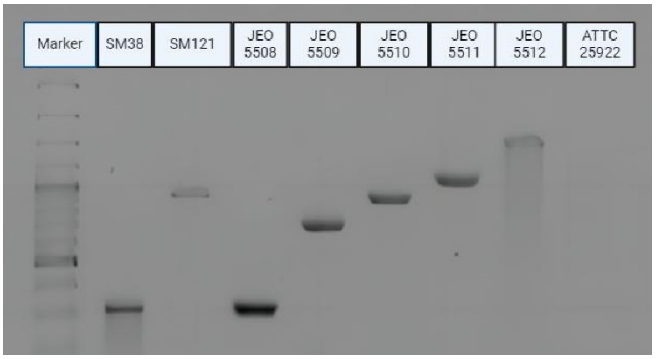

Figure 3. *Gel electrophoresis results showing PCR products for the detection of colistin resistance genes. SM38 and SM121 are the tested isolates; JEO5508–JEO5512 are reference strains carrying the colistin resistance genes mcr-1 to mcr-5; ATCC 25922 is the negative control*

3.4. Relationship between phenotypic and genotypic cephalosporin resistance in *Salmonella* isolates

Extended-spectrum beta-lactamases (ESBLs) are a group of enzymes produced by bacteria that can hydrolyze and inactivate a wide range of beta-lactam antibiotics, including penicillins, cephalosporins, monobactams, and carbapenems. ESBL-producing isolates are of particular concern because they not only break down many beta-lactam antibiotics but are also often associated with resistance to other antibiotic groups. Several studies have demonstrated a close link between ESBL-producing isolates and quinolone-resistant isolates [27]. In this study, we detected seven *Salmonella* isolates with an ESBL-producing phenotype: 6 out of 59 (10.2%) isolates recovered in 2018 and 1 out of 42 (2.4%) isolates recovered in 2023 (**Figure 4**).

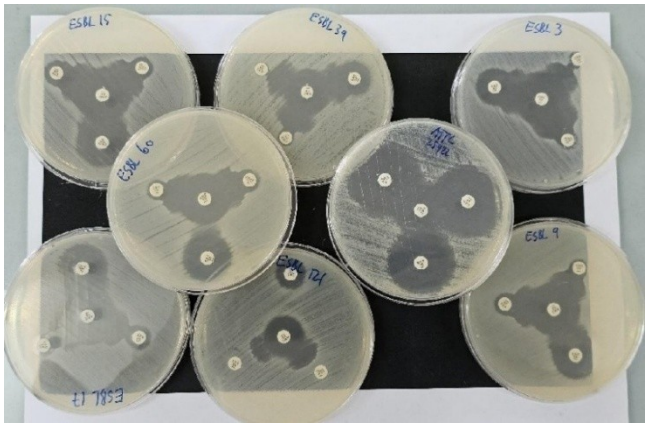

Figure 4. *ESBL-producing phenotype of Salmonella isolates on Mueller Hinton agar plates*

We also tested for the presence of genes responsible for ESBL production (*blaTEM, blaSHV, blaCTX-M*). The results showed that five isolates carried the *blaCTX-M* gene while two isolates harbored both *blaCTX-M* and *blaTEM* genes, no isolates carried the *blaSHV* gene. These findings are consistent with previous studies indicating that *blaCTX* is the most common ESBL gene in Vietnam, followed by *blaTEM*, and the *blaSHV* is rarely detected [28, 29]. For example, Cam Thi Thu Ha et al. (2023) reported that 6 out of 41 (14.63%) *Salmonella* isolates recovered from pork were ESBL producers. Among these, one isolate carried the *blaCTX-M-9* gene, three isolates carried the *blaTEM* gene, one isolate carried both *blaCTX-M-1* and *blaTEM* genes, and one isolate harbored three genes *blaCTX-M-1, blaCTX-M-8/25, blaTEM* [14]. Similarly, Truong Huynh Anh Vu et al. (2021) found that 25 out of 43 (58.14%) *Salmonella* isolates carried ESBL genes of the *blaTEM* group and 4 out of 43 (9.30%) carried the *blaCTX* group, no *blaSHV* genes were detected [17]. Globally, the prevalence of ESBL genes among *Salmonella* isolates remains low; for example, in Thailand, 8 out of 237 (3.4%) *Salmonella* isolates tested positive for ESBL genes, while in Laos, 2 out of 86 (1.2%) isolates were ESBL-positive [21].

In this study, we found a close relationship between the phenotype and genotype of colistin and cephalosporin resistance in the isolated *Salmonella* spp. All isolates exhibiting colistin or cephalosporin resistance phenotypes were found to carry the corresponding resistance genes. This finding contrasts with some previous reports where isolates displayed resistance phenotypes but lacked detectable resistance genes [30], or conversely, carried resistance genes without exhibiting the corresponding resistance phenotypes [31].

4. CONCLUSIONS

Salmonella spp. isolates recovered in 2018 and 2023 showed high resistance rates to tetracycline (83.3% and 79.7%, respectively), sulfonamides (78.6% and 83.1%), and ampicillin (71.4% and 67.8%). In 2018, 2.4% of *Salmonella* isolates were resistant to ceftazidime, while the ceftazidime resistance rate among isolates recovered in 2023 was 10.2%. The results also showed that in 2018, *Salmonella* isolates were completely susceptible to amoxicillin/clavulanic acid; however, by 2023, the resistance rate had increased to 35.6%. All tested *Salmonella* spp. isolates remained fully susceptible to imipenem and norfloxacin.

The colistin resistance rate was low (2 out of 110 isolates, 1.98%); the isolate recovered in 2018 carried the *mcr-1* gene, and the isolate recovered in 2023 carried the *mcr-3* gene. These are common colistin resistance genes reported in Vietnam.

Seven *Salmonella* isolates exhibited an ESBL phenotype, in which 6/59 (10.2%) isolates were obtained in 2018 and 1/42 (2.4%) isolates were obtained in 2023. Of these, 5 isolates carried the *blaCTX-M* gene, two isolates carried both the *blaCTX-M* and *blaTEM* genes, and no isolates carried the *blaSHV* gene.

ACKNOWLEDGMENTS

We would like to thank the International Cooperation Center for Agricultural Research and Development - CIRAD France for providing a part of labs consumable for the study.

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