

RESEARCH ARTICLE

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Antibiotic Resistance in *Salmonella* Isolated from Ho Chi Minh City (Vietnam) and Difference of Sulfonamide Resistance Gene Existence in Serovars

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Abstract

In this study, a total of 228 raw meat samples (pork: 76, beef: 76, chicken: 76) and 301 raw seafood samples (fish: 199, shrimp: 67, squid: 35) were collected randomly at traditional markets in Ho Chi Minh City (Vietnam). In meat, the ratio of *Salmonella* spp. was 70.61% (161/228). Among the contaminated meat samples, pork was infected with a ratio of 90.79% (69/76) while the contamination ratios in beef and chicken were 43.42% (33/76) and 77.63% (59/76), respectively. *Salmonella* contamination was detected in fish (40.20%), shrimp (7.46%) and squid (17.14%). Because of sulfonamide group is used in *Salmonella* treatment, the study focused on sulfonamide resistance. In fresh seafood comparison, there were 32.56%, 40% and 10% *Salmonella* showing resistance to sulfamethoxazol in fish, squid and shrimp, respectively. In fresh meat comparison, there were 31.58%, 16.67% and 55.56% *Salmonella* showing resistance to sulfamethoxazol in pork, beef and chicken, respectively. Interestingly, there were 21 serovars including 19 identified serovars including *S. Kentucky* (8), *S. Agona* (2), *S. Infantis* (4), *S. Saintpaul* (1), *S. Indiana* (1), *S. Braenderup* (1), *S. Potsman* (2) and 2 unidentified serovars showing different phenotype to this antibiotic. Among the 21 serovars, only 23.81% strains carried both genes (*su1*, *su2*). For the *su1* gene, 61.9% strains were presented while *su2* occupied at a lower rate than *su1* with the rate of 52.38%. The study is very interesting and useful to go more functional analysis in sulfonamide resistance.

Keywords: *Salmonella*, sulfonamide resistance, *su1* and *su2* genes, multiplex Polymerase chain reaction (m-PCR), serovars, raw meat, seafood

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INTRODUCTION

Food safety is one of the issues that we have been particularly concerned about for a long time. This is also one of significant factors for the sustainable development of socio-economic system, and public health in Vietnam. Therefore, the need for strengthened prevention, surveillance and management, of foodborne disease is extremely important at the present and future. This makes a significant contribution to economic development, poverty reduction and international integration. *Salmonella* spp. are the most widely distributed causes of foodborne outbreaks in human.¹ They are the foodborne hazards that are responsible for the highest annual burden and the largest number of deaths globally. Globally, there were estimated that millions of people fall ill from eating contaminated food every year, resulting thousands of deaths. *Salmonella* spp. are found in everywhere (including water, soil, insects, animal feces, raw meats, poultry, seafood, and on factory and kitchen surfaces), distributing differently depending on geographic location.^{2,3} In Vietnam, more than one-third of meat samples from Ho Chi Minh City (HCMC) had *Salmonella* bacteria. The prevalence of *Salmonella* spp. in 25 g meat samples from HCMC was 71.8% (chicken), 70.7% (pork), and 62.2% (beef).

The survival of *Salmonella* not only depends on the water activity (aw) of the environment or food but also on other factors such as matrix composition and storage temperature. The main causes of this bacterium contamination in low-moisture foods are poor sanitation practices, substandard facilities, equipment design, and improper maintenance.⁴ The rise of *Salmonella* antibiotic resistance in food has been a global concern in recent decades because of the overuse of antibiotics for treating human and animal diseases. The inappropriate use of antibiotics in humans and animals make a great pressure on selection, leading to the presence and spread of antibiotics-resistant bacteria strains. Approximately 30 - 90% of the used antibiotics are not metabolized and exist in the environment.⁵ The habit of antibiotic usage in livestock production, prevention from diseases for decades is one of the main causes of increasing occurrence of antibiotic resistant *Salmonella* spp.^{6,7} In Vietnam,

antimicrobials are often found in both commercial pig and poultry rations. A study estimated in-feed consumption of antimicrobials extrapolated from a retail survey of commercial feeds in 77 mg per kg of chicken produced.⁸ A study of medium-sized chicken farms estimated that chickens consumed 57 mg per kg of animal produced.⁹ However, that study was based on a small sample of 6 farms. Consequently, it is difficult to control infections, prevention and illness treatment, which gives rise to the loss of human, economy and brings about serious consequences to the society.¹⁰⁻¹³ The emergence of antibiotic resistance in bacteria raises the global problems in public health. Although there were many researches on *Salmonella* using the natural extracts to improve immunology and anti *Salmonella* activities,¹⁴⁻¹⁸ the traditional antibiotics were still used. One of antibiotic group has a high rate is sulfonamides. These antibiotics have bacteriostatic activity and the mechanism of action involved in the synthesis of tetrahydrofolic acid. Sulfonamide inhibits dihydropteroate synthetase while trimethoprim inhibits dihydrofolate reductase.¹⁹ The resistance of *Salmonella* to sulfonamides is due to the *sul* gene family. The *sul1*, *sul2* and *sul3* genes are three main genes in which the most common *sul1* gene is found in the majority of serovars such as *Salmonella* Enteritidis, *Salmonella* Hadar, *Salmonella* Heidelberg, *Salmonella* Orion, *Salmonella* Rissen, *Salmonella* Agona, *Salmonella* Albany, *Salmonella* Derby, *Salmonella* Djugu and *Salmonella* Typhimurium. The *sul1* gene is located on translocation genetic elements such as class I integrons or on the plasmids, however, the *sul2* gene is located on the plasmids. Some of the antibiotic resistance genes of this group have been found to be *dhfr* and *dfr* located closely to *sul1* and *sul3* in an integron, plasmids or islands on the *Salmonella* genome.²⁰

As the above state, the study aimed to evaluate the sulfonamide resistance characteristics of *Salmonella* strains isolated from raw meat and seafood samples at conventional markets in Ho Chi Minh City (Vietnam). The results would contribute to the science about some antibiotic resistance characteristics in *Salmonella* spp. isolated from food in Vietnam as well as the world.

MATERIALS AND METHODS

Materials

Sample collection and preservation

228 raw meat samples (pork: 76, beef: 76, chicken: 76) and 301 raw seafood samples (fish: 199, shrimp: 67, squid: 35) were collected eventually at conventional markets in the districts of Ho Chi Minh City. 150 g -1.500 g of each sample were collected for the study. The samples were transported aseptically to the laboratory as soon as possible to be immediately analyzed. It should not let longer than 12 hours.

Salmonella spp. isolation and identification

Salmonella spp. isolation was based on ISO 6579-1:2017. To enrich bacteria for isolation, 25 g samples were placed in sterile bags (BagPage 400 mL, Interscience), added 225 mL buffer peptone water (BPW). Homogenization was performed using Stomacher method (Seward) at 230 rpm/30 seconds and incubated at 37°C in 18 ± 2 hours. Then, bacteria from cultures were spread on the selective rappaport vassiliadis medium supplied with soya (RV) and kauffmann tetrathionate novobiocin (MKTtn), then were let at 41.5°C in 24 ± 3 hours and 37°C in 24 ± 3 hours, respectively. The pure colonies were spread on xylose lysine deoxycholate (XLD) agar and mannitol lysine crystal violet brilliant green agar (MLCB) before incubated at 37°C in 24 ± 3 hours. The suspected colonies were picked up for checking *Salmonella* spp. by PCR using *invA* gene. The protocol was followed as the previous study.²¹ The same strains that showed many serovars with the sulfonamide resistance were used to the detect *sul1* and *sul2* by PCR to clarify or find out more whether *sul1*, *sul2* or both could affect the resistance as many previous studies concluded before.

Evaluation of sulfamethoxazole susceptibility

Sterile antibiotic disks that had 6 mm-diameter were immersed in sulfonamide

(Oxoid/10 µg/mL). Inhibition zones were recorded and evaluated according to the guidance of CLSI 2018 (M100-S28).²²

Resistant gene detection by multiplex PCR (m-PCR)

Multiplex PCR ingredients included AmpliTaq Gold 2 UI; 0.2 mM dNTP; 1.5 mM MgCl₂; 1X buffer; 0.5 µL each primer (0.625 µM); 5 µL DNA template and de-ionized water up to 25 µL.

The PCR program was set up at 95°C/10 minutes for thermal cycle. Then, PCR was performed continuously for 30 cycles. One cycle included 95°C/30 seconds, 55°C/60 seconds and 72°C/60 seconds and 72°C/7 minutes.

PCR products were run on gel agarose 1.5% containing 1 µg/mL ethidium bromide in TBE. The duration was 35 - 40 minutes in 100V and 100 mA. The primer pairs were *sul1* (forward: 5' - TCACCGAGGACTCCTTCTTC - 3', reverse: 5' - CAGTCCGCCTCAGCAATATC - 3') and *sul2* (forward: 5' - CCTGTTTCGTCCGACACAGA - 3', reverse: 5' - C1TGTTTCGTCCGACACAGA - 3'), giving the 331 bp and 435 bp PCR products, respectively.²³

RESULTS

Salmonella spp. Isolation and determination

Analysis methods were based on the guidelines of ISO 6579-1:2017 for detection of *Salmonella* spp. in raw meat samples originated from markets illustrated in Table 1.

The suspected colonies of *Salmonella* spp. were confirmed by PCR using *invA* gene as a target for detection. The incorporated *invA* gene results revealed that among collected meat samples, there were 161 samples infected by *Salmonella* spp. occupying 70.61% (161/228). Pork and chicken had the approximate proportions at 90.79% (69/76) and 77.63% (59/76). The lowest proportion of contamination occurred in beef samples with the rate of 43.42% (33/76). Based on the results, there were high contamination in

Table 1. The percentage of *Salmonella* spp. detection in raw meat samples

Samples	Quantity	Positive		Negative	
		Quantity	Percentage (%)	Quantity	Percentage (%)
Pork	76	69	90.79	7	9.21
Beef	76	33	43.42	43	56.58
Chicken	76	59	77.63	17	22.37
Total	228	161	70.61	67	29.39

Table 2. The percentage of *Salmonella* spp. detection in raw seafood samples

Samples	Quantity	Positive		Negative	
		Quantity	Percentage (%)	Quantity	Percentage (%)
Fish	199	80	40.20	119	59.80
Shrimp	67	5	7.46	62	92.54
Squid	35	6	17.14	29	82.86
Total	301	91	30.23	73	69.77

Table 3. Antimicrobial susceptibility of *Salmonella* spp. in raw meat

Pork		Chicken		Beef		Total	
Quantity	Percentage	Quantity	Percentage	Quantity	Percentage	Quantity	Percentage
12	31.58	20	55.56	3	16.67	35	38.04

raw meat collected in traditional markets in Ho Chi Minh city (Vietnam). Similarly to the confirmation of suspected colonies of *Salmonella* spp. in meat samples, the contamination in raw seafood was also detected and presented in Table 2.

Antimicrobial susceptibility of *Salmonella* spp. strains

Salmonella spp. showed resistance to sulfonamide antibiotics in the study (Table 3 and 4). The resistance was seen in all cases of *Salmonella* isolated from raw meat and seafood. From Table 3, the ratios of sulfonamide resistance in pork and chicken were higher than beef samples.

For the seafood samples, fish is a good condition for microbial growth. Therefore, there were many strains of *Salmonella* contaminated in fish as seen in Table 2. As the statement, *Salmonella* strains resist to sulfonamide more than other sources such as squid and shrimp (Table 4).

The results suggested it should be careful when handling fishes to prevent contamination and resistance. In order to find out the resistance due to the same gene or other elements, the study screen some serovars and detect sulfonamide resistance genes.

Detection of sulfonamide resistant genes

After checking sulfonamide resistance in *Salmonella*, the sulfonamide resistant genes were detected in serovars so that the phenotype and genotype involving resistance could be clarified partly. There were unidentified 2 serovars and 19 identified serovars of *Salmonella* including S. Kentucky (8), S. Infanis (4), S. Potsdam (2), S. Agona

(2), S. Indiana (1), S. Saintpaul (1), S. Braenderup (1) gave the phenotype as presented in Table 5. To find out which genes involving the phenotype, PCR reaction was performed to identify *sul1* and *sul2*. The results were presented in Fig. 1 and summarized in Table 5. Among the 19 identified serovars and 2 unidentified serovars, only 5 strains carried both genes (*sul1*, *sul2*) at the same time, accounting for 23.81% (5/21). For the *sul1* gene, 13/21 strains were present (61.9%), *sul2* was present at a lower rate than *sul1* with the rate of 52.38% (11/21). Kentucky carried *sul1* and *sul2* with the highest frequency around 38.46% and 45.45%, respectively. In the study, *sul1* was common in 19 serovars. However, the resistance was not only due to *sul1* or *sul2* involvement but also the other elements such as the plasmids, integrons or gene cassette location. Actually, the same serovar showed the phenotype and genotype difference such as there were 8 serovars belonging to S. Kentucky in which there were 2 serovars having antibiotic sensitivity while others were resistance (Fig. 1 and Table 5). Surprisingly, there was no *sul1* or *sul2* detected in S. Indiana (pork) and S. Agona (fish) although they were resistant to sulfamethoxazole. The study will drive to more functional analysis in future.

DISCUSSION

From the results in Table 1, it might be that living conditions of these animals affect the situation of contamination in meat. The different dissemination in pork, chicken and beef might

Table 4. Antimicrobial susceptibility of *Salmonella* spp. in raw seafood

Fish		Squid		Shrimp		Total	
Quantity	Percentage	Quantity	Percentage	Quantity	Percentage	Quantity	Percentage
14	32.56	2	40	1	10	17	29.31

be due to the meat components or structure that can affect the contamination. More samples and mechanisms should be studied to aid for the contamination prevention.

The difference in *Salmonella* prevalence for different food groups could be caused by a variety of reasons. One of the main reasons is that the slaughter of livestock and poultry is usually carried out in unhygienic places such as the yard or slaughter tools are unsterilized knives, buckets and pots as well as the water sources are contaminated.

Furthermore, meat of cattle and poultry after slaughtering are transported by motorbike and uncovered by anything. Free sale in the market without being covered or stored for a long time along with customers who come into contact with the meat when selecting, make meat more susceptible to contaminate contaminated with *Salmonella* spp. by contacting with feces, skin, feathers, nails, meat cutters, trays, air, soil, water in the slaughterhouse, and from clothing, and limbs of slaughterhouse personnel. Additionally, according to Folster et al²⁴, the difference in the prevalence of *Salmonella* spp. is possible that this bacterium is widespread in the environment and plays an important role in the infection between pathogens. Furthermore, *Salmonella* spp. distribution is very different depending on each country and region. As a result, different

food sources may be contaminated with strains of *Salmonella* spp. with different rates.

The resistance was seen in all cases of *Salmonella* isolated from raw meat and seafood (Table 3 and 4). From the Table 3, the ratios of sulfonamide resistance in pork and chicken were higher than beef samples. Probably, beef was not used sulfonamide as casual as pork and chicken.

There were many strains of *Salmonella* contaminated in fish as seen in Table 2 with the highest resistant rate than other seafood sources such as squid and shrimp (Table 4). Probably, fish is a good condition for microbial growth that is the opportunity for antibiotic resistance. However, many mechanisms should be study more to understand deeply that is important to prevent bacterial contamination together with antibiotic resistance.

A high prevalence of sulfonamide resistance was observed in *Salmonella* isolated from animal products in many countries. One hundred percent sulfonamide resistance was reported in isolates originated from pigs in Thailand,²⁵ Malaysia²⁶ and Japan²⁷; from broilers in Japan²⁸ and Iran.²⁹ High sulfonamide resistance rates were also reported in isolates originated from broilers in Japan (92.5% to 100%)^{30,31} and pigs (100%)²⁵ and from a combination of poultry and swine isolates (68% to 70%) in Thailand.³² A study in China found that isolates originated

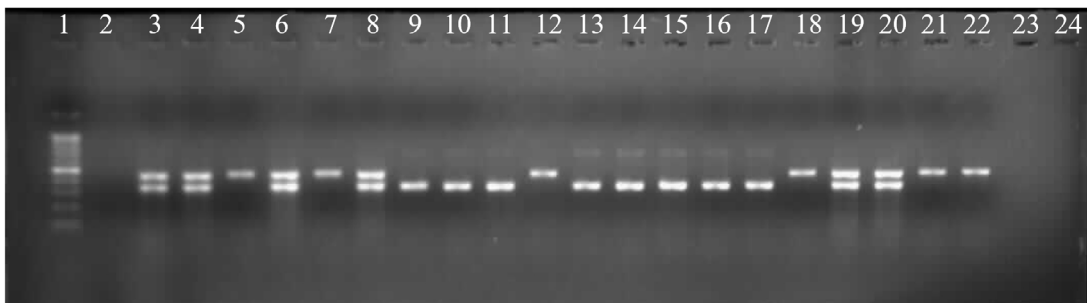


Fig. 1. PCR products of 21 serovars. (1): ladder 100bp; (2): negative control; (3): positive control; (4 - 24): serovars in which *S. Indiana* (pork) and *S. Agona* (fish) were the wells of 23 and 24 without any product.

Table 5. The detection of *sul1* and *sul2* genes in serovars

Sources		Serovar	Phenotype	<i>sul1</i>	<i>sul2</i>
Pork	1	S. Kentucky	S	+	+
	2	S. Indiana	R	-	-
Beef	3	S. Infantis	R	+	-
Chicken	4	S. Agona	R	-	+
	5	S. Infantis	R	+	-
	6	S. Potsdam	R	-	+
	7	S. Kentucky	R	+	-
Fish	8	S. Kentucky	R	+	-
	9	S. Kentucky	R	-	+
	10	S. Saintpaul	R	+	+
	11	S. Braenderup	R	+	+
	12	S. Kentucky	R	+	+
	13	S. Kentucky	R	+	-
	14	S. Potsdam	R	-	+
	15	S. Kentucky	R	-	+
	16	S. Infantis	R	+	-
	17	S. Agona	R	-	-
	18	S. Infantis	S	+	-
	19	OMF:1,z ₆ :UT	R	+	+
	20	7:1,z ₆ :UT	R	+	-
	21	S. Kentucky	S	-	+

S: Susceptible and R: Resistant

from broilers, ducks, geese and pigs exhibited the resistance rates of 93.8%, 20.0%, 16.7% and 52.5%, respectively.³³ Resistance to sulfonamides is usually associated with the acquisition of resistance genes such as *sul1*, *sul2* and *sul3*.³⁴ Among *Salmonella* spp. isolates obtained from beef samples collected from retail markets in Vietnam in 2009, the resistance to sulfonamides was found in 39.7% (25/63 isolates) and 80.0% (20/25) were *sul1* positive.³⁵ Between 2007-2008, 110 isolates were obtained from meat (chicken, turkey and pork) from retail stores in Canada.³⁶ There were 71% (78/110) showed resistance to sulphonamides. The *sul1* gene was found in 5 isolates while *sul2* expressed in 3 isolates and the *sul3* gene was only found in one (pork) isolate. Among 88 *Salmonella* spp. strains isolated from retail meats and street foods in Malaysia, the sulfonamide resistant percentage was 63.6%.³⁷ Among of these resistant strains, there were 32 strains those were positive for *sul1* and *sul2* while there were 5 strains carrying *sul1*, and 14 strains which were positive for *sul2*. These sulfonamide-

resistance genes are commonly associated with mobile genetic elements, particularly class 1 integrons that can also relate to other antibiotic resistance. These factors facilitate the wide dissemination of sulfonamide resistance in bacteria.³⁸

CONCLUSION

Salmonella spp. detected in raw meats and seafood collected in traditional markets in Ho Chi Minh city (Vietnam). The isolated *Salmonella* strains showed sulfonamide resistance in meat and seafood, but the high contamination was seen in chicken, fish and pork. The *sul1* gene was common in the identified serovars but resistance was due to many elements besides *sul* genes such as the plasmids, integrons or gene cassette location. There was no *sul1* or *sul2* detected in one serovar of isolated S. Indiana (pork) and one serovar of isolated S. Agona (fish) although they were resistant to sulfamethoxazole. This study brings out the interesting and useful points to explore the sulfonamide resistance in the future.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTIONS

THAV is proposal investigator, designed, performed experiment. THAV and NHKT did the data analysis and wrote the manuscript. NHKT did the supervision. CVH and HYH supported the research.

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DATA AVAILABILITY

The data obtained in the study is available from the corresponding author and first author on reasonable request.

ETHICS STATEMENT

Not applicable.

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