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Preliminary study on phenotype and genotype of antibiotic resistance in *Salmonella* enterica subsp. enterica serotype *Typhimurium* Isolated from Chicken Samples in Malaysia

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Abstract

Salmonellosis is a significant public health concern around the world. The issue of antibiotic resistance, particularly in non-typhoidal *Salmonella* (NTS), is a global threat to human and animal health. Thirty-five *Salmonella* enterica subsp. enterica serotype *Typhimurium* (*S. Typhimurium*) isolated from various chicken samples were tested for antimicrobial-resistant profiling using the disc diffusion method. The strains were further examined for the presence of the resistance genes by using polymerase chain reaction (PCR) according to published protocols. Phenotype resistance profiling revealed that 22.9% (8 out of 35) isolates showed resistance to five antibiotics used in this study. All the penta-resistant isolates carried the blaTEM, floR, strA, and tetA gene which encoded for ampicillin, chloramphenicol, streptomycin, and tetracycline resistance. Only one isolate (1 out of 35) was found to contain the sulfonamides resistance, sulA gene. Nine isolates were found susceptible to all antibiotics tested and do not harbor any of the resistant associated genes. These findings provide evidence that the presence of resistance genes contributes to the phenotypic resistance profile, and thus could give rise to the emergence of the drug-resistant strain of *Salmonella*.

1. INTRODUCTION

Salmonella is one of the most common foodborne pathogens worldwide (Abatcha et al., 2018). This pathogen causes mild self-limiting gastroenteritis to severe infections, with diarrhea as its main symptom. Non-typhoidal *Salmonella* (NTS) plays a significant role in human salmonellosis which is transmitted through the consumption of contaminated foods of animal origin (Sodagari et al., 2020). In Malaysia, previous report by Abdullah and Ismail (2021), showed that food poisoning cases among school children in Terengganu most likely to be associated with egg consumption compared with rice consumption. Furthermore, *Salmonella enteritidis* and *Salmonella java* were the commonest NTS serovar identified from invasive *Salmonella* infections among children with resprtaory and gastroenteritis symptoms, respectively based on retrospective study conducted in Sarawak from 2011 to 2016 (Mohan et al., 2019). To date, more than 2500 serovars of *Salmonella enterica* have been identified, however *S. Typhimurium* and *Enteritidis* are the most common serovars associated with human infections worldwide (Hendriksen et al., 2011).

During the last few decades, there have been increasing reports on the resistance of non-typhoidal *Salmonella* to a range of antimicrobials (Sodagari et al., 2020). *Salmonella Typhimurium* has emerged as the most important serovar of public health importance related to antibiotic resistance issues (Wang et al., 2019). Increased incidence of resistance in *S. Typhimurium* can be attributed to multi-resistant *S. Typhimurium* phage type DT104 which has been reported in human and various animal species in the early 1980s (Carlson et al., 1999). This multidrug-resistant strain is characterized by penta-drug resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline which is identified as the ACSSuT type of resistance (Faldynova, et al., 2003). Several authors reported the incidence of human salmonellosis due to multidrug-resistant strain including *S. Typhimurium* DT104 in various geographical locations (Bacci et al., 2012). Most of the *S. Typhimurium* strain isolated from livestock, predominantly cattle in the United Kingdom and several European countries during the 1990s had the ACSSuT-resistance pattern (Poppe et al., 1998). Sameshima et al (2000) reported that penta-resistant *S. Typhimurium* was identified among isolates isolated from cattle, poultry, and environmental samples during the period 1973 to 1999 throughout Japan. Based on these findings, cattle are the primary reservoir for penta-resistant

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S. Typhimurium DT104. However, this pathogen also has been reported to cause infections in humans either through direct contamination from infected animals or consumption of unpasteurized milk and contaminated pork products (Poppe, et al., 1998).

The emergence of multidrug-resistant *S. Typhimurium* become a serious challenge in treating severe infections (Wang et al., 2019). Misuse of antibiotics is the most important factor contributing to the high resistance to antibiotics in pathogenic bacteria including *S. Typhimurium (*Wang et al., 2019). However, other intrinsic factors which involved horizontal gene transfer found on mobile genetic elements might contribute to the rapid dissemination of multidrug-resistant bacteria across the food chain (Wang et al., 2019). These factors might play an important role in the evolution and emergence of multidrug-resistant *S. Typhimurium* strains, which serve as a complex serovar involving a broad host range spectrum (Paul et al., 2016). The presence of resistance-associated genes encoding for streptomycin, chloramphenicol, tetracycline, ampicillin, and sulfamethoxazole located within the chromosomal genomic island has been linked to the insurgence of multidrug-resistant strain (De Vito et al., 2015). To date, data on the distribution of resistance genes and the relatedness with phenotype resistance profile among the multidrug-resistant *S. Typhimurium* isolates from livestock in Malaysia is still scarce. *Salmonella Typhimurium* is among the most prevalent serovars found in chicken and chicken meat based on samples received at Veterinary Research Institute (VRI) for several years (Khoo et al., 2015; Roseliza et al., 2020). Previous studies revealed a high percentage of *S. Typhimurium* (70-83%) isolated from chicken meat showed multidrug-resistant with a common resistant profile to sulfamethoxazole, sulfonamides, and tetracycline (Roseliza et al., 2013; Khoo et al., 2015). On the other hand, 3.17% of *S. Typhimurium* isolated from chicken were found to carry the plasmid-mediated resistant gene (mcr-1) which is associated with colistin resistance, contributing to the worst antibiotic-resistant threat to food safety (Roseliza et al.,2020b). Therefore, this study was conducted to obtain preliminary data on the presence of resistant genes and their association with the antibiotic resistance profile of *S. Typhimurium* isolated from chicken.

2. MATERIALS AND METHODS

2.1 Bacterial isolates

The 35 *S. Typhimurium* isolates included in this study were available stock culture collection in Bacteriology Laboratory, Veterinary Research Institute identified from diagnostic cases submitted from 2009 to 2021. The bacteria were isolated from cloacal swabs, environmental swabs, and pooled organs from national surveillance and monitoring sampling throughout Malaysia. All the isolates were revived from maintenance media using 5% ox blood agar with an overnight incubation at 37°C. The pure bacterial culture was re-confirmed as *S. Typhimurium* by performing slide agglutination tests with specific O:4, O:12, H:i, and H:2 antisera according to White-Kauffmann classification (World Health Organization (WHO) Collaborating Centre for Reference and Research *Salmonella*, 2007) and the cultures were kept in maintenance media for further use.

2.2 Antibiotic sensitivity test

Antibiotic sensitivity test was conducted using the disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI, 2016)) standard protocol toward five selected antibiotics ampicillin (10 µg), chloramphenicol (30 µg), streptomycin (10 µg), sulfonamides (300 µg), and tetracycline (30 µg). Escherichia coli ATCC 25922 was used as the quality control reference strain. The inhibition zones were measured and scored as susceptible, intermediate, or resistant according to the breakpoint for *Salmonella* in Clinical and Laboratory Standards Institute (CLSI) guidelines.

2.3 Genomic DNA extraction

The *S. Typhimurium* bacterial DNA was extracted by boiling method from an overnight culture grown on blood agar (Roseliza, et al., 2020b). A few colonies were transferred into a 1.5 ml Eppendorf tube containing 100 µl sterile distilled water. The suspension was placed in a thermal block set at 95 °C for 10 minutes. After cooling to room temperature, the suspension was centrifuged at 12,470 x g for 5 minutes and 5 µl of the supernatant was used as a PCR DNA template.

2.4 Detection of resistant genes by Polymerase Chain reaction (PCR)

The simplex PCR was performed in a Mastercycler Nexus GSX1 (Eppendorf, Hamburg, Germany) machine using 25 µl ul of reaction mix comprising 12.5 µl of 2x MyTaq™ Mix (Bioline, UK), 1 µl of each 10 µM forward, and reverse primers (Apical Scientific, Malaysia), 5 µl of DNA template and appropriate volume of sterile distilled water. The list of published primers and amplification cycles to detect the targeted resistant genes were presented in Table 1. Amplified PCR products were electrophoresed through the 1.5% agarose gel stained with SYBR™ Safe DNA Gel Stain (Invitrogen, USA) and visualized using a gel documentation system (Uvitec, UK). The molecular size of PCR products was compared with the 100bp DNA HyperLadderTM (Bioline, UK). The reference strain *Salmonella enterica* subsp. enterica serovar *Typhimurium* NCTC 13348 (typical for penta-resistant DT104) with resistance to ampicillin, aminoglycosides, tetracycline, and

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sulfonamides was used as the positive control. Statistical analysis using the Pearson Chi-square was performed to determine the association between the presence of the resistance gene with phenotype profile of *S. Typhimurium*.

3. RESULTS

In total, the antibiotic sensitivity test showed that 62.8% of the isolates were found highly resistant to tetracycline and sulfonamides, 57.1% were resistant to ampicillin and streptomycin, and 42.8% were resistant to chloramphenicol. Resistance gene encoding for ampicillin *(blaTEM*) was highly detected in 26 out of 35 (74.2%) isolates tested, followed with *tetA* (68.5%), *strA* (62.8%), *floR* (37.1%), and the least detected sulA (2.9%). 22.8% (8 out of 35) *S. Typhimurium* showed a penta-resistant profile which contained gene encoding for ampicillin (*blaTEM*), chloramphenicol (*floR*), streptomycin (*strA*), and tetracycline (*tetA*). All the penta-resistant isolates lacked the *sulA* gene encoding for sulfonamides (Table 2). However, the *sulA* gene was only detected in one *S. Typhimurium* (isolate ID 18) with phenotype resistant to three antibiotics tested. Seven isolates (20%) showed resistance to at least four antibiotics, 11 isolates (31.4%) showed resistance to at least two antibiotics while nine isolates (25.7%) were susceptible to all five antibiotics (Table 3). None of the resistant genes was detected among all the susceptible *S. Typhimurium* isolates. In this study, four isolates (isolate ID 19, 20, 21, and 24) were found to carry the blaTEM resistance gene but demonstrated a susceptible profile by antibiotic sensitivity test to ampicillin. In addition, four isolates (isolates ID 12, 14, 15, and 23) showed phenotypic resistance to chloramphenicol, however, none of those isolates carry the *floR* resistance gene. Five isolates showed intermediate resistance profile to chloramphenicol, streptomycin, and sulfonamides but genotype profiles varied among those isolates.

Statistical analysis using Pearson Chi-Square (Table 4) showed a significant association between the presence of *blaTEM*, *floR*, *strA*, and *tetA* resistance gene with phenotype antibiotic resistance profile with $p < 0.05$. However, for *sulA* gene statistical analysis revealed that there is no association between the resistance gene with antibiotic resistance profile with $p > 0.05$ (0.435).

4. DISCUSSION

A large percentage of *S. Typhimurium* isolates were resistant to multiple antibiotics in agreement with previous reports by other researchers (Bacci et al., 2012; Ahmed & Shimamoto, 2012). In this finding we found eight *S. Typhimurium* isolates exhibited penta resistant profile ACSSuT which is commonly related to DT104 phage type. In the recent past, *S. Typhimurium* DT104 is primarily found in cattle, however, this pathogenic strain has been frequently isolated from swine, poultry, and other domestic animals (Rajashekara et al., 2000; Leekitcharoenphon et al., 2016). We further found, however, that all the penta-resistant isolates were isolated from apparently healthy chickens without a history of clinical signs recorded in the sample submission form during submission to VRI for further serotyping.

Table 1: Simplex PCR primers for the detection of corresponding resistance genes.

Antimicro-bial	Gene	Forward and reverse primer sequence (5' – 3')	Amplic-on size (bp)	Reference
Ampicilin	*blaTEM F* *blaTEM R*	GCA CGA GTG GGT TAC ATC GA GGT CCT CCG ATC GTT GTC AG	310	Hur *et al*., 2011 Faldynova *et al*., 2003 Carlson *et al*., 1999
Chloramp henicol	*floR F* *floR R*	CTG AGG GTG TCG TCA TCT AC GCT CCG ACA ATG CTG ACT AT	673	Abatcha *et al*., 2018
Streptomy cin	*strA F* *strA R*	CTT GGT GAT AAC GGC AAT TC CCA ATC GCA GAT AGA AGG C	548	Hur *et al*., 2011 Levings *et al*., 2005 Gebreyes and Altier, 2002
Sulfonami des	*sulA F* *sulA R*	CAC TGC CAC AAG CCG TAA GTC CGC CTC AGC AAT ATC	360	Gebreyes and Altier, 2002
Tetracycli ne	*tetA F* *tetA R*	GCT ACA TCC TGC TTG CCT TC CAT AGA TCG CCG TGA AGA GG	210	Ng *et al*., 2001

Rajashekara et al (2000) explained that infection with DT104 might not produce clinical disease in the infected chicken, but infection with DT104 strain particularly in laying hens might transmit to humans through intact egg shells and meat. The data presented here suggest that DT104 is present in the poultry industry in the country as early as 2012. Although these penta-resistant strains were found to carry only blaTEM, floR, strA, and tetA resistance genes, their close relationship to DT104 through phenotyping analysis may signify its potential public health and economic importance. The first report on *S. Typhimurium* which ACSSuT-resistant pattern in Malaysia involved *S. Typhimurium* isolates retrieved from human and various animal species including chicken published in 2010. A previous finding by Benacer et al (2010) revealed that four out of 47 isolates tested exhibited a DT104 resistance pattern. High rates of resistance among the *S. Typhimurium* isolates also were indicated with the presence of resistance genes and integrons (Benacer et al., 2010).

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Table 2: Antibiotic resistance profiles and resistance gene in penta-resistant *S. Typhimurium* isolated from chicken.

	Antibiotics									
Isolates ID	Ampicillin AMP10		Chloramphenicol C30		Streptomycin S10		Sulfonamides S300		Tetracycline TE30	
	AST	*bla*$_{TEM}$	AST	*floR*	AST	*strA*	AST	*sulA*	AST	*tetA*
1	R	/	R	/	R	/	R	X	R	/
2	R	/	R	/	R	/	R	X	R	/
3	R	/	R	/	R	/	R	X	R	/
4	R	/	R	/	R	/	R	X	R	/
5	R	/	R	/	R	/	R	X	R	/
6	R	/	R	/	R	/	R	X	R	/
7	R	/	R	/	R	/	R	X	R	/
8	R	/	R	/	R	/	R	X	R	/

AST: Antibiotic sensitivity test result
R: Resistance
/: Gene detected
X: Gene not detected

Table 3: *Salmonella Typhimurium* isolates with phenotype resistant profile to at least two antibiotics tested.

	Antibiotics									
Isolates ID	Ampicillin AMP10		Chloramphenicol C30		Streptomycin S10		Sulfonamides S300		Tetracycline TE30	
	AST	*bla*$_{TEM}$	AST	*floR*	AST	*strA*	AST	*sulA*	AST	*tetA*
9	R	/	R	/	MS	X	R	X	R	/
10	R	/	R	/	R	/	R	X	S	/
11	R	/	MS	/	R	/	R	X	R	/
12	S	/	R	X	R	/	R	X	R	/
13	R	/	R	/	R	/	R	X	S	/
14	R	/	R	X	R	/	R	X	R	/
15	R	/	R	X	R	/	R	X	R	/
16	R	/	R	/	R	/	R	X	R	/
17	R	/	S	X	R	X	S	X	R	/
18	R	/	S	X	S	/	R	/	R	/
19	S	/	S	X	R	/	R	X	R	/
20	S	/	S	X	R	/	R	X	R	/
21	S	/	S	X	R	/	R	X	R	/
22	R	/	S	X	R	/	MS	X	R	/
23	S	/	R	X	R	/	R	X	S	X
24	S	/	S	X	S	/	R	X	R	/
25	R	/	S	X	S	X	S	X	R	X
26	R	/	MS	X	S	X	S	X	R	/
27	R	/	S	/	MS	/	R	X	S	/
28	S	X	S	X	S	X	S	X	S	X
29	S	X	S	X	S	X	S	X	S	X
30	S	X	S	X	S	X	S	X	S	X
31	S	X	S	X	S	X	S	X	S	X
32	S	X	S	X	S	X	S	X	S	X
33	S	X	S	X	S	X	S	X	S	X
34	S	X	S	X	S	X	S	X	S	X
35	S	X	S	X	S	X	S	X	S	X
36	S	X	S	X	S	X	S	X	S	X

AST: Antibiotic sensitivity test result
R: Resistance
MS: Intermediate resistance
S: Sensitive
/: Gene detected
X: Gene not detected

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Table 4: Statistical analysis summary for each antibiotic.

Antibiotics	Resistant gene	Pearson Chi-square value	P value
Ampicillin	*bla*$_{TEM}$	16.154	p= 0.00, p<0.05
Chloramphenicol	*floR*	21.581	p= 0.00, p<0.05
Streptomycin	*strA*	21.581	p= 0.00, p<0.05
Sulfonamides	*sulA*	0.608	p= 0.435, p>0.05
Tetracycline	*tetA*	19.863	p= 0.00, p<0.05

- P<0.05 show significant association

Resistance to traditional antibiotics such as tetracycline, ampicillin, and sulfonamides was observed in this study, in agreement with previously reports by other authors (Adesiji et al., 2014; Hu et al., 2020). The high resistance rate to these traditional antibiotics has also been reported in other developing countries as it still been used in human therapy due to their low cost and availability (Adesiji et al., 2014). In Malaysia, a total of 47 *S. Typhimurium* isolates isolated from various animal species demonstrated a multidrug-resistant profile with high resistance to tetracycline, sulfonamides, ampicillin, and chloramphenicol (Benacer et al., 2010). Long-term exposure to antibiotics has been known as one of the important factors attributed to antibiotic resistance in bacteria. The previous finding revealed that under short-term antibiotic pressure, susceptible *S. Typhimurium* strains can gradually develop resistance to tetracycline in addition to acquired resistance gene through horizontal transfer from other microbes (Peng et al., 2018). This finding also explained that *S. Typhimurium* barely lost its antibiotic resistance genes throughout the period of study, which indicated relatedness between high resistances to traditional antibiotics which have been used in food-producing animals for a long time (Peng et al., 2018). High resistance to streptomycin in several *Salmonella* serovars including *S. Typhimurium* reported in Brazil by Rodrigues et al (2020) can be evidence of streptomycin, a broad-spectrum aminoglycoside widely used as a growth promoter in poultry and swine. Extensive use of certain antibiotics over a long period of time can trigger antibiotic resistance to naïve bacterial strain, in addition with the presence of associated resistance genes. Genotyping analysis revealed that these isolates carried resistance genes blaTEM, floR, strA, and tetA is consistent with the DT104 reference strain used in this study. However, the sulA gene encoding for sulfonamides resistance was not detected in the majority of the isolates tested. It is suggested that the sulA gene was least detected among the isolates might be due to the genetic heterogeneity among *S. Typhimurium* isolates.

Besides that, it is claimed that strains collected from the same location and time also contain different resistant gene profiles (Gebreyes & Altier, 2002). On the other hand, the presence of integron, a well-defined structure consisting of an integrase gene, which catalyzes the integration of new genes on conjugative plasmid might lead to the easy transfer of those resistance genes within and between bacterial species, leading to the rapid evolution of multidrug resistance profile (Gebreyes and Altier, 2002). Since most of the isolates do not harbor the sulA gene, it is hypothesized that this might also happen due to the loss survival of chromosomal pieces in the environment or selective expression of resistance genes by *Salmonella* strains outside the animal host (Gebreyes and Altier, 2002). Plasmid compatibility plays an important factor in the expansion of antimicrobial resistance genes in *Salmonella*, McMillan et al (2019) demonstrated that plasmids of the same incompatibility group are unlikely to persist in the same isolate. Moreover, four isolates that were susceptible to ampicillin also were found to confer the blaTEM gene. It is suggested that this condition might due to the some antimicrobial genes do not express in bacteria in-vitro but under certain conditions might turn to express in-vivo (Adesiji et al., 2014).

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