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Antibiotic resistance and characterisation of *Salmonella* Typhimurium isolated from chicken from slaughtering house

[1]Aishah, E., [2]Safiyyah, S., [1]Zaini, N. A. M., [1,3]*Sahilah, A. M. and [4]Azuhairi, A. A.

[1]Department of Food Science, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia
[2]Universiti Teknologi MARA, Cawangan Negeri Sembilan, Kampus Kuala Pilah, 72000 Kuala Pilah, Negeri Sembilan, Malaysia
[3]Innovation Centre for Confectionery Technology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia
[4]Department of Community Health, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

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Abstract

Salmonella is one of the major food pathogens in the broiler industry. Subtyping of *Salmonella* is critical to monitor and trace its existence in chicken processing lines. Therefore, the present work evaluated the antibiotic resistance and characterisation of *Salmonella* Typhimurium isolated from the chicken slaughterhouse. A total of 1,100 colonies of tentative *Salmonella* were isolated and identified using biochemical tests and polymerase chain reaction (PCR). From these 1,100 colonies, 474 were identified as *Salmonella* spp., while 18 were *S.* Typhimurium. Antibiotic resistance against nine types of antibiotics was examined for the 18 isolates of *S.* Typhimurium, namely amoxicillin (10 µg), ampicillin (10 µg), chloramphenicol (30 µg), gentamicin (10 µg), penicillin (10 µg), streptomycin (10 µg), sulfamethoxazole (25 µg), tetracycline (30 µg), and vancomycin (30 µg). It was found that these isolates were resistant to at least four antibiotics or more, while the multiple antibiotic resistance (MAR) index value was 0.64. All *S.* Typhimurium isolates were resistant to amoxicillin, ampicillin, penicillin, and vancomycin, followed by tetracycline (55.6%, n = 10/18), gentamicin (44.4%, n = 8/18), chloramphenicol (38.9%, n = 7/18), streptomycin (27.8%, n = 5/18), and sulfamethoxazole (11.1%, n = 2/18). A total of 12 antibiograms were observed; A1 - A12. Plasmid's size ranged from 3 to > 24 kbp, and seven plasmid profiles (P1 - P7) were observed, while Enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) showed 12 ERIC-PCR fingerprinting (E1 - E12). GelCompar II software discriminated the *S.* Typhimurium into four clusters and five single isolates at 80% similarity. The antibiogram, plasmid profiling, and ERIC-PCR fingerprinting revealed significant genetic heterogeneity in *S.* Typhimurium strains indicating that consumers could face high contamination risk from consuming chickens obtained from the studied slaughterhouse.

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Introduction

Salmonellosis is one of the most common infectious diseases transmitted by *Salmonella*-contaminated foods, and can cause food-borne illness in humans. *Salmonella* are Gram-negative short rods measuring about 0.7 - 1.5 µm by 2 - 5 µm, non-sporulating, catalase positive, and oxidase negative. *Salmonella* can ferment sugars with gas production, produce H_2S, and are motile due to peritracheal flagella, except for *S.* Pullorum and *S.* Gallinarum, which are non-motile (Plym and Wierup, 2006). These bacteria are either aerobic or facultative anaerobic, and similar to other mesophilic bacteria, *Salmonella* can thrive at a temperature range between 5 and 45°C. Their optimum growth temperature is 37°C, and thus, *Salmonella* can greatly multiply in foods that have been contaminated and left at room temperature (Tessari *et al.*, 2012). *Salmonella* naturally live in the intestinal tract of animals,

*Corresponding author.
Email: sahilah@ukm.edu.my

including chicken, swine, cattle, sheep, avian, and pet animals (Demirbilek, 2018). As intestinal inhabitants in numerous animal species, *Salmonella* can spread by infected animal faecal material and urine, and subsequently contaminating the environment. After leaving their habitats, these bacteria can disseminate and contaminate water, foods, and inanimate objects, providing opportunities for a new infection to fester. According to Tessari *et al*. (2012), contamination of poultry products (meat and eggs) destined for human consumption may occur either at the slaughterhouse, during food preparation, or by cross-contamination with materials from poultry with intestinal and systemic infections.

The current version of the nomenclature shows that *S.* Typhimurium is different from *Salmonella* Typhi due to their O and H antigens. A specific serovar for *S.* Typhi is designated as [9,12, Vi/d/–]. *S.* Typhi does not have a phase II antigen, whereas *S.* Typhimurium is designated as [1,4,5,12/i/1,7] (Smith *et al*., 2016). *S.* Typhi can cause prolonged fever, known as enteric fever. Meanwhile, the non-typhoidal *Salmonella* (NTS) and *S.* Typhimurium can cause infection in humans with predominant symptoms of fever, abdominal cramps, diarrhoea, vomiting, and headache, and the onset symptoms can be observed within six to 96 hours (one to four days) (Smith *et al*., 2016; Gong *et al*., 2022). The foodborne non-typhoidal *Salmonella* is estimated to cause over 93 million cases of gastroenteritis annually, and 155,000 deaths globally (Majowicz *et al*., 2010). *S.* Typhimurium usually infects humans, and is considered a primary cause of foodborne disease in developed countries, and responsible for considerable morbidity and occasionally death, especially in immunocompromised patients (Casin *et al*., 1999). Literature has shown that *S.* Typhimurium infection can occur from improperly handled food contaminated by animals' faeces or humans carrying the bacteria (Harish and Menezes, 2015). The major reservoir of *S.* Typhimurium is poultry, including meat and eggs (Dar *et al*., 2017). *S.* Typhimurium is also commonly associated with fruits, vegetables, and peanut products.

Antibiotic resistance in *Salmonella* is driven mostly by the use of antibiotics in animal husbandry or food-producing animals. The antibiogram or antibiotic resistance profile is a reflection of the length of time an antibiotic has been used. World Health Organization (WHO, 2021) reported an increase in antibiotic resistance as the causative agents of infectious diseases in humans, animals, and the environment, and this has caused major issues in clinical microbiology. The increase in antimicrobial resistance has narrowed the potential uses of antibiotics for the treatment of microbial infections in humans and animals (Angulo *et al*., 2004). Antibiogram has another function; it can be used as one of the phenotyping characterisations for bacterial subtyping. In epidemiological studies, bacterial subtyping is vital for monitoring the clonal circulating in different locations. Besides antibiogram, plasmid profiling and other DNA-based PCR analyses, such as Enterobacterial repetitive intergenic consensus (ERIC), are also useful in bacterial subtyping. Bacterial subtyping is critical for determining the pathogenic profile of bacteria, detecting new pathogenic strains, and linking cases in epidemiological studies. Therefore, the present work aimed to provide essential insights into the resistance profiles of *S.* Typhimurium, highlighting the critical role of monitoring and tracing *S.* Typhimurium typing in the chicken processing line.

In the present work, the prevalence of antibiotic resistance *S.* Typhimurium isolated from a chicken slaughterhouse was identified and evaluated. We isolated and identified 474 strains of tentative *Salmonella* spp. using random sequence target and *fli*C target genes in the chicken carcasses and environment areas around the slaughterhouse. The antibiogram and ERIC-PCR amongst *S.* Typhimurium isolates were also examined.

Materials and methods

Sampling

The project was approved by the UKM Animal Ethical Committee (UKMAEC) of Universiti Kebangsaan Malaysia (approval no.: FST/2019/SAHILAH/25-SEPT./1037.-SEPT.-2020-NAR-CAT2). Samples were procured from halal chicken slaughtering house, in Semenyih, Selangor, Malaysia (GPS 2.9692774682140315, 101.83764240143056) in February, March, and October 2020. Swab samples (n = 7) were collected from the chicken crate (1), chicken hook (1), scalding tank (1), defeathering (1), rinsing track (1), chicken cutter (1), and drain (1). For water samples (n = 4), samples were collected from the scalding tank (1), rinsing track water (1), spin chiller water (1), and drain water (1). Samples from chicken carcasses were collected from two carcasses, and six samples were

taken from each (n = 6 × 2) carcass: thigh (2), wing (2), and breast (2). All samples were stored in an ice box prior to being analysed in the laboratory on the same day.

Swab and water sample preparation

Swab and water samples were taken according to Fuzihara *et al*. (2000) and isolation method of *Salmonella* spp. and *S*. Typhimurium, as described by ISO 6579 (ISO, 2017). Briefly, 40 mL of buffered peptone water (BPW) was used, and swab was aseptically dipped into it to moisten. Then, an area of 25 cm^2 was swabbed. Swabbed cotton was lodged into the 40 mL BPW, immediately taken to the laboratory, pre-enriched with 160 mL of BPW (to a total of 200 mL), and shaken using a stomacher (Seward™, UK), followed by incubation at 37°C for 24 h in an incubator (Memmert, GY). For the water samples, 100 mL of water was taken and immediately transported to the laboratory. These water samples were then mixed with 100 mL of BPW (to a total of 200 mL), and incubated at 37°C for 24 h.

Following incubation, 0.1 mL of pre-enriched swab and water samples were enriched with 10 mL of Rappaport-Vassiliadis (RVS) broth (Oxoid, UK). The solution was incubated at 42°C for 24 h. Swab and water samples were then plated in triplicates onto MacConkey, *Salmonella Shigella* (SS), and Xylose Lysine Deoxycholate agar (XLD) agar plates. The selective agars were incubated at 37°C for 24 h (ISO 6579) (ISO, 2017). Then, 1 mL of each swab and water sample was enriched in 10 mL of Muller-Kauffmann Tetrathionate-Novobiocin (MKTTn) broth (Oxoid, UK). The solution was incubated at 37°C for 24 h. Swab and water samples were then plated in triplicates, as mentioned above. The selective agars were incubated at 37°C for 24 h (ISO, 2017). From each MacConkey, SS, and XLD agar plates, five presumptive colonies were taken and grown into Luria Bertani (LB) broth (Oxoid, UK). The LB broth mixtures were then incubated at 37°C for 24 h. All presumptive colonies were examined for triple sugar iron (TSI), citrate, and catalase for biochemical confirmation.

Chicken sample preparation

Similar to the above, the isolation of *Salmonella* spp. and *S.* Typhimurium were as described by ISO 6579 (ISO, 2017). The pre-enrichment process was conducted by adding 25 g meat samples with 225 mL of BPW, and these samples were homogenised using a stomacher. The homogenised samples were incubated at 37°C for 24 h. Then, 0.1 mL of pre-enriched samples were enriched with 10 mL of RVS broth, and incubated at 42°C for 24 h. Another 1 mL of homogenised sample was added to 9 mL MKTTn broth, and incubated at 37°C for 24 h. All of these solutions were then streaked onto MacConkey, SS, and XLD agar plates in triplicate. All inoculated plates were incubated at 37°C for 24 h. From each triplicate agar, five presumptive colonies were taken and grown into LB broth at 37°C for 24 h. Those colonies were then examined for TSI, citrate, and catalase for biochemical confirmation.

DNA extraction

The boiling method was used to extract the DNA of presumptive colonies (De Medici *et al*., 2003). Briefly, 1 mL of aliquot from each tube containing the overnight culture was put into a microcentrifuge tube (Eppendorf, GY), and centrifuged at 13,000 *g* for 5 min. The resulting supernatant was discarded, while the pellet was added to 300 µL of sterile distilled water, and vortexed. The mixture was centrifuged for the second time at 13,000 *g* for 5 min. The resulting supernatant was carefully discarded, while the pellet was added to 200 µL of sterile distilled water, and vortexed. The produced mixture was boiled in boiling water for 10 min, then cooled at -20°C for 10 min, and centrifuged at 13,000 *g* for 5 min. The final supernatant was transferred into a new microcentrifuge tube, and used as a template for PCR amplification.

Oligonucleotide primers

The oligonucleotide primers used were referred to Thung *et al*. (2016). Three sets of primers were used: (i) ST11 (5'- GCCAA CCATT GCTAA ATTGG CGCA – 3') and ST15 (5' – GGTAG AAATT CCCAG CGGGT ACTGG – 3') to detect *Salmonella* spp. by targeting random sequence (429 bp) (Soumet *et al*., 1999); (ii) ENTF (5'- TGTGT TTTAT CTGAT GCAAG AGG -3') and ENTR (5'- TGAAC TACGT TCGTT CTTCT GG-3') to detect *S.* Enteritidis target gene *SdfI* (304 bp) (Alvarez *et al*., 2004); and (iii) Fli15 (5' – CGGTG TTGCC CAGGT TGGTA AT – 3') and Typ04 (5' - ACTGG TAAAG ATGGC T – 3') to detect *S*. Typhimurium target gene *fliC* (620 bp) (Soumet *et al*., 1999). Positive controls were *S*. Enteritidis ATCC 13076 and *S*. Typhimurium ATCC 14028.

Polymerase chain reaction (PCR)

DNA amplification was carried out in a 25 µL reaction mixture containing 12.5 µL master mix, 5.0 µL DNA template, and 0.3 µL forward and reverse primers each (ENTF and ENTR, ST11 and ST15, and Fli15 and Typ04) with a concentration of 10 µM and 5.5 µL sterile distilled water. The negative control was similarly prepared, but did not contain the DNA template. The PCR reactions were carried out in a thermal cycler (Analytik Jena Flexcyler^2, GY), starting with an initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation for 30 s at 95°C, 1-min annealing at 53°C, 15-s elongation at 72°C, and final extension at 72°C for 5 min. Gel electrophoresis was completed in a 1.5% (w/v) agarose in 1X TAE (40 mM Tris-OH), 20 mM acetic acid, and 1 mM of EDTA, pH 7.6 at 120 V for 30 min, pre-stained with RedSafe nucleic acid stain (JH Science, USA), and visualised and photographed using a UV transilluminator (Syngene, UK). A 100 bp ladder (Genedirex, CN) was used as a DNA size marker.

Antibiotic resistance

Antibiotic resistance tests were examined on Mueller-Hinton agar (MHA) (Oxoid, UK) using the disc diffusion method described by Bauer *et al*. (1966). The resistance of the bacteria was tested against nine antibiotic discs (µg/disc) (Oxoid, UK). These isolates were tested for amoxicillin (10 µg), ampicillin (10 µg), chloramphenicol (30 µg), gentamicin (10 µg), penicillin (10 µg), streptomycin (10 µg), sulfamethoxazole (25 µg), tetracycline (30 µg), and vancomycin (30 µg). These inoculums were spread evenly on the surface of the agar plate by swabbing in three directions using a sterile cotton swab. To ensure no contamination, antibiotic discs were applied firmly on the agar surface within 15 min of inoculation, and incubated at 37°C for 24 h. The sensitivity or resistance of each isolate towards each antibiotic was determined by measuring the diameter of the inhibition zone around the antibiotic disc.

Multiple antibiotic resistance (MAR) index

The MAR index of the isolates was calculated and interpreted according to Lee and Wendy (2011) using the following formula: MAR index = X / (Y × Z), where X = total number of antibiotic resistance cases, Y = the total number of antibiotics used in the study, and Z = the total number of bacterial isolates. A high risk of exposure to the antibiotics administered to the animals will be denoted by a MAR index value of higher than 0.2 (Al-Dulaimi *et al.*, 2019).

Plasmid profiling

Plasmid profiling began by extracting the plasmid using the FavorPrep™ Plasmid Extraction Mini Kit (Favorgen, TW) following the manufacturer's instructions. From the overnight culture, 5 mL of overnight culture was lysed, centrifuged, plasmid-bound, washed, column-dried, and eluted. Then, 10 µL of plasmid extracts were added to 2 µL of 6× loading dye (NEB, US), and pipetted in and out to mix. These extracts were loaded into the well of a 1.5% (w/v) agarose in 1X TAE (40 mM Tris-OH, 20 mM acetic acid, and 1mM of EDTA; pH 7.6) at 100 V for 40 min, pre-stained with RedSafe nucleic acid stain (JH Science, USA), and viewed and photographed with UV transilluminator (Syngene, UK). For the DNA marker, a lambda *Hind*III DNA ladder was used.

ERIC-PCR

ERIC-PCR was performed following the methods of Safiyyah *et al*. (2021). The primers used were ERIC1R (5'-CAC TTA GGG GTC CTC GAA TGT A -3') and ERIC2 (5'- AAG TAA GTG ACT GGG GTG AGC G-3') (Versalovic *et al*., 1991). The assay was carried out in 25 µL solution containing 12.5 µL of Vazyme rapid *Taq* mastermix (Vazyme, CN), 5 µL of DNA template, 1 µL each of 10 µM forward and reverse primers, and 5.5 µL of distilled water. The PCR reaction was carried out using a thermal cycler (Analytik Jena Flexcyler^2, GY) with pre-denaturation at 95°C for 7 min, followed by 35 cycles of denaturation at 95°C for 30 s. Annealing was performed at 52°C for 30 s, and extension was performed at 72°C for 5 min. The final extension was conducted at 72°C for 10 min. The amplicons were analysed by electrophoresis in a 1.5% (w/v) agarose in 1X TAE (40 mM Tris-OH), 20 mM acetic acid, and 1 mM of EDTA, pH 7.6 at 120 V for 30 min, pre-stained with RedSafe nucleic acid stain (JH Science USA), and visualised and photographed using a UV transilluminator (Syngene, UK). A 1 kb ladder (Genedirex, CN) was used as a DNA size marker.

Data interpretation

The DNA band patterns produced on the gel image were analysed according to Al-Dulaimi *et al*. (2019). These clusters were based on unweighted

pairwise grouping with arithmetic significance (UPGMA), as well as the Dice similarity coefficient analysed through the dendrogram software Gel ComparII (Applied Math, Kortjik, Belgium).

Result and discussion

The isolation of *Salmonella* spp. and *S.* Typhimurium were conducted in a halal chicken slaughtering house in Semenyih, Selangor. Sampling were swabs, water samples, and chicken carcasses for three interval times in February, March, and October 2020. Isolation of *Salmonella* spp. and *S.* Typhimurium were conducted as described by ISO 6579 (ISO, 2017). In each sampling, 23 samples were collected, resulting in 69 samples to be examined for the presence of *Salmonella* spp. From the 1,175 isolates of *Salmonella* spp. isolated using selective agar, 537 presumptive colonies were positive for citrate, TSI, and catalase tests. Then, the PCR method was used to identify *Salmonella* spp. and *S.* Typhimurium in the samples. Based on the analysis, 474 isolates were positive for *Salmonella* spp., 18 samples were S. Typhimurium, and no *S.* Enteritidis was detected. Therefore, the prevalence of *Salmonella* spp. was 86.96% (*n* = 20/23), 65.22% (*n* = 15/23), and 52.12% (*n* = 12/23) for first, second, and third samplings, respectively, as shown in Table 1. While for *S.* Typhimurium, the prevalence was 30.43% (*n* = 7/23), 0% (*n* = 0/23), and 8.70% (*n* = 2/23) for the first, second, and third samplings, respectively. On average, the prevalence of *Salmonella* spp. prevalence was high, 68.12% (*n* = 47/69), and these findings were contradicted by Fuzihara *et al.* (2000), who reported only 41% prevalence in slaughterhouses. The variations between these results might have been due to the time of sampling and sources of chicken supplied to the slaughterhouse. The Semenyih slaughterhouse started its operation at 4 am, and ceased at 10 am. As sampling was conducted between 7 - 8 am, it could increase the possibility of *Salmonella* spp. detection in the slaughterhouse. As various poultry farms also sourced these chickens, it could be another contributing factor that led to the high prevalence of *Salmonella* spp., as various poultry farms could practice varied hygienic practices, thus affecting the microbial community and *Salmonella* spp. loads.

Table 1. Prevalence of *Salmonella* spp. and *S.* Typhimurium isolates obtained from poultry slaughterhouse in Semenyih, Selangor, Malaysia.

Sample	Sample number	Sample positive for *Salmonella* spp. (%)	Sample positive for *S.* Typhimurium (%)	Isolated *S.* Typhimurium strain
1st sampling				
Swab	7	100.00 (7/7)	0 (0/7)	
Water	4	100.00 (4/4)	50.00 (2/4)	ST1, ST2, ST3
Chicken before washing	6	100.00 (6/6)	50.00 (3/6)	ST9, ST10, ST11, ST15, ST16
Chicken after washing	6	50.00 (3/6)	33.33 (2/6)	ST12, ST13, ST14, ST17, ST18
Total	23	86.96 (20/23)	30.43 (7/23)	
2nd sampling				
Swab	7	28.57 (2/7)	0 (0/7)	
Water	4	25.00 (1/4)	0 (0/4)	
Chicken before washing	6	100.00 (6/6)	0 (0/6)	
Chicken after washing	6	100.00 (6/6)	0 (0/6)	
Total	23	65.22 (15/23)	0 (0/23)	
3rd sampling				
Swab	7	71.43 (5/7)	0 (0/7)	
Water	4	75.00 (3/4)	0 (0/4)	
Chicken before washing	6	33.33 (2/6)	0 (0/6)	
Chicken after washing	6	33.33 (2/6)	33.33 (2/6)	ST4, ST5, ST6, ST7, ST8
Total	23	52.17 (12/23)	8.69 (2/23)	
Grand Total	**69**	**68.12 (47/69)**	**13.04 (9/69)**	

As mentioned earlier, 18 *S.* Typhimurium were detected using PCR analysis; thus, the attention was shifted to characterise those isolates since *S.* Typhimurium is a prominent *Salmonella* serovar in chicken (Dar *et al*., 2017). The isolates were tested with nine antibiotics in six different groups: β-lactam (3), chloramphenicol (1), aminoglycoside (2), sulphonamide (1), tetracycline (1), and glycopeptide (1). Antibiotics resistance test showed that *S.* Typhimurium was resistant to at least four of the nine antibiotics, as shown in Table 2.

Table 2. Typing of *Salmonella* Typhimurium isolates by antibiograms, plasmid profiling, and enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) obtained from poultry slaughterhouse in Semenyih, Selangor, Malaysia.

Strain no.	MAR	Antibiotic resistance pattern	Antibiograms	Plasmid profile	ERIC-PCR fingerprinting	Strain profile
ST1	0.78	AcAmGPSTV	A1	P1	E1	1
ST2	0.56	AcAmGPV	A2	ND	Cluster1, E2	2
ST3	0.67	AcAmGPSV	A3	ND	Cluster 1, E3	3
ST4	0.56	AcAmGPV	A2	P2	E8	4
ST5	0.67	AcAmGPTsV	A4	P3	Cluster 2, E6	5
ST6	0.56	AcAmCPV	A5	P2	E4	6
ST7	0.44	AcAmPV	A6	P3	E11	7
ST8	0.67	AcAmCGPV	A7	P2	Cluster 2, E5	8
ST9	0.78	AcAmCGPTV	A8	P2	Cluster 4, E12	9
ST10	0.44	AcAmPV	A6	P2	Cluster 4, E12	10
ST11	0.56	AcAmPTV	A9	P3	Cluster 4, E12	11
ST12	0.56	AcAmPTV	A9	P4	Cluster 4, E12	12
ST13	0.67	AcAmCPTV	A10	P4	Cluster 4, E12	13
ST14	0.67	AcAmCPTV	A10	P5	Cluster 4, E12	14
ST15	0.67	AcAmCPTV	A10	P6	Cluster 4, E12	15
ST16	0.67	AcAmPSTV	A11	P6	Cluster 3, E9	16
ST17	1.00	AcAmCGPSTsTV	A12	P7	E7	17
ST18	0.67	AcAmPSTV	A11	P7	Cluster 3, E10	18

Ac: amoxicillin, Am: ampicillin, C: chloramphenicol, G: gentamicin, P: penicillin, S: streptomycin, Ts: sulfamethoxazole, T: tetracycline, and V: vancomycin. ND: not detected.

All isolates were found to be resistant to amoxicillin, ampicillin, penicillin, and vancomycin. Simultaneously, resistance toward tetracycline was 55.7% (*n* = 10/18), followed by gentamycin at 44.4% (*n* = 8/18), chloramphenicol at 38.9% (*n* = 7/18), streptomycin at 27.8% (*n* = 5/18), and sulphamethoxazole 11.1% (*n* = 2/18). Antibiogram showed 12 profiles, namely A1 to A12, with antibiogram A10 found as the frequent pattern (AcAmCPTV) shown by three isolates (ST13, ST14, and ST15). Only one isolate (ST17) was resistant to all nine antibiotics tested (AcAmCGPSTsTV, A12). Antibiogram represented similar patterns were A2 (ST2, ST4), A6 (ST7, ST10), A9 (ST11, ST12), and A11 (ST16, ST18). For antibiograms of A1, A3, A5, A7, and A8, they showed a single isolate in each pattern. Among all antibiograms, the A6 antibiogram (AcAmPV) was the most susceptible isolate with only four antibiotics resistance. Antibiograms A2 and A9 showed five resistant antibiotics, which were AcAmGPV and AcAmPTV, respectively. Antibiogram A11 consisted of six resistant antibiotics, which were AcAmPSTV.

All isolates of *S.* Typhimurium assessed in the present work were resistant toward penicillin, mirroring the finding of Mir *et al.* (2022), who reported that all *S.* Typhimurium isolated from chicken meat is resistant toward penicillin and tetracycline. However, for tetracycline, the analysis was contrasted with Mir *et al.* (2022), whose study found that 55.6% and no antibiotic tested for tylosin, erythromycin, and tiamulin. Resistance toward

penicillin was expected due to its rampant use in chicken-rearing facilities, as it is proven to be highly effective in treating sinusitis and chronic respiratory disease in poultry. The effectiveness of penicillin has been demonstrated by Jammoul and El Darra (2019), who revealed that chicken samples collected from Lebanese farms contained antibiotic residues of amoxicillin and penicillin G above the Maximum Residue Limit (MRL) set by the European Union. Guidelines have been promulgated to stop the overuse of antimicrobial agents in chicken to avoid the development of resistant *Salmonella*. Past research has shown that penicillin inhibited cell wall synthesis, and has been found ineffective against Gram-negative bacteria as the outer membrane causes the cell wall to be extra thick (Hopwood, 2007; Peterson and Kaur, 2018).

In the present work, all *S.* Typhimurium samples were tested against two other β-lactam antibiotics, such as amoxicillin and ampicillin. Results showed that all isolates of *S.* Typhimurium were resistant to both antibiotics. *S.* Typhimurium is resistant to β-lactam antibiotic due to the β-lactam antibiotic sharing a similar structure to penicillin-binding protein (PBP) substrates (peptidoglycan precursors), thus allowing the antibiotic to associate and cause acylation of the active site serine that leads to its inhibition (Peterson and Kaur, 2018). The resistance of all *S.* Typhimurium to all β-lactams antibiotics led to a high multiple antibiotic resistance (MAR) value. The MAR index of all 18 isolates of *S.* Typhimurium was recorded at 0.64, indicating a high risk of exposure to those antibiotics received by the animals. As stated earlier, the MAR index values higher than 0.2 indicated a high risk of exposure to the antibiotics administered to the animals (Al-Dulaimi *et al*., 2019). However, the practice of antibiotic administration of the poultry in the slaughterhouse was uncertain as no detailed information was investigated.

Plasmid profiling is one of several useful methods used to determine the link or absence of bacterial strains containing plasmid DNA with antibiotic resistance. It is a genotyping method that extracts bacterial plasmid DNA and electrophiles. Results revealed that *S.* Typhimurium contained plasmids ranging from 4 to > 24 Kb, as shown in Figure 1. Due to the varying sizes of plasmids, seven plasmid profiles were observed among the isolates, named as P1 - P7. Five isolates showed the most profiles (P2) of the *S.* Typhimurium, which contained > 24 Kb size plasmid. Profiles with single isolates were P1 and P5, while for every P4, P6, and P7, two isolates were found in each profile. P3 profiles had three of the same isolates. This finding correlated with the results of previous studies by Khasa *et al*. (2018), which showed that *S.* Typhimurium harboured plasmids from 90 to 120 kb in size. However, it was discovered in the present work that plasmid profiling was not very helpful in subtyping *S.* Typhimurium isolates, as opposed to the antibiogram method that managed to discriminate 12 antibiograms.

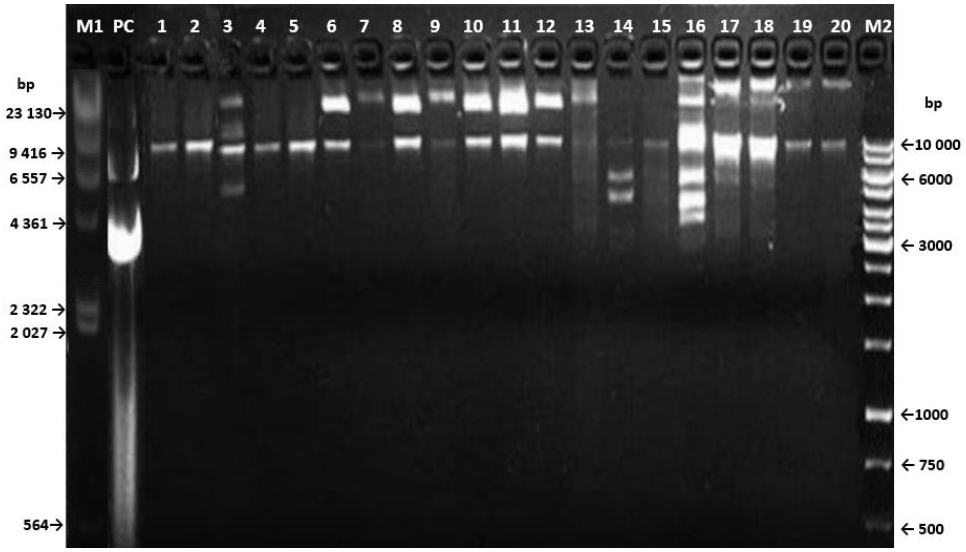

Figure 1. Plasmid profiles of 18 *Salmonella* Typhimurium isolates. Lane M1: Lambda hind marker: Lane PC: positive control; plasmid DNA *Escherichia coli* RR1 (Sigma, US); Lanes 1 - 2: positive control; *Salmonella* Typhimurium ATCC1308 DNA without plasmid (ND), *S.* Enteritidis DNA (ND); Lanes 3 - 20: *S.* Typhimurium isolates ST1 - ST18; Lane M2: 1 kb Marker (Smobio, TW); and ND: not detected.

As indicated in Table 3, 89% (*n* = 16/18) of *S.* Typhimurium isolates harboured plasmids, while 11% (*n* = 2/18) of isolates did not. Antibiotic resistance of isolates with plasmids and without plasmids were found to be similar in resisting amoxicillin, ampicillin, penicillin, and vancomycin, recorded at 100% resistant. However, more isolates containing plasmids were resistant compared to those without plasmids. This finding suggested that the resistance of *S.* Typhimurium isolates may be encoded on plasmids and chromosomal DNA. This finding was supported by Rychlik *et al*. (2006), who reported that the antibiotic resistance gene for ampicillin of *S.* Typhimurium is chromosomally located in the *blapsE-I* gene and plasmid-encoded in the *bla*TEM gene. Resistance genes for chloramphenicol, streptomycin, tetracycline, and sulphonamides are similarly located both in chromosome and plasmid (Rychlik *et al*., 2006). However, in the present work, all isolates resistant to chloramphenicol and tetracycline had plasmid, while for streptomycin, one of five isolates resistant to streptomycin without plasmid. This finding suggested that in this isolate, the streptomycin resistance gene was located in the chromosome. However, this discovery was not corroborated by another method like the detection of gene *aadA2* by PCR. Resistance plasmids, also known as R plasmids, are the plasmids that carry genes that provide resistance to various antibiotics such as chloramphenicol and tetracycline, and heavy metals such as mercury. Most R plasmids consist of two components: the resistance transfer factor (RTF) and resistance (R) genes. Thus, the link of R-plasmids with *Salmonella* virulence genes is significant in epidemiological studies. The virulence plasmid of *S.* Typhimurium has demonstrated its capability of independent conjugative transfer (Ahmer *et al*., 1999).

Table 3. Antibiotic resistance and plasmid occurrence of *Salmonella* Typhimurium isolates obtained from poultry slaughterhouse in Semenyih, Selangor, Malaysia .

Antibiotic	Number of resistant isolate	Number of resistant *S.* Typhimurium isolate	
		With plasmid (16)	Without plasmid (2)
Amoxicillin (Ac)	18	16 (100%)	2 (100%)
Ampicillin (Am)	18	16 (100%)	2 (100%)
Chloramphenicol (C)	7	7 (43.75%)	0 (0%)
Gentamycin (G)	8	6 (37.5%)	2 (100%)
Penicillin (P)	18	16 (100%)	2 (100%)
Streptomycin (S)	5	4 (66.67%)	1 (50%)
Sulphamethoxazole (Ts)	2	2 (12.5%)	0 (0%)
Tetracycline (T)	10	10 (62.5%)	0 (0%)
Vancomycin (V)	18	16 (100%)	2 (100%)

The plasmid profile and antibiogram in the present work were different for each isolate, as shown in Table 2. The same antibiogram did not show the same profiles of the plasmid, as exemplified by isolates ST13, ST14, and ST15 that indicated similar antibiograms but different plasmid profiles. This result shared similarity with Făgărăşan *et al.* (1997), which showed no parallelism between antibiotic resistance and plasmid profile because multiple plasmid profiles were found for the same resistance pattern.

Next, subtyping of the *S.* Typhimurium isolates using enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) was conducted. The ERIC-PCR is a popular tool for phylogenetic analysis and comparison of bacterial strains. This method targets DNA elements spread in different regions, and those separated by various distances depending on the length of existing DNA, and it could be achieved without needing prior information about the nucleotide sequence (Sahilah *et al*., 2005). The dendrogram of ERIC-PCR of *S.* Typhimurium is shown in Figure 2. Based on Figure 2, ERIC-PCR managed to successfully discriminate 18 isolates of S. Typhimurium into 12 profiles and four clusters with five single isolates at 80% similarity. Also, the ERIC-PCR results showed the genetic variation of the *S.* Typhimurium strains that were isolated from the same

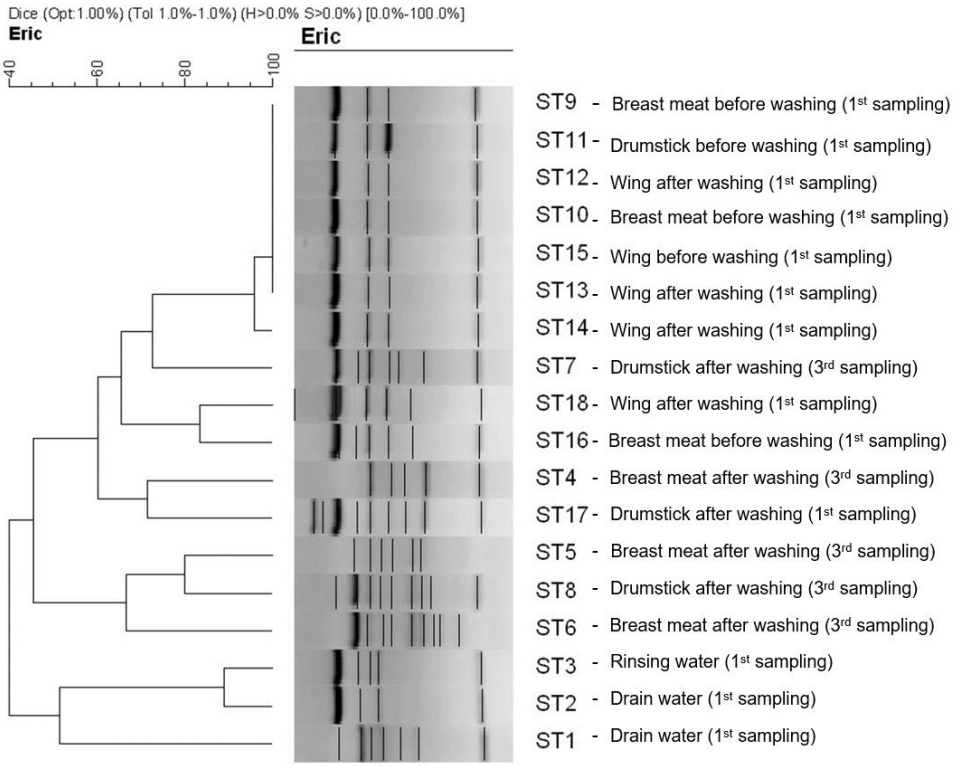

Figure 2. Dendrogram of *Salmonella* Typhimurium isolates (ST1 - ST18) by ERIC-PCR analysis. Clustering is based on unweighted pairwise grouping with arithmetic significance (UPGMA) as well as the Dice similarity coefficient.

slaughterhouse. The genetic variation exhibited by *S.* Typhimurium isolates can be found in samples and the heterogeneity given against *S.* Typhimurium strains in the samples. Some previous studies also used the ERIC-PCR method in observing the comparison and relationship of bacterial isolates from different samples. Anjay *et al*. (2015) used the ERIC-PCR for molecular typing of *S.* Typhimurium and *S.* Enteritidis serovars from diverse origins. It revealed similar genotypes among human isolates and isolates from foods of animal origin, water, vegetables, and sewage, thus indicating that the transmission of this important zoonotic pathogen is through food and between humans and animals.

These findings corroborated Fendri *et al*. (2013), which revealed that the heterogeneity or diversity given by *Salmonella* strains through ERIC-PCR not only occurred based on the sampling location but also within the same sample type. The ERIC-PCR analysis of *Salmonella* conducted by Herrera-Sánchez *et al*. (2020) also revealed that strains of these bacteria in the same group may be genetically related to each other, and these strains could be discriminated based on their source of isolation. The *Salmonella* spp. strains exhibited resistance to multiple antibiotics, as well as multiple genes associated with them, and the ERIC-PCR method was shown to be a helpful technique in generating clusters with biological significance (Herrera-Sánchez *et al*., 2020).

Conclusion

A combination of antibiotic resistance, plasmid profiles, and ERIC-PCR showed heterogeneity of *S.* Typhimurium isolates. Alarmingly, the high value of the MAR index highlights the potential risk faced by consumers due to antibiotic resistance. The presence of *S.* Typhimurium in slaughterhouses must be diligently observed to prevent the possibility of food poisoning if temperature abuse occurs along the food supply chain.

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