Prevalence and antibiotic resistance of *Salmonella* Enteritidis and *Salmonella* Typhimurium in raw chicken meat at retail markets in Malaysia

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ABSTRACT Salmonellosis is one of the major food-borne diseases in many countries. This study was carried out to determine the occurrence of *Salmonella* spp., *Salmonella* Enteritidis, and *Salmonella* Typhimurium in raw chicken meat from wet markets and hypermarkets in Selangor, as well as to determine the antibiotic susceptibility profile of *S.* Enteritidis and *S.* Typhimurium. The most probable number (MPN) in combination with multiplex polymerase chain reaction (mPCR) method was used to quantify the *Salmonella* spp., *S.* Enteritidis, and *S.* Typhimurium in the samples. The occurrence of *Salmonella* spp., *S.* Enteritidis, and *S.* Typhimurium in 120 chicken meat samples were 20.80%, 6.70%, and 2.50%, respectively with estimated quantity varying from <3 to 15 MPN/g. The antibiogram testing revealed differential multi-drug resistance among *S.* Enteritidis and *S.* Typhimurium isolates. All the isolates were resistance to erythromycin, penicillin, and vancomycin whereas sensitivity was recorded for Amoxicillin/Clavulanic acid, Gentamicin, Tetracycline, and Trimethoprim. Our findings demonstrated that the retail chicken meat could be a source of multiple antimicrobial-resistance *Salmonella* and may constitute a public health concern in Malaysia.

Key words: *Salmonella*, most probable number PCR, chicken meat, prevalence, antimicrobial susceptibility

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INTRODUCTION

Salmonella is an important cause of food-borne disease in humans throughout the world and is a significant cause of morbidity, mortality, and economic loss (Lin et al., 2014; Sallam et al., 2014). Among the more than 2,500 *Salmonella* serotypes, *Salmonella* Enteritidis and *Salmonella* Typhimurium are the most frequent serovars associated with human illness (Rodpai et al., 2013). Human *S.* Enteritidis cases are mostly associated with the consumption of contaminated eggs and poultry meat, while *S.* Typhimurium cases with the consumption of contaminated pork, poultry, and beef meat (Spector and Kenyon, 2012; Park et al., 2014). Prevalence of *S.* Enteritidis and *S.* Typhimurium in meat has been reported in many countries (de Freitas et al., 2010; Shah and Korejo, 2012; Rodpai et al., 2013). In Malaysia, while there have been numerous reports on the prevalence of *S.* Enteritidis and *S.* Typhimurium in food, very limited information has been published on *S.* Enteritidis and *S.* Typhimurium in retail raw chicken meat.

Rapid and accurate methods with shorter turnaround time (1 to 2 days) for the detection of *S.* Enteritidis and *S.* Typhimurium would significantly reduce the resources required in routine laboratory operations. Hence, multiplex polymerase chain reaction (**mPCR**) is undoubtedly useful to be a specific and sensitive method, which involves more than one pair of primers allowing a simultaneous detection and identification of different specific DNA sequences in a single-tube reaction (Chen et al., 2012). Saeki et al. (2013) reported that mPCR assay showed high specificity for the simultaneous detection and differentiation of *Salmonella* spp., *S.* Enteritidis, and *S.* Typhimurium in chicken meat. However, mPCR was limited to qualitative determination of bacterial pathogens. To be able to quantify the microorganism in a sample, the most probable number (**MPN**) method has been used. Thus, mPCR in association with the MPN method is usually used for detection and enumeration of food-borne pathogens (Chai et al., 2007; Kuan et al., 2013).

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The occurrence of antimicrobial resistance among zoonotic *Salmonella* is an increasing problem and has become a serious health hazard worldwide (Singh et al., 2013). Importantly, multi-drug resistant strains involved in human salmonellosis have been identified in commercial retail meats, and high levels of extended-spectrum beta-lactamase producing *Salmonella* have also been reported, particularly in poultry meat (EFSA and ECDC, 2012). Lately, widespread overuse and misuse of antibiotics in developing countries has contributed an increasing trend of drug resistance level of *Salmonella* (Ikwap et al., 2014). Geidam et al. (2012) reported high prevalence of multi-drug resistant strains of *Salmonella* was detected in the poultry environment in Selangor (centre of Peninsular Malaysia) region. Therefore, the objective of this study was to determine the prevalence of *Salmonella* spp., *S.* Enteritidis, and *S.* Typhimurium in raw chicken meat samples at retail markets in Selangor area of Malaysia using the MPN-mPCR method. Additionally, the antibiotic susceptibility profiles of *S.* Enteritidis and *S.* Typhimurium were also investigated.

MATERIALS AND METHODS

Sample Collection

From June to December 2014, a total of 120 samples of raw chicken meat (wings = 40; breast = 40; drumsticks = 40) were purchased randomly from wet markets and hypermarkets in Selangor, Malaysia. Wet markets were open markets that sold both live and processed chickens. Meanwhile, hypermarkets were enclosed markets that sold chickens chilled of frozen, supplied mostly by large integrated poultry companies. The collected samples were transported directly to the laboratory for analysis by using an ice box.

Enrichment and MPN Method

Enrichment and MPN method was performed as described by Pui et al. (2011). The sample (10 g) was homogenized with 90 mL of sterile buffered peptone water (Merck, Darmstadt, Germany) for 1 min using a Bag-Mixer 400P stomacher machine (Interscience, Saint-Nom-la-Bretèche, France). The suspension was then diluted 10-fold serially to 1,000-fold. Three-tube MPN method was carried out by transferring each dilution (1 mL) into triplicate MPN tubes containing 10 mL of Rappaport-Vasiliadis broth (Merck, Darmstadt, Germany). All the tubes were incubated under aerobic conditions at 37°C for 24 h. After incubation, the turbidity of the MPN tubes were examined prior to genomic DNA extraction.

Genomic DNA Extraction

The genomic DNA of bacterial cultures from the turbid MPN tubes was extracted by using boiled-cell method (Chai et al., 2007). A 1 mL of each broth was centrifuged at 12,000 × *g* for 5 min. The resulting pellet was resuspended in 500 μL of sterile distilled water and boiled for 10 min. Then, the mixture was cooled at −20°C for 10 min and centrifuged at 12,000 × *g* for 5 min. The supernatant containing DNA was used as template for the multiplex PCR.

mPCR Amplification

mPCR was conducted by using 3 sets of primers: i) ST11 (5′-GCCAA CCATT GCTAA ATTGG CGCA-3′) and ST15 (5′-GGTAG AAATT CCCAG CGGGT ACTGG-3′) for detection of *Salmonella* spp. 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′) for detection of *S.* Enteritidis targeting *SdfI* gene (304 bp) (Alvarez et al., 2004); and iii) Fli15 (5′-CGGTG TTGCC CAGGT TGGTA AT-3′) and Typ04 (5′-ACTGG TAAAG ATGGC T-3′) for detection of *S.* Typhimurium targeting the *fliC* gene (620 bp) (Soumet et al., 1999). Positive controls (*S.* Enteritidis ATCC 13076 and *S.* Typhimurium ATCC 14028) used in the multiplex PCR assay were obtained from the Institute for Medical Research, Malaysia. Amplification of DNA was performed in 25 μL reaction mixtures containing 2 μL DNA template, 5 μL 5× PCR buffer, 0.5 μL 10 m*M* deoxynucleotide triphosphate, 2.5 μL 25 m*M* $MgCl_2$, 0.5 μL (0.2 μ*M* for ST11 and ST15, 1.2 μ*M* for Fli15, Typ04, ENTF, and ENTR) primer and 14.2 μL sterile distilled water. The mixture was then treated with 0.3 μL (1.5 U) *Taq* DNA polymerase. The PCR reactions were carried out in a Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA) with the following condition: initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 45 s, annealing at 53°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 7 min. A 3 μL aliquot of the amplified PCR products were electrophoresed on 1.5% (wt/vol) agarose gel at 100 V for 40 min. Subsequently, the gel was stained with ethidium bromide and visualized under UV light using the Gel Documentation System (SynGene, Frederick, MD). A DNA fragment of 100-bp (Vivantis Technologies, Selangor, Malaysia) was included in each gel as molecular weight marker.

Serotyping

Presumptive *S.* Enteritidis and *S.* Typhimurium isolates were further confirmed by slide agglutination test using polyvalent 'O' and 'H' antisera (BD, Franklin Lakes, NJ). The isolates were serotyped at Veterinary Research Institute, Ipoh, Malaysia according to the

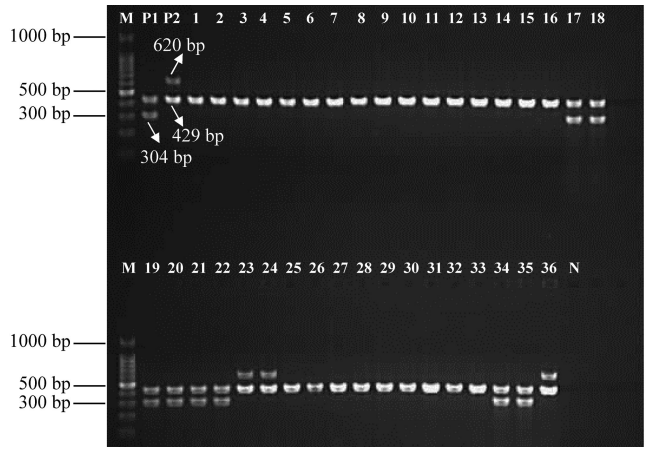

Figure 1. Representative amplification of random sequence, *SdfI* gene, and *fliC* gene for identification of *Salmonella* spp. (429 bp), *Salmonella* Enteritidis (304 bp), and *Salmonella* Typhimurium (620 bp) in chicken meat samples.

Kauffmann-White classification scheme using a battery of somatic and flagellar antisera (OIE Terrestial Manual, 2008).

Antibiotic Susceptibility Test

Antibiotic susceptibility of the putative isolates was tested by using disc diffusion method described in the Clinical and Laboratory Standards Institute (CLSI, 2008). The microorganisms were cultured aerobically in 10 mL Tryptic Soy Broth (Merk, Darmstadt, Germany) at 37°C for 24 h. The cultures were swabbed with sterile non-toxic cotton swab on Mueller-Hinton agar plates (Merk, Darmstadt, Germany) and left to dry for 2 to 4 min. The antimicrobial sensitivity discs (Oxoid, Hamphire, United Kingdom) were then placed on the culture by using a Disk Diffusion Dispenser (Oxoid). Antibiotic discs tested were Amoxycillin (30 μg), Ampicillin (10 μg), Amoxicillin/Clavulanic acid (30 μg), Cephazolin (30 μg), Ceftazidime (30 μg), Ciprofloxacin (5 μg), Erythromycin (15 μg), Gentamicin (10 μg), Kanamycin (30 μg), Penicillin (10 μg), Nalidixic acid (30 μg), Streptomycin (10 μg), Tetracycline (30 μg), Trimethoprim (5 μg), and Vancomycin (30 μg). After incubation at 37°C for 24 h, the size of the inhibition zone was measured and the level of susceptibility (sensitive, intermediate, or resistant) was determined. *Escherichia coli* (ATCC 25922) was used as a control. The multiple antibiotic resistance (**MAR**) index was calculated by using the formula: a/b, where 'a' represents the number of antibiotics to which a particular isolate was resistant and 'b' the total number of antibiotics tested (Krumperman, 1983).

Statistical Analysis

All measurements were carried out in triplicate. Minitab (v. 14) statistical package (Minitab Inc., State College, PA) was used. For all analysis, P value < 0.05 was considered significant.

RESULTS

The target genes specific to *Salmonella* spp., *S.* Enteritidis and *S.* Typhimurium produced PCR products 429 bp, 304 bp, and 620 bp in size, respectively. The PCR products were obtained clearly distinguished by agarose gel electrophoresis (Figure 1).

The prevalence of *Salmonella* spp., *S.* Enteritidis, and *S.* Typhimurium in chicken meat from wet markets and hypermarkets are summarized in Table 1. Out of 120 samples examined, the prevalence of *Salmonella* spp., *S.* Enteritidis, and *S.* Typhimurium were 20.80% (n = 25), 6.70% (n = 8), and 2.50% (n = 3), respectively. The contamination rates of *Salmonella* spp. was found to be the most predominant in wet markets (26.70%) compared with hypermarkets (15.00%) ($P < 0.05$). In addition, the prevalence of *S.* Enteritidis from wet markets (10.00%) was slightly higher than those from hypermarkets (3.33%) ($P < 0.05$). Low detection of *S.* Typhimurium was observed using MPN-mPCR method, indicating no significant difference between wet markets and hypermarkets.

In this study, chicken breast was the important reservoir for *Salmonella*, with prevalence rate of 47.50% (n = 40) (Table 1). From the MPN-PCR method, the highest concentration of *Salmonella* spp., *S.* Enteritidis, and *S.* Typhimurium were 15.0 MPN/g, 3.6 MPN/g, and 3.6 MPN/g, respectively (Table 2).

Antibiotic susceptibility testing was carried out for *S.* Enteritidis (n = 8), and *S.* Typhimurium (n = 3).

Table 1. Prevalence of *Salmonella* spp., *Salmonella* Enteritidis, and *Salmonella* Typhimurium in chicken meat samples using MPN-mPCR method.

	Wet markets				Hypermarkets			
		No. of Positive Sample				No. of Positive Sample		
Chicken part	n[1]	*Salmonella* spp.	*S.* Enteritidis	*S.* Typhimurium	n	*Salmonella* spp.	*S.* Enteritidis	*S.* Typhimurium
Wing	20	5 (25.0%)[2]	1 (5.0%)	0 (0.0%)	20	2 (10.0%)	0 (0.0%)	0 (0.0%)
Breast	20	7 (35.0%)	4 (20.0%)	1 (5.0%)	20	5 (25.0%)	1 (5.0%)	1 (5.0%)
Drumstick	20	4 (20.0%)	1 (5.0%)	1 (5.0%)	20	2 (10.0%)	1 (5.0%)	0 (0.0%)
Total	60	16 (26.7%)	6 (10.0%)	2 (3.3%)	60	9 (15.0%)	2 (3.3%)	1 (1.7%)

[1]n = number of samples.
[2]Percentage = percentage of positive samples.

Table 2. Concentration of *Salmonella* spp., *Salmonella* Enteritidis, and *Salmonella* Typhimurium (MPN/g) in chicken meat samples using MPN-mPCR method.

	Wet markets									Hypermarkets								
	Salmonella spp.			*S.* Enteritidis			*S.* Typhimurium			*Salmonella* spp.			*S.* Enteritidis			*S.* Typhimurium		
Chicken part	Min[1]	Med[2]	Max[3]	Min	Med	Max	Min	Med	Max	Min	Med	Max	Min	Med	Max	Min	Med	Max
Wing	<3	<3	15.0	<3	<3	3.6	<3	<3	<3	<3	<3	3.6	<3	<3	<3	<3	<3	<3
Breast	<3	<3	15.0	<3	<3	3.6	<3	<3	3.6	<3	<3	15.0	<3	<3	3.6	<3	<3	3.6
Drumstick	<3	<3	3.6	<3	<3	3.6	<3	<3	3.6	<3	<3	3.6	<3	<3	3.6	<3	<3	<3

[1]Min = minimum MPN/g value.
[2]Med = median MPN/g value.
[3]Max = maximum MPN/g value.

Table 3. Antimicrobial susceptibility pattern of *Salmonella* Enteritidis, and *Salmonella* Typhimurium isolated from chicken meat samples tested by disc diffusion method.

		Antibiogram pattern of *S.* Enteritidis and *S.* Typhimurium		
Antimicrobial agent	No. of isolates tested	Resistant (%)	Intermediate (%)	Sensitive (%)
Amoxycillin (AML30)	11	3 (27.27)	–	8 (72.73)
Ampicillin (AMP10)	11	8 (72.73)	3 (27.27)	–
Amoxicillin/Clavulanic acid (AMC30)	11	–	–	11 (100)
Cephazolin (KZ30)	11	3 (27.27)	2 (18.18)	6 (54.55)
Ceftazidime (CAZ30)	11	–	3 (27.27)	8 (72.73)
Ciprofloxacin (CIP5)	11	3 (27.27)	8 (72.73)	–
Erythromycin (E15)	11	11 (100)	–	–
Gentamicin (CN10)	11	–	–	11 (100)
Kanamycin (K30)	11	–	3 (27.27)	8 (72.73)
Penicillin (P10)	11	11 (100)	–	–
Nalidixic acid (NA30)	11	1 (9.09)	3 (27.27)	7 (63.64)
Streptomycin (S10)	11	1 (9.09)	3 (27.27)	7 (63.64)
Tetracycline (TE30)	11	–	–	11 (100)
Trimethoprim (W5)	11	–	–	11 (100)
Vancomycin (VA30)	11	11 (100)	–	–

Table 4. The antibiotic resistance profile patterns and multiple antibiotic resistance (MAR) index of *Salmonella* Enteritidis and *Salmonella* Typhimurium isolated from chicken meat samples.

Isolate no.	*Salmonella* serovar	Retail market	Antibiotic resistance profiles	MAR index[1]
1	*S.* Enteritidis	Wet market	AMPEPNAVA	0.33
2	*S.* Enteritidis	Wet market	AMPEPSVA	0.33
3	*S.* Enteritidis	Wet market	AMPEPVA	0.27
4	*S.* Enteritidis	Wet market	AMPEPVA	0.27
5	*S.* Enteritidis	Wet market	AMPEPVA	0.27
6	*S.* Enteritidis	Wet market	AMPEPVA	0.27
7	*S.* Enteritidis	Hypermarket	AMPEPVA	0.27
8	*S.* Enteritidis	Hypermarket	AMPEPVA	0.27
9	*S.* Typhimurium	Wet market	AMLKZCIPEPVA	0.40
10	*S.* Typhimurium	Wet market	AMLKZCIPEPVA	0.40
11	*S.* Typhimurium	Hypermarket	AMLKZCIPEPVA	0.40

[1]MAR index = number of resistance antibiotics/total number of antibiotics tested.
AML - Amoxycillin; AMP - Ampicillin; KZ - Cephazolin; CIP - Ciprofloxacin; E - Erythromycin; P - Penicillin; NA - Nalidixic acid; S - Streptomycin; VA - Vancomycin.

All the isolates were resistance to erythromycin, penicillin, and vancomycin (Table 3). However, low level of resistance was observed to nalidixic acid (9.09%) and streptomycin (9.09%). As can be seen in the profile (Table 3), four antibiotics amoxicillin/clavulanic acid, gentamicin, tetracycline, and trimethoprim were found to be 100% effective, whereas low level of sensitivity was showed by cephazolin (54.55%). Based on the resistance pattern, *S.* Typhimurium isolates exhibited the highest MAR index value of 0.40 (Table 4). Meanwhile, MAR index of *S.* Enteritidis isolates were observed at 0.27 and 0.33. Multi-drug resistant (**MDR**) *S.* Enteritidis and *S.* Typhimurium isolates displayed resistance to at least three antibiotics (erythromycin, penicillin, and vancomycin) were most often observed (Table 4).

DISCUSSION

The prevalence of *Salmonella* spp. in this study was found to be similar to the results reported by Ng et al. (2013), where the contamination rates of *Salmonella* spp. from wet markets (43.00%) was higher than those from hypermarkets (32.00%). In comparison between the two types of shops, the levels of *Salmonella*

incidence from hypermarkets was lower than that from wet markets. This may suggest that hypermarkets had food handlers of better personal hygiene and better sanitary condition in the food processing environment as compared to wet markets. Oscar (2004) suggested that lower *Salmonella* prevalence from hypermarkets could be due to the storage temperature, which identified as an important risk factor of pathogen survival and growth. According to Kuan et al. (2013), the difference in holding time has a remarked effect on variation in prevalence from wet markets and hypermarkets.

Slaughtered poultry are well known reservoirs of *Salmonella*. Thus, our finding is consistent with that of Lin et al. (2014) who showed that *Salmonella* prevalence of 25.66% (n = 113) on retail chicken meat. It also proposed that cross-contamination during processing and cutting or workers during retailing and marketing could be attributed to the high prevalence of *Salmonella* in chicken meat. High levels of *Salmonella* prevalence on retail chickens have been reported in Thailand (57.00%, n = 72) (Padungtod and Kaneene, 2006), China (53.59%, n = 515) (Yang et al., 2010), and Vietnam (42.91%, n = 268) (Thai et al., 2012). In contrast, low *Salmonella* prevalence rates were found in Morocco (0.42%, n = 1,200) (Bouchrif et al., 2009). This observation suggested that several factors such as differences in country and origin, type of meat samples, sampling seasons, slaughterhouse sanitation, and isolation method may also influenced the differences in prevalence.

The MPN-mPCR method used to quantify the microbial load can facilitate the enumeration of *Salmonella* spp., *S.* Enteritidis, and *S.* Typhimurium in retail chicken meat samples in a short time. Low counts of these food-borne pathogens in this study are less likely to cause salmonellosis. However, it is important to note that chicken meat products, by their nature, undergo extensive processing and handling during their production can increase the risk of contamination. According to Yang et al. (2010), *Salmonella* contamination was common in retail meats (chicken, beef, lamb, and pork), which could be a potential vehicle for transmitting *Salmonella* to humans. Hence, implementation and maintenance of some control measures such as hazard analysis and critical control point and good manufacturing practices, as well as further strengthen the education of food processors will be necessary, in order to reduce the risk of salmonellosis.

High resistance of *S.* Enteritidis and *S.* Typhimurium to erythromycin, penicillin, and vancomycin in our study suggest that these antibiotics are widely used in this region. Resistance to erythromycin and penicillin has been reported as the most common resistance profile in retail meat products (Sallam et al., 2014). In another case, Singh et al. (2013) have found that resistance to penicillin and vancomycin was 100% in poultry and poultry environments. This could be due to improper usage or overuse of a particular antimicrobial causing resistance to occur. In contrast, the resistance to nalidixic acid and streptomycin were found to be lower. Similarly, low levels of resistances to nalidixic acid (13.36%) and streptomycin (10.62%) were also observed, particularly among isolates (*S.* Enteritidis and *S.* Typhimurium) recovered from retail chicken meats (Yang et al., 2010). In this study, there were no *S.* Enteritidis and *S.* Typhimurium isolates resistant to amoxicillin/clavulanic acid, gentamicin, tetracycline, and trimethoprim. Previously, Dong et al. (2014) reported that all the *Salmonella* isolates (n = 83) displayed 100% sensitive to amoxicillin/clavulanic acid, whilst 98.80% to gentamicin and 92.77% to tetracycline.

On the other hand, our findings demonstrate that MDR strains of *S.* Enteritidis and *S.* Typhimurium are prevalent in both retail markets. This might lead to human infections with food-borne antimicrobial resistant pathogen, and probably create an enormous challenge to treatment of *Salmonella* infection in humans and animals in Malaysia.

Various reports on the risk factor associated with the occurrence of MDR *Salmonella* isolates have been published. Fashae et al. (2010) reported that the appearance of MDR *Salmonella* isolates correlates positively with the indiscriminate use of antibiotics at recommended doses or at sub-therapeutic doses as feed additives in poultry farm. In addition, genetic and biochemical mechanisms may have significantly contributed to the emergence of MDR strains of *Salmonella*, and thus preserve their drug resistance genes and enhance their survivability. On the contrary, Giraud et al. (2006) had reported that modifications of topoisomerase targets, increased efflux activity, and topoisomerase protection by the plasmid-encoded protein might be used to combat resistance. Therefore, the finding of this study indicated that retail raw chicken meat acts as a reservoir for harboring multi-drug resistance *Salmonella*, which can be a problem and a major food safety concern for public health. Thus, it is necessary for developing effective intervention strategies, as well as employing natural biocontrol agents such as bacteriophages to ensure the safety of our food supplies.

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