



Antimicrobial-resistant *Salmonella* spp. isolated from retail farmed shrimp in Kuala Lumpur

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Abstract

Antimicrobial resistant *Salmonella* is recognized as a potential food safety threat and its prevalence in farmed shrimps is very concerning. However, information about antimicrobial resistant *Salmonella* in retail farmed shrimps in Malaysia is very limited. Therefore, this study was aimed at determining the level of *Salmonella* contamination of farmed shrimps at selected retails in Kuala Lumpur and antibiotic resistance pattern of *Salmonella* isolated from retail farmed shrimps. Farmed shrimp samples were collected from selected supermarkets and indoor markets. *Salmonella* was detected by conventional methods. All presumptive colonies of *Salmonella* were subjected to antimicrobial susceptibility test. A high rate of *Salmonella* contamination was detected ($n = 9$, 60%) with an estimated *Salmonella* load ranging between 0 and $>800 \times 10^8$ cfu/ml. All isolates (100%) were resistant to at least one antimicrobial agent, and 17 isolates (94.4%) were multidrug-resistant. 2 isolates (11.1%) were resistant to all eight types of antimicrobial agents. High rates of resistance were observed for erythromycin (100%), doxycycline (77.8%), tetracycline (72.2%), nalidixic acid (61.1%), ampicillin (55.6%) and chloramphenicol (44.4%). Findings from the present study provide insight on antibiotic resistant *Salmonella* contamination in retail farmed shrimps and suggest its potential food safety risk to the public. These data are valuable to warrant further investigation of risk management and public health strategies.

Keywords: Antimicrobial resistant, *Salmonella*, Farmed shrimp, Retail

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1. Introduction

Shrimp is appreciated given its uniqueness in taste and texture. Thus, it is unsurprising that shrimp is one of the most popular seafood throughout the world. The popularity has led to high demand from both local and international markets and driven rapid growth in shrimp agriculture industry. In order to fulfill the great capacity, many shrimp farmers shifted to more intensive farming system. While the new system has brought about significant yield improvement, disease outbreaks have frequently arose causing substantial economic losses to the farmers. Antibiotics have often become the quick solution when dealing with shrimp disease issue. Nonetheless, the uncontrolled use of antibiotics including the banned ones in shrimp farming will give rise to the development of antibiotic resistance pathogens.

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Salmonella has been identified as one of the major microbial contributors to shrimp import detention in the United States (Wan Norhana et al., 2010). Whereas in the European region, *Salmonella* was recorded as the second main cause of shrimp import rejection under microbial contamination category between 1999 and 2002 (Huss et al., 2004). *Salmonella* has been notorious for causing foodborne illness. People infected with *Salmonella* will likely to develop food poisoning symptoms, however, to a vulnerable population, *Salmonella* infection can be lethal (CDC, 2016). In addition to the emergence of antimicrobial resistant *Salmonella*, it will certainly become a serious food safety threat to the public (CDC, 2015).

Realizing the urgency of the issue, several researches (Carvalho et al., 2013; Banerjee et al., 2012; Jeyasekaran and Ayyappan, 2002; Wan Norhana et al., 2001; and Bhaskar et al., 1995) have intensively investigated the prevalence of antibiotic resistant *Salmonella* in shrimp at farming sites and majority of the findings indicated *Salmonella* was widespread. However, there was limited information regarding the antimicrobial resistance patterns of *Salmonella* in farmed shrimps at retail level, especially in Malaysia. Thus, the objectives of this study are to determine the level of *Salmonella* contamination in retail farmed shrimp at selected retails in Kuala Lumpur and to study the antimicrobial resistance pattern of *Salmonella* isolated from retail farmed shrimps.

2. Materials and methods

2.1. Sample collection

According to data provided by Roslan et al. (2016), there are nine hypermarkets and 28 indoor markets situated in Kuala Lumpur (Figure 1). A 10% of retailers were selected based on the above-mentioned data. Samples were collected from three indoor markets (WMC, WMT and WMS) and two hypermarkets (HMA and HMB) with three replicates for each market. Shrimp were sampled and contained in sterile poly-bags, labeled and stored in the ice box. Samples were collected and transferred to the laboratory for microbiological analysis on the same day.

2.2. Detection and enumeration

Each sample (25 g) was homogenized with 225 ml of buffered peptone water (OXOID, England) in a stomacher bag for one minute and incubated for 18-20 h at 35 °C as pre enrichment. After incubation, 1 ml of the pre enriched broth was added into 10 ml of Rappaport-Vassiliadis (RV) broth (OXOID, England) followed by serial dilution up to 10⁻⁸ and mixed well, then incubated at 35 °C for 24 h. 0.1 ml of RV broth cultures were spread onto Hektoen Enteric Agar (HEA) (OXOID, England) and incubated at 35 °C for 24 h. Incubated agar plates were placed on colony counter to record the number of presumptive *Salmonella* colonies exhibited as blue-green to blue and black colonies on the selective agar. Presumptive colonies were then streaked onto nutrient agar plates (OXOID, England) and incubated for 24 h at 35 °C to obtain pure colonies prior to antimicrobial susceptibility test.

2.3. Antimicrobial susceptibility test

Antimicrobial susceptibility of *Salmonella* was tested using Kirby-Bauer disk diffusion method using Mueller-Hinton agar (OXOID, England) based on National Committee for Clinical Laboratory Standards (CLSI, 2017) guidelines. Pure colonies of *Salmonella* cultured on nutrient agar were suspended in normal saline (0.85%) until turbidity was comparable to 0.5 McFarland turbidity standards. Next, the suspension was swabbed onto Mueller-Hinton agar then antimicrobial discs were fixed onto the *Salmonella* seeded agar surface. Antimicrobial agents used in this test and their corresponding concentrations are as follow: Doxycycline 30 µg, Gentamicin 10 µg, Tetracycline 30 µg, Ampicillin 10 µg, Chloramphenicol 30 µg, Nalidixic acid 30 µg and (Oxoid, England). After incubation for 24 h at 37 °C, the diameter of the zone of inhibition of each antimicrobial disc on agar was measured and the results were interpreted according to interpretive criteria provided by CLSI (2017).

2.4. Statistical analysis

All data obtained from the analyses were entered into Microsoft Excel to generate descriptive information including charts and graphs. Minitab 16 was used for further statistical analysis with differences accepted as significant at values of $p \leq 0.05$.

3. Results and discussion

3.1. Prevalence and enumeration of *Salmonella* spp. in retail farmed shrimps

Out of 15 shrimp samples, 9 (prevalence rate of 60%) were positive for *Salmonella* spp. *Salmonella* spp. was

Sampling location	First sampling			Second sampling			Third sampling		
	Rep. 1	Rep. 2	Avg.	Rep. 1	Rep. 2	Avg.	Rep. 1	Rep. 2	Avg.
Hyper-market (HMA)	$>8 \times 10^4$	$>8 \times 10^4$	$>8 \times 10^4$	$<1 \times 10^2$	$<1 \times 10^2$	$<1 \times 10^2$	$>8 \times 10^4$	$>8 \times 10^4$	$>8 \times 10^4$
Hyper-market (HMB)	$<1 \times 10^2$	$<1 \times 10^2$	$<1 \times 10^2$	$>8 \times 10^{10}$	$>8 \times 10^{10}$	$>8 \times 10^{10}$	$<1 \times 10^2$	5×10^8	5×10^8
Wet market (WMA)	$<1 \times 10^2$	$<1 \times 10^2$	$<1 \times 10^2$	$<1 \times 10^2$	$>8 \times 10^4$	$>8 \times 10^4$	$<1 \times 10^2$	$<1 \times 10^2$	$<1 \times 10^2$
Wet market (WMB)	$<1 \times 10^2$	$<1 \times 10^2$	$<1 \times 10^2$	1×10^9	5×10^9	3×10^9	$>5 \times 10^{10}$	3×10^{10}	3×10^{10}
Wet market (WMC)	$<1 \times 10^2$	$<1 \times 10^2$	$<1 \times 10^2$	7×10^7	9×10^{10}	5×10^{10}	$<1 \times 10^2$	1×10^9	1×10^9

recovered from all five different sampling locations. Table 1 presents the screening and enumeration of *Salmonella* spp. in retail farmed shrimps in the current study. This is in accord with the study conducted by Nguyen *et al.* (2016) where the team detected a high rate of *Salmonella* contamination in raw shrimps (49.1%) sold in fresh markets of Ho Chi Minh City, Vietnam. Contrary, in China, Yang *et al.* (2015) reported lower (13%) prevalence of *Salmonella* in retail raw shrimp samples. On the other hand, 5.71% of raw shrimps from seven randomly selected markets in Dhaka city tested by Hossain *et al.* (2012) were positive for *Salmonella*. In Iran, Rahimi *et al.* (2011) also reported low percentage (1.8%) of *Salmonella* in fresh shrimps. Comparing to data obtained in 1995, Arumugaswamy and colleagues discovered a prevalence rate of 25% in raw shrimp samples collected from various retail markets and shops surrounding Kajang, Serdang and Kuala Lumpur. Although Arumugasamy *et al.* (1995) did not mention the specific types of shrimp that was being studied, it is worthy to suggest the possibility of growing trend in the prevalence rate of *Salmonella* spp. in raw shrimps within two decades in Malaysia.

Despite the absence of international agreement on microbiological specification for *Salmonella* in food including shrimp and its related products, several countries such as the United States (US), the European Union (EU), Australia, New Zealand and China (including Hong Kong) have taken initiative to impose regulatory requirement on raw and ready-to-eat shrimps in order to protect public from major Salmonellosis outbreak (Wan Norhana *et al.*, 2010). Generally, zero tolerance policy is applied. For instance, Australia, New Zealand, and China require the absence of *Salmonella* in 25 g of raw shrimp sample. On average, enumeration data in this investigation showed a heavy load of *Salmonella* spp. in both indoor markets and hypermarkets, which far exceeded beyond the well-recognized zero tolerance limits. Therefore, this finding could indicate a substantial food safety risk to the public.

The root cause to *Salmonella* spp. contamination in raw shrimps can be vague. While Amagliani *et al.* (2012), Lunestad and Barlaug (2009), and Dalsgaard *et al.* (1995) emphasized that *Salmonella* present in either aquatic environment or its associated food products as a result of fecal contamination during farming stage, some researchers (Wan Norhana *et al.*, 2001; Bhaskar *et al.*, 1998, 1995; Reilly *et al.*, 1992; and Iyer and Varma, 1990) argued that *Salmonella* is a part of the natural aquatic microflora in shrimp farming environment, thus rationalizing the prevalence of the pathogen in raw farmed shrimps at later stages of supply chain. The latter



Figure 1: Sampling locations of retail farmed shrimp in Kuala Lumpur

conclusion is supported by the WHO who declared *Salmonella* as “geonotic” disease and removal of foodborne zoonoses from the food chain can be very challenging (Wan Norhana et al., 2010). Besides upstream factors, downstream controls are also a critical determinant in *Salmonella* load in retail farmed shrimps. Sloppy distribution, unhygienic retail environment, mishandling, cross contamination during transportation or storage and temperature abuse are among the typical contributors (Dib et al., 2014; Carrasco et al., 2012; Wan Norhana et al., 2010; and Panisello et al., 2000).

By studying the findings from previous similar studies (Begum et al., 2010; and Minami et al., 2010), fresh market is expected to have higher load of *Salmonella* in raw shrimp compared to supermarket because the latter type of retailer tend to have better resources, facilities and environment in lowering risk of contamination. Surprisingly, the results from the current study are contradicting against previous findings (Begum et al., 2010; and Minami et al., 2010). Average *Salmonella* spp. count in samples obtained from hypermarket is higher than the indoor market ($p > 0.05$). Even though hypothesis testing did not yield statistical significance, it could be attributed to the relatively small sampling size in this study.

Besides, it can be observed from the data that load of *Salmonella* spp. in the samples did not exhibit a steady trend. In one sampling location, *Salmonella* spp. was found absent and highly prevalent at different sampling dates. High load of *Salmonella* spp. from one particular date gave drastic rise to total contamination load. Hence, it is inconclusive to reflect *Salmonella* spp. contamination in raw farmed shrimps in hypermarket was more severe than indoor market. Minami et al. (2010) encountered similar *Salmonella* prevalence trend in their study, in which *Salmonella* was detected in the first batch of sampling but not in the second batch. The team was supposing that these retailers are engaging to the same suppliers or transportation service, then the inconsistency in the trend might suggest cross-contamination during these stages. Nonetheless, the findings from the current study provide preliminary insight into current *Salmonella* spp. contamination status in farmed shrimps at the retail stage that warrants further investigation. The uncertainties in current study should be overcome by increasing sampling size to develop a more established contamination trend and to yield more meaningful statistical hypothesis testing outcome.

3.2. Antimicrobial resistance pattern of *Salmonella* isolated from retail farmed shrimps

Table 2 and Figure 2 summarize the resistance pattern of 18 *Salmonella* spp. isolates against eight antimicrobial drugs tested in the present study. The prevalence rate of antimicrobial-resistant *Salmonella* spp. was high

Table 2: Antimicrobial resistance of *Salmonella* spp. isolates from farmed shrimp in each sampling location

Sampling location	Antimicrobial agents*							
	CN	TE	NA	CIP	E	DO	AMP	C
Hypermarket (HMA) (n = 3)	0	2	1	(2) ^a	3	2	(1)	1
Hypermarket (HMB) (n = 4)	(1)	2	3	1(3)	4	2(1)	2(1)	1(1)
Wet market (WMC) (n = 1)	0	1	0	0	1	1	0	(1)
Wet market (WMT) (n = 5)	2(1)	4	3(1)	1(4)	5	4	5	3
Wet market (WMS) (n = 5)	1	4	4	1(2)	5	5	3	3(1)

Note: ^a The number with parentheses indicate intermediate resistance; * CN: gentamycin, TE: tetracycline, NA: nalidixic acid, CIP: ciprofloxacin, E: erythromycin, DO: doxycycline, AMP: ampicillin, and C: chloramphenicol.

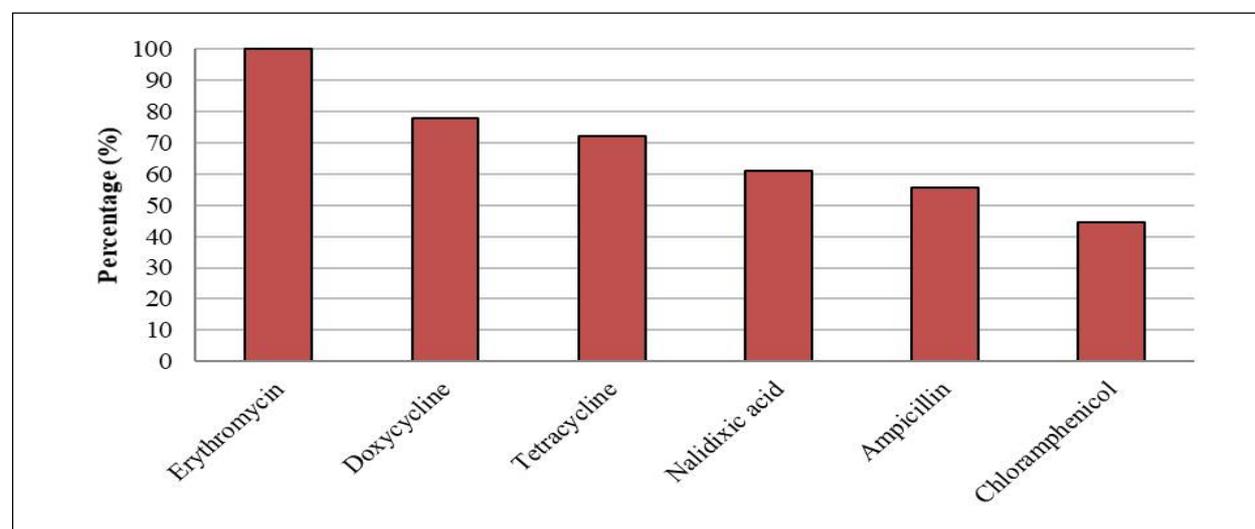


Figure 2: Percentage of resistant, *Salmonella* spp. isolates from retail farmed shrimp to commercial antibiotics

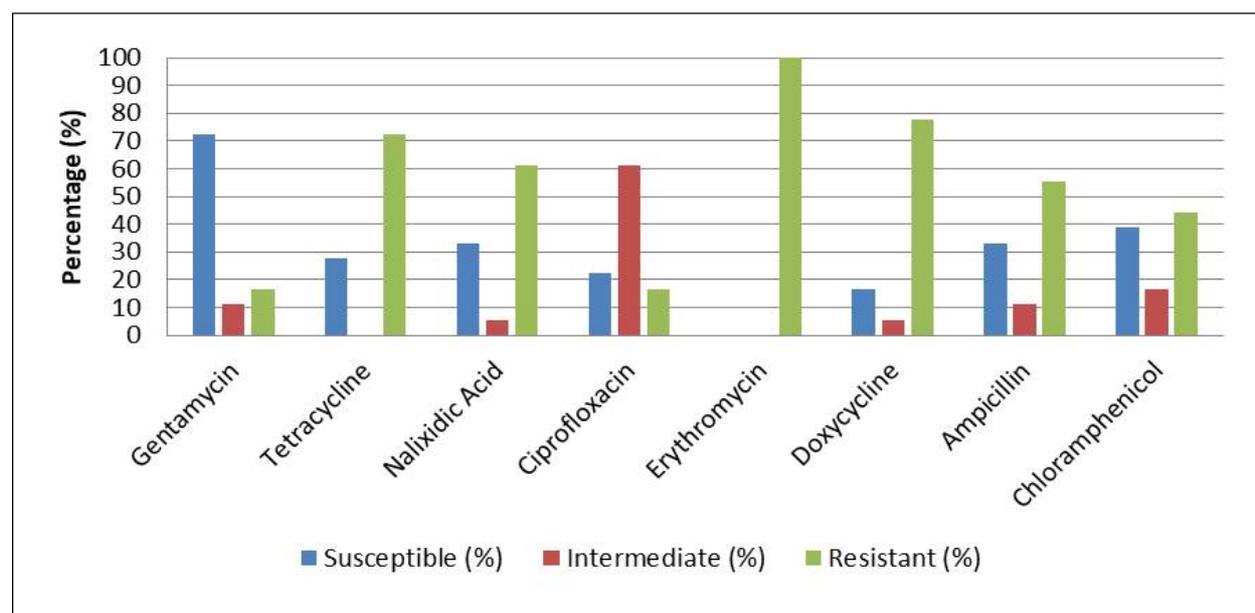


Figure 3: Percentage of resistant, intermediate and susceptible *Salmonella* spp. isolates from retail farmed shrimp to commercial antibiotics

(100%); all of the *Salmonella* spp. isolates were resistant to at least one antimicrobial drug. High levels of resistance were found to erythromycin (100%), doxycycline (77.8%), tetracycline (72.2%), nalixidic acid (61.1%), ampicillin (55.6%), and chloramphenicol (44.4%). There were 16.7% of isolates resistant towards gentamycin and ciprofloxacin. It is crucial to highlight the high prevalence rate of antimicrobial resistance found in this study upon comparison against data from other countries. Even though most *Salmonella* isolates recovered by Hossain et al. (2012) and Wan Norhana et al. (2001) were also resistant to erythromycin, however, in contrast, Hossain and the team's (2012) isolates were all (100%) susceptible to chloramphenicol, ciprofloxacin and doxycycline. Also, Nguyen et al. (2016) and Woodring et al. (2012) reported overall lower occurrence rate of antimicrobial resistance in *Salmonella* isolated from shrimp retailers in Vietnam and Thailand respectively compared to the present study. In addition, Rahimi et al. (2013) even reported zero incidence of antimicrobial resistance in the *Salmonella* isolates obtained from retail raw shrimp samples in Iran.

The susceptibility of a bacterium to a given antibiotic is categorized as intermediate when its growth is inhibited in vitro by a concentration of the drug that is associated with an uncertain therapeutic effect (CLSI, 2017). Notably, in the present study, high prevalence rate of intermediate resistance was observed in ciprofloxacin.

The majority of the researchers (Yang et al., 2015; Zhang et al., 2015; and Hossain et al., 2012; and Cabello, 2006) linked prevalence of antimicrobial resistance in *Salmonella* to abuse of antimicrobial in aquaculture and agriculture either to promote growth or therapeutic purpose. Hossain and the team (2012) further suggest that the use of probiotics in shrimp farming can contribute to the development of antimicrobial resistance. It is also worth to note that ampicillin is rarely used in shrimp farming (Hossain et al., 2012). Therefore, ampicillin resistance in *Salmonella* spp. recovered in this study could be acquired from other sources (Hossain et al., 2012). As highlighted by Zhang et al. (2015), ciprofloxacin is commonly used as the front-line drug that is prescribed to treat Salmonellosis in adults. Therefore, the emergence of ciprofloxacin-resistant *Salmonella* (16.7%) could lead to serious medical complication.

Multidrug resistance is defined as the resistance to more than one type of antimicrobial drugs (Zhang et al., 2015). 94.4% ($n = 17$) *Salmonella* spp. isolates from the present study were multidrug-resistant (Figure 3). Notably, two isolates were resistant to all eight antimicrobial agents. These particular two isolates were recovered from wet market, WMT and WMS. The high prevalence rate of multidrug-resistant *Salmonella* spp. is another critical highlight in this study as this phenomenon will limit therapeutic options for clinical cases that require the prescription of antimicrobial agents. This is especially crucial to the vulnerable population such as the elderly, infants and immunocompromised individuals since the delay in receiving effective treatment will significantly increase their risk of mortality.

4. Conclusion

This study leads to a preliminary insight of current antimicrobial-resistant *Salmonella* spp. contamination status in raw farmed shrimp at retail level. In conclusion, the prevalence rate of *Salmonella* spp. was found to be very high at retail level. Therefore, raw consumption should be avoided and farmed shrimps should be thoroughly cooked before serving to minimize health risk associated with *Salmonella* contamination. Hypermarkets which were perceived to be more hygienic were just as likely to be contaminated as those bought from the more austere indoor market. On the other hand, based on the antibiotic resistance pattern obtained from the present study, the prevalence rate of multidrug resistant *Salmonella* spp. in retail farmed shrimps is very high. Further investigation is warranted to confirm and expand upon the food safety risks reflected in the findings from the present study. Besides, surveillance programs need to be implemented to closely monitor on antibiotic use in aquaculture and agriculture as well as the microbiological status of farmed shrimps. Information from these investigations and monitoring programs are valuable to establish science-based public health policy and to develop effective interventions such as Hazard Analysis Critical Control Point (HACCP) programs in farmed shrimp supply chain.

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