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Prevalence of virulent resistant *Salmonella enterica* strains from sushi and sashimi samples in Malaysia

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Abstract. *Salmonella enterica* is one of the leading causes of human foodborne infections. The objectives of this study are to investigate *S. enterica* prevalence in sushi and sashimi in Malaysia, to determine the presence of virulence genes and the antimicrobial resistance profiles of isolated *S. enterica*. In the 200 samples tested, 16% were positive for *S. enterica*. Sixty-six percent of the *S. enterica* isolates harboured at least one virulence gene and the most common virulence gene was *sifA* (37.5%). Antibiotic susceptibility testing showed 65.6% (21/32) of the isolates to be resistant to at least one antibiotic tested, with sulfamethoxazole resistance as the most common (50%). Resistance to the drugs-of-choice (fluoroquinolones and third-generation cephalosporin) for severe salmonellosis were also detected – ceftriaxone (25%), ceftazidime (28.1%) and ciprofloxacin (9.4%). Two isolates (9.5%) were resistant to all antibiotic tested while 12 isolates (37.5%) exhibited multi-drug resistance (MDR) with 10 different MDR profiles. Most of the isolates presented MDR profiles-AP, AUG, FOX, NA (penicillins, beta-lactams, cephems and quinolone) with or without the addition of other drugs. In conclusion, the high rate of *S. enterica* prevalence in the sampled sushi and sashimi warrants increased safety measures for sushi and sashimi preparation.

INTRODUCTION

Ready-to-eat (RTE) foods refer to foods that do not require further preparation such as washing, cooking or heating to eliminate or reduce the levels of microorganisms of concern to acceptable levels (EC 2005; FDA, 2009). RTE foods include raw animal foods that are cooked according to the U.S. Food and Drug Administration (FDA) guidelines, raw fruits and vegetables, fruits and vegetables that are cooked for hot-holding, plant food, substances derived from plants, dry fermented meat products and thermally processed low-acid food packages (FDA, 2009).

The convenience of RTE foods complements the busy lifestyles of modern families, and with improved quality and flavors, RTE foods have become an integral part of many cuisines. In Malaysia, sushi and sashimi are popular RTE delicacies. In addition, sushi and sashimi are considered permissible ("halal") by the Muslims, thus these foods are eaten by the multi-ethnic Malaysian population leading to increased consumption.

To meet this demand, sushi and sashimi are easily available from most supermarkets, hypermarkets, retail chain stores and restaurants located in the Klang Valley (Kuala Lumpur metropolitan area). This has given rise to food safety concerns as reports of outbreaks of foodborne illnesses have been associated with sushi or sashimi consumption (Food and Environmental Hygiene Department HKSAR, 2000; Barralet *et al.*, 2004; CDC, 2012, 2015).

Salmonellosis, caused by the *Salmonella* bacterium, is one of the principal causes of foodborne illness often associated with sushi and sashimi consumption in Australia (Barralet *et al.*, 2004) and USA (CDC, 2012; 2015). Fish and fish products (Amagliani *et*

al., 2012) can be sources of *Salmonella* infection although poultry, meat products (Thai *et al.*, 2012; Lin *et al.*, 2014) and eggs are generally recognized as primary food vehicles for human salmonellosis (Howard *et al.*, 2012).

The *Salmonella* genus is divided into two major species – *S. bongori* and *S. enterica*. The latter species can be subdivided into six subspecies – enterica, salamae, arozonae, diarizonae, houtenae and indica (Malorny *et al.*, 2011). *S. enterica* is frequently reported to be associated with human infections, with more than 2500 serological variants described in the Kauffmann-White scheme (Grimont & Weill, 2007).

Each *Salmonella* serotype differs in their growth and survival characteristics. It generally grows optimally between 35 and 37°C, with a growth range of 2 to 54°C (Andino & Hanning, 2015). It can survive under frozen and dried states for long periods (Murray *et al.*, 1999). *Salmonella* species are naturally present in any raw food products as they are widely dispersed in nature in water, soil, plants and animals (Carrasco *et al.*, 2012). The bacterium is transmitted to human through ingestion of foods that are directly contaminated by animal feces or cross-contaminated by other sources such as farm equipment (Gorski *et al.*, 2011).

Salmonella possesses a large number of defensive and offensive virulence factors that are associated with its pathogenicity. Yoon and co-workers (2011) reported more than 300 genes including known and novel virulence factors via integrating multiple transcriptomic and proteomic datasets. The investigations also showed varying distribution of virulence genes in *Salmonella* serovars (Murugkar *et al.*, 2003; Saroj *et al.*, 2008). In this study, 12 virulence genes responsible for different functions were selected, i.e., intracellular survival (*spiA*, *pagC*, *msgA)*, adhesion (*sipB*, *spaN)*, invasion (*tolC*, *lpfC*, *sopB*, *prgH)*, iron acquisition (*iroN*, *sitC)* and filamentous structure formation (*sifA*) for molecular investigation (Skyberg *et al.*, 2006; Mezal *et al.*, 2014).

The abuse of antibiotics in foods of animal origin pose a significant threat to public health as it contributes to the development of antibiotic-resistant bacteria that can be passed on to human (White *et al.*, 2001). In the case of *Salmonella*, foodborne disease in human caused by antibiotic-resistant isolates is well documented (Economou & Gousia 2015). Resistant against fluoroquinolones and third-generation cephalosporin which are important for the treatment of severe salmonellosis is of utmost concern (Cui *et al.*, 2009). In addition, multidrug-resistance (MDR) with resistant to at least one agent in three or more antimicrobial categories has been noted in several *Salmonella* serovars (Brichta-Harhay *et al.*, 2011; Magiorakos *et al.*, 2012).

By far, no data has yet been reported on the isolation of *S. enterica* from retailed sushi and sashimi samples in Malaysia. Hence, the objectives of this study are to characterize the presence of virulence genes and antibiotic resistance patterns of *S. enterica* isolated from sushi and sashimi sampled from different food retail outlets in Malaysia.

MATERIALS AND METHODS

Sample collection and isolation of *Salmonella* species

From August to December 2014, 200 retailed RTE sushi (n=149) and sashimi (n=51) were collected from different food retail outlets in the Klang Valley, Malaysia. Sushi samples consisted of rice with various toppings included marine fishes, fish roe, squid, octopus, jellyfish, edible seaweed, scallop, egg, crab stick, cherry shrimp, prawn and clam. Sashimi samples consisted only of seafood - salmon, tuna, yellow tail, squid and scallop cut into slivers. All the ready-packaged food samples were packed into another sterile (autoclaved) plastic bags and transported in a portable cooler pre-cleaned with 70% (v/v) alcohol. Food samples were analyzed immediately upon arrival at the laboratory.

The external surfaces of sample packagings were wiped with 70% (v/v) alcohol. Each sample was aseptically weighed in an analytical balance and 25 g

was homogenized in 225 mL of buffered peptone water (Oxoid, UK) using a Stomacher Bagmixer 400W (Interscience, France). The mixture was incubated for 16 hours at 37°C with agitation. On the following day, 100 µL of pre-enriched homogenate was then transferred into 10 mL of Rappaport–Vassiliadis Soya broth followed by incubation at 42°C for 24 h. A loopful of the culture was plated onto CHROMagar *Salmonella* Plus (CHROMagar, Paris, France) and incubated overnight at 37°C. The presumptive *Salmonella* spp. colonies (mauve) from each food sample were randomly selected and purified using nutrient agar for further confirmation by molecular analysis.

DNA extraction and identification of *Salmonella enterica*

Total genomic DNA was prepared using an adapted in-house boiling method (Puah *et al.*, 2016) and stored at -20°C for further investigations. All presumptive colonies were confirmed by DNA amplification using the polymerase chain reaction (PCR) for *Salmonella enterica* with primers targeting the *iroB* gene (Table 1) (Baumler *et al.*, 1997).

DNA amplification of virulence genes

The presence of 12 virulence genes - *tolC*, *sopB*, *lpfC*, *prgH*, *spaN*, *sipB*, *pagC*, *spiA*, *msgA*, *sitC*, *iroN*, *sifA* – were investigated by singleplex PCR using primers as described in published studies (Table 1). PCR was performed using TopTaq Master Mix Kit (Qiagen, Germany) according to the manufacturer's protocol. DNA amplification reaction was carried out in a PCR mixture that contained 25 µL TopTaq Master Mix, 0.2 µM forward primer, 0.2 µM reverse primer, 100 ng of DNA template, RNase-free water and 100 ng DNA. The mixture was denatured at 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products were then electrophoresed in a 1.5% (w/v) agarose gel.

Antimicrobial susceptibility testing

The susceptibility of the *Salmonella* isolates to antimicrobials was tested by the disc diffusion method on Mueller-Hinton agar using commercial antibiotic disks (Oxoid, UK) according to CLSI guidelines (CLSI, 2013). *Escherichia coli* ATCC 25922 was used as quality control strains in each run. Twelve antimicrobials were tested – ampicillin (10 µg), amoxicillin/clavulanic acid (10 µg), cefoxitin (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), gentamicin (10 µg), kanamycin (30 µg), streptomycin (10 µg), tetracycline (30 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), sulfamethoxazole (25 µg) and chloramphenicol (30 µg). Interpretation of inhibition zones was based on manufacturer's and CLSI guidelines (CLSI, 2013).

RESULTS AND DISCUSSION

Isolation and prevalence of isolates

S. enterica was detected in 32 samples (16%) from the 200 food items. The high contamination rate could have arisen from the natural aquatic environment, during inappropriate storage or during preparation/handling. The majority of *S. enterica* contamination was from sashimi samples at 25.5% (13/51) while sushi samples showed a contamination rate at 12.8% (19/149). Of the 19 contaminated sushi samples, 18 samples were topped with fish and fish products including clam (n=1), jelly fish (n=1), scallop (n=1), yellow tail fish (n=1), octopus (n=2), squid (n=2), tuna (n=2), fish roe (n=3) and salmon (n=5).

Taken together, fish and fish products were found in most contaminated sushi and sashimi samples (96.9%, 31/32). The results from this study concurred with a previous published report which indicated that fish and fish products were commonly at risk for *Salmonella* contamination (Allshouse *et al.*, 2004). The presence of *Salmonella* in fish and fish products was reported to vary widely in countries such as India (Kumar *et al.*, 2009), Thailand (Minami *et al.*, 2010), China (Yan *et al.*, 2010), Spain (Herrera *et al.*, 2006) and Malaysia (Budiati *et al.*, 2013).

Although data on the prevalence of *Salmonella* in sushi and sashimi is limited,

Table 1. Primer nucleotide sequences for DNA amplification and amplified amplicon sizes of the *iroB* gene and 12 virulence genes of *Salmonella enterica*

Gene	Upstream primer sequence (5'-3')	Downstream primer sequence (5'-3')	Amplicon size (bp)	Reference
iroB	TGCGTATTCTGTTTGTCGGTCC	TGCGTATTCTGTTTGTCGGTCC	606	Baumler *et al.*, 1997
tolC	TACCCAGGCGCAAAAAGAGGCTATC	CCGCGTTATCCAGGTTGTTGC	161	Skyberg *et al.*, 2006
sopB	CGGACCGGCCAGCAACAAAACAAGAAGAAG	TAGTGATGCCCGTTATGCGTGAGTGTATT	220	
lpfC	GCCCC GCCTGAAGCCTGTGTTGC	AGGTCGCCGCTGTTTGAGGTTGGATA	641	
prgH	GCCCGAGCAGCCTGAGAAGTTAGAAA	TGAAATGAGCGCCCCTTGAGCCAGTC	756	
spaN	AAAAGCCGTGGAATCCGTTAGTGAAGT	CAGCGCTGGGGATTACCGTTTTG	504	
sipB	GGACGCCGCCCGGGAAAAACTCTC	ACACTCCCGTCGCCGCCTTCACAA	875	
pagC	CGCCTTTTCC GTGGGGTATGC	GAAGCCGTTTATTTTTGTAGAGGAGATGTT	454	
spiA	CCAGGGGTCGTTAGTGTATTGCGTGAGATG	CGCGTAACAAAGAACCCGTAGTGATGGATT	550	
msgA	GCCAGGCGCACGCGAAATCATCC	GCGACCAGCCACATATCAGCCTCTTCAAAC	189	
sitC	CAGTATATGCTCAACGCGATGTGGGTCTCC	CGGGGCGAAAATAAAGGCTGTGATGAAC	768	
iroN	ACTGGCACGGCTCGCTGTCGCTCTAT	CGCTTTACCGCCGTTCTGCCACTGC	1205	
sifA	TTTGCCGAACGCGCCCCCACACG	GTTGCCTTTTCTTGCGCTTTCCACCCATCT	449	

Table 2. Virulence gene profiles among 32 *Salmonella enterica* isolated from sushi and sashimi samples

Virulence gene	Sushi n=19 (%)	Sashimi n= 13 (%)	Total n=32 (%)
tolC	2 (10.5)	2 (15.4)	4 (12.5)
sopB	2 (10.5)	2 (15.4)	4 (12.5)
lpfC	2 (10.5)	4 (30.8)	6 (18.8)
prgH	2 (10.5)	3 (23.1)	5 (15.6)
spaN	2 (10.5)	2 (15.4)	4 (12.5)
sipB	2 (10.5)	3 (23.1)	5 (15.6)
pagC	2 (10.5)	2 (15.4)	4 (12.5)
spiA	1 (5.3)	2 (15.4)	3 (9.4)
msgA	0	2 (15.4)	2 (6.3)
sitC	3 (15.8)	1 (7.7)	4 (12.5)
iroN	1 (5.3)	3 (23.1)	4 (12.5)
sifA	8 (42.1)	4 (30.8)	12 (37.5)

there are three well-documented outbreaks in Western countries (Barralet *et al.*, 2004; CDC, 2012; 2015). In 2004, 13 cases of *Salmonella* Singapore infection were associated with sushi roll consumption purchased from Queensland (Barralet *et al.*, 2004). In 2012, consumption of sushi made with raw yellow tuna, known as Nakaochi Scrape imported from India were identified as a source of *S. Bareilly* and *S. Nchanga* infections (CDC, 2012). Recently, another outbreak strain of *S. paratyphi* B variant L (+) tartrate (+), formally known as *S. Java* infected 53 people eating sushi containing raw tuna (CDC, 2015). Furthermore, ongoing results of investigations after the outbreak showed that *S. Newport* and *S. Weltevreden* were detected in unopened frozen ground tuna imported from Indonesia (CDC, 2015). All of these cases indicate that the raw fish ingredients in the preparation of sushi and sashimi determine the microbiological quality of the end product.

Virulence potential

A total of 32 representative isolates of each food sample was randomly selected for further analysis. Of the 32 isolates, 65.6% carried at least one virulence gene from the 12 target genes tested. *sifA* was the most prevalent virulence gene and it was detected in 37.5% of all isolates, followed by *lpfC* (18.8%), *prgH* (15.6%), *sipB* (15.6%), *sitC* (12.5%), *iroN* (12.5%), *tolC* (12.5%), *sopB* (12.5%), *spaN* (12.5%), *pagC* (12.5%), *spiA* (9.4%) and *msgA* (6.3%) (Table 2). These results suggest that *S. enterica* isolated from sushi and sashimi samples in this study are potentially virulent and can therefore cause infection in the host. Akiyama and co-workers (2011) demonstrated that the presence of similar virulence genes among seafood isolates and clinical isolates of *Salmonella* have the ability to cause human disease. For example, the most common virulence gene detected in this study was *sifA*, which is located in *Salmonella* pathogenicity island-2. Its role in pathogenicity mechanisms is well elucidated as *sifA* deletion mutants are defective for replication within macrophages as well as strongly attenuated for virulence in mice (Beuzón *et al.*, 2000; LaRock *et al.*, 2015).

Susceptibility of *Salmonella enterica* to antimicrobials

The susceptibility of the 32 *S. enterica* isolates was evaluated against 14 antimicrobial agents encompassing nine antibiotic classes (Tables 3 and 4). Most of the *S. enterica* isolates (65.6%, 21/32) were resistant to at least one antimicrobial drug (Table 4). The high rate of 65.6% observed was probably due to the use of antibiotic agents in aquaculture feed as reported by

Table 3. Frequency of antimicrobial resistance in *Salmonella enterica* isolated from sushi and sashimi samples

Antimicrobialagents	Disk concentration (µg)	Number of resistant isolates		Total n=32 (%)
		Sushi n=19 (%)	Sashimi n=13 (%)	
Ampicilin (AP)	10	6 (31.6)	4 (30.8)	10 (31.3)
Amoxicillin/clavulanic acid (AUG)	10	4 (21.1)	3 (23.1)	7 (21.9)
Cefoxitin (FOX)	30	4 (21.1)	3 (23.1)	7 (21.9)
Ceftazidime (CAZ)	30	6 (31.6)	3 (23.1)	9 (28.1)
Ceftriaxone (CRO)	30	5 (26.3)	3 (23.1)	8 (25.0)
Gentamicin (CN)	10	3 (15.8)	0	3 (9.4)
Kanamycin (K)	30	3 (15.8)	3 (23.1)	6 (18.8)
Streptomycin (S)	10	5 (26.3)	3 (23.1)	8 (25.0)
Tetracycline (TE)	30	3 (15.8)	6 (46.2)	9 (28.1)
Ciprofloxacin (CIP)	5	3 (15.8)	0	3 (9.4)
Nalidixic acid (NA)	30	6 (31.6)	2 (15.4)	8 (25.0)
Trimethoprim/sulfamethoxazole (SXT)	1.25/23.75	3 (15.8)	5 (38.5)	8 (25.0)
Sulfamethoxazole (SF)	25	7 (36.8)	9 (69.2)	16 (50.0)
Chloramphenicol (C)	30	3 (15.8)	5 (38.5)	8 (25.0)

other investigators (Yang *et al.*, 2015; Zhang *et al.*, 2015). Resistance was observed most commonly to sulfamethoxazole (50%), followed by ampicilin (31.3%), ceftazidime (28.1%), tetracycline (28.1%), ceftriaxone (25%), streptomycin (25%), nalidixic acid (25%), trimethoprim/sulfamethoxazole (25%), chloramphenicol (25%), amoxicillin/clavulanic acid (21.9%), cefoxitin (21.9%), kanamycin (18.8%), gentamicin (9.4%) and ciprofloxacin (9.4%) (Table 3). The high rate (65.6%) of antimicrobial resistance in *S. enterica* isolates detected in this study was in agreement with previous reports of *Salmonella* isolated from seafood in China (Yang *et al.*, 2015) and India (Deekshit *et al.*, 2012) who recorded the antimicrobial resistance levels of 66% and 67.5% respectively.

The most remarkable result of this study was the high frequency of *S. enterica* isolates (37.5%) with multiple drug resistance (Table 4). The most prevalent MDR isolates showed resistance to penicillins, beta-lactams, cephems and quinolone (AP, AUG, FOX, NA) with or without addition of other groups (Table 4). Two isolates showed resistance to the full panel of antibiotics tested. Observations in this study are in accordance with other studies where the incidences of MDR *Salmonella* are now reported frequently worldwide. These MDR strains have been isolated from various sources including food animals (Abraham and others 2014), meat and dairy product (Ahmed & Shimamoto, 2014), street foods (Thong & Modarressi, 2011) as well as seafood (Yang *et al.*, 2015). It is also noteworthy that 9.4 to 28.1% of *S. enterica* isolates shown in Table 3 were resistant to extended-spectrum cephalosporins (cefoxitin, ceftazidime and ceftriaxone) and fluoroquinolones (ciprofloxacin), where both are used in clinical salmonellosis treatment (Cui *et al.*, 2009).

Antimicrobial resistance is often associated with infection. Helms and co-workers (2002) reported that fatality rates of *Salmonella* infections caused by antimicrobial-resistant strain are higher than those infections caused by antimicrobial-susceptible *Salmonella* strains. Moreover, worldwide spread of *Salmonella* resistant strains via the food chain is an evolving public health challenge (Amagliani *et al.*, 2012). Hence, this study demonstrates that RTE sushi and sashimi may act as reservoirs for antimicrobial-resistant *S. enterica* and this prompts significant concerns in food safety.

Table 4. Antimicrobial resistance patterns of 21 *Salmonella enterica* isolated from sushi and sashimi samples

Number of antimicrobials	Resistance patterns	Classes of antibiotic in pattern	Isolates		Total isolates n=21 (%)
			Sushi n=9	Sashimi n=12	
One antibiotic	SF	1	2	3	5 (23.8)
	CAZ	1	1	1	2 (9.5)
Two antibiotics	CAZ, NA	2	1	0	1 (4.8)
	CAZ, SF	2	1	0	1 (4.8)
Three antibiotics	CAZ, S, NA	3	1	0	1 (4.8)
	TE, SXT, C	3	0	1	1 (4.8)
	AP, AUG, FOX	3	0	1	1 (4.8)
	TE, SXT, SF, C	3	0	2	2 (9.5)
Four antibiotics	AP, AUG, FOX, NA	4	1	0	1 (4.8)
	CAZ, CRO, TE, C	3	0	1	1 (4.8)
Six antibiotics	AP, CAZ, K, S, TE, SF	5	0	1	1 (4.8)
Nine antibiotics	AP, AUG, FOX, CRO, K, S, NA, SXT, SF	6	0	1	1 (4.8)
	AP, AUG, FOX, K, TE, NA, SXT, SF, C	8	0	1	1 (4.8)
Fourteen antibiotics	AP, AUG, FOX, CAZ, CRO, CN, K, S, TE, CIP, NA, SXT, SF, C	9	2	0	2 (9.5)

CONCLUSIONS

In the present study, *S. enterica* contamination was detected in 16% (32/200) of the sushi and sashimi samples. *Salmonella* virulence factor, *sifA* gene required for filamentous structure formation was the most prevalent at 37.5%, thus confirming the possible pathogenic capacity of these foods. Resistance to at least one antibiotic was detected at a high rate of 65.6% and sulfamethoxazole resistance was the most common at 50%. Twelve of the isolates (12/32) exhibited multiple drug resistance. In conclusion, the results of this study demonstrate the presence of *S. enterica* isolates carrying antimicrobial resistance and virulence genes in sushi and sashimi samples in the Klang Valley, Malaysia. This first report of *S. enterica* in sushi and sashimi samples in Malaysia highlights the importance for safer fish/fish products and food preparation.

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