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FULL PAPER

Occurrence and antibiotic resistance of *Salmonella* spp. in raw beef from wet market and hypermarket in Malaysia

[1*]Tan, C.W., [1]Noor Hazirah, M.Z., [1]Shu'aibu, I., [1]New, C.Y., [1]Malcolm, T.T.H., [1]Thung, T.Y., [3]Lee, E., [3]Wendy, R.D. [1,3]Nuzul, N.J., [2,3]Noor Azira, A.M., [2]Ungku Fatimah, U.Z.A., [1,4]Rukayadi, Y., [4]Rinai, R. and [1,3]Son, R.

[1]*Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia*
[2]*Department of Food Service and Management, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM Serdang, Serdang, Selangor Darul Ehsan, Malaysia*
[3]*Food Safety and Food Integrity, Institute of Tropical Agriculture and Food Security (ITAFoS), Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia*
[4]*Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia*

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Abstract

Salmonellae are highly pathogenic foodborne bacteria able to cause infection even at low doses. Infection by *Salmonella* from contaminated foods leads to gastrointestinal disease known as salmonellosis. Raw beef can be a source of human infection if the meat products are not properly handled, stored or cooked. This study aimed to investigate the prevalence and concentration of *Salmonella* in the raw beef sold at wet markets and hypermarkets in Serdang, Selangor, Malaysia, using MPN-PCR and MPN-plating on Xylose Lysine Deoxycholate (XLD) medium. In addition, *Salmonella* isolates recovered from the samples were tested for antibiotics susceptibility using Kirby-Bauer antibiotic susceptibility testing. The incidence of *Salmonella* in the raw beef samples using plating and PCR methods were 64.63% (53/82) and 17.07% (14/82) respectively. The microbial concentration of *Salmonella* in raw beef samples ranged between 3-4600 MPN/g by MPN-plating and 3-30 MPN/g by MPN-PCR approach. All isolates were found to be susceptible to imipenem, gentamicin, kanamycin, and chloramphenicol but resistant to cephalothin. It can be deduced from the results that raw beef can be a reservoir for *Salmonella* infection and the use of cephalothin (30 μg) in the treatment of infection due to these strains could be ineffective. Preventive measures such as proper temperature control as well as proper handling of raw beef in the market place are crucial to the minimization of any potential health hazard posed by this foodborne pathogen.

1. Introduction

Salmonellae are rod-like Gram-negative bacteria that cause salmonellosis, one of the most important foodborne illnesses worldwide. *Salmonella* is described as highly pathogenic bacteria as their infective dose is as low as 108 to 10^4cells (Perreten, 2005). Salmonellosis is a self-limiting infection that does not normally require antibiotic medication. However, when the disease worsens, antibiotic therapy could be recommended for the patient (CDC, 2017). In Malaysia, Salmonellae have been recognized as the culprits for some of the food poisoning outbreaks (ISID, 2013; Karim *et al*., 2017). Outbreaks of food poisoning are probably due to cross-contamination, improper handling, inappropriate storage temperature and inadequate cooking which encourage the growth of these pathogens in food.

Meat contributes an important proportion in the human diet. It is classified into red and white meat depending on the concentration of myoglobin, a substance in the muscle fiber of animals that is responsible for the meat color. Oxymyoglobins are meat components responsible for the red color of animal flesh and are developed upon exposure of myoglobin to oxygen (Mancini and Hunt, 2005). However, the redness of flesh also depends on the animal species, ages, and fiber types. Generally, cow, sheep, buffalo, goat, pork, and horses are categorized as red meat animals.

*Corresponding author.
Email: *chiawanq@gmail.com*

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Data on prevalence and outbreak involving *Salmonella* reported were usually those associated with poultry, meat, eggs and dairy product (Dominguez *et al*., 2009; Gantois *et al*., 2009; Minami *et al*., 2010; Chong *et al*., 2017). The innermost part of the meat of animals is considered sterile unless the animal is infected with pathogens. The contamination of meat by pathogenic microbes is likely due to cross-contamination at various processing steps in the slaughterhouse and marketplace, transportation of the meat, poor packaging and after cooking.

Antibiotics are widely used in the animal farm to treat, prevent and control infections and promote growth (Marshall and Levy, 2011). The excessive use and misuse of antibiotics in livestock are among the public health concerns as the antibiotic residues could be passed along the food chain which eventually makes the bacteria in the food chain resistant to them. Animals are the main reservoir for many zoonotic pathogens and the extensive use of antibiotics as growth promoters as well as therapeutic agents in the animal farm can promote the evolution of antibiotic-resistant strains. For instance, several food animals such as poultry and cattle have been reported to serve as reservoirs for multidrug-resistant Salmonellae (Yoke-Kqueen *et al*., 2008; Arslan and Eyi, 2010; Thung *et al*., 2016).

The purpose of this study was to determine the occurrence of *Salmonella* in raw beef samples obtained from various wet markets and hypermarkets in Serdang, Selangor, Malaysia using MPN-Plating and MPN-PCR. Antibiotic susceptibility testing was performed on the isolates recovered from the raw beef samples to determine their tendencies for multiple antibiotic resistance.

2. Materials and methods

2.1 Sample collection

A total of eighty-two beef samples were purchased from different wet markets (n=41) and hypermarkets (n=41) in Serdang, Selangor, Malaysia. All samples were collected in a sterile plastic bag, placed into a cold storage box with ice packs and transported to the laboratory. Samples were analysed immediately upon arrival to the laboratory.

2.2 Sample processing

Each sample was processed individually on a sterilized cutting board. Beef samples were cut and weighed approximately 10 g into a stomacher bag and added with 90 mL of buffered peptone water (BPW; Oxoid, UK). The mixture was then homogenized for 60 s using a Stomacher Lab-Blender 400 (Seward Medical, UK).

2.3 Enumeration of Salmonella using the Most Probable Number (MPN) method

The homogenized sample in the stomacher bag (10^{-1} dilution) was serially diluted by transferring 1 mL into a tube containing 9 mL of BPW to make a 10^{-2} dilution and this action continued until 10^{-4} dilution was reached. Each dilution was then transferred into a set of 3-tubes MPN. The MPN tubes were incubated at 37°C overnight. Following the incubation, MPN tubes were examined for growth and turbidity and proceeded for confirmation on plating and PCR.

2.3.1 MPN-plating

Positive MPN tubes were confirmed by streaking on Xylose Lysine Deoxycholate (XLD; Oxoid, UK) agar plates. The agar plates were incubated at 37°C overnight. Agar plates yielding presumptive *Salmonella* colonies (black centers) were confirmed positive. The presumptive colonies were picked from each agar plate and streaked on Tryptic Soy Agar (TSA; Oxoid, UK) for purification and later stored as working culture.

2.3.2 MPN-PCR

DNA extraction was carried out using boiled cell method (Pui *et al*., 2011). From the positive MPN tubes, 1 mL was transferred into a 1.5 mL microcentrifuge tube. The suspension was centrifuged at 12,000 x *g* for 3 mins. The supernatant was discarded, and the pellet was re-suspended in 500 µL of sterile distilled water. The suspension in the tube was heated on a hot dry bath (Labnet, USA) at 100°C for 10 mins and then cooled immediately at -20°C for another 10 mins. The suspension was centrifuged at 12,000 x *g* for 1 min prior to use. The supernatant was used as the DNA template.

PCR detection of *Salmonella* in raw beef samples was performed using a pair of primer (SIGMA, USA) with the forward sequence of 5'-ATC GCT GAC TTA TGC AAT CG-3' and reverse sequence of 5'- CGG GTT GCG TTA TAG GTC TG-3' specifically designed to amplify a 204 bp fragment of *ompC* genes in the *Salmonell*a spp. (Kwang *et al*., 1996).

A total of 25 µL reaction mixture was prepared for the amplification and consisted of 7.5 µL of 5×PCR buffer, 2.0 µL of 25 mM $MgCl_2$, 0.5 µL of 10 mM dNTPs, 0.25 µL of 10 µM *ompC* primers, 1.5 U of *Taq* polymerase, 2 µL of DNA template and finally made up to 25 µL with sterile distilled water. PCR was carried out in the thermocycler, GeneAmp® PCR System 2700 (Applied Biosystem,USA) with the following conditions: initial denaturation at 95°C for 3 mins, 35 cycles each of

denaturation at 95°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 7 mins. In order to visualize the PCR products, 5 µL of the PCR products were loaded onto an ethidium bromide-stained 1.2% agarose gel prepared using 0.5× TBE buffer. The products were subjected to gel electrophoresis at 100 V for 30 mins and viewed under the UV-transilluminator using gel documentation system (SynGene, UK).

2.4 Antibiotic susceptibility testing

Antibiotic susceptibility testing was conducted on the confirmed isolates of *Salmonella* from the samples by Kirby-Bauer method (Bauer *et al*., 1966). *Salmonella* isolates kept in the stock culture were subcultured aerobically in Muller Hinton broth (MHB; Oxoid, UK) at 37°C for overnight. The cultures were swabbed evenly onto Mullen Hinton agar (MHA; Oxoid, UK) plate and left to dry for 5 mins. Antibiotic disks were pressed down lightly to ensure attachment on the agar surface. The plates were incubated in inverted positions at 37°C for 24 h. A total of twelve types of antibiotic susceptibility test disks, namely imipenem (10 µg), nalidixic acid (30 µg), cephalothin (30 µg), ciprofloxacin (5 µg), ceftriaxone (5 µg), sulphamethoxazole/ trimethoprim (25 µg), tetracycline (30 µg), ceftazidime (30 µg), gentamicin (10 µg), streptomycin (10 µg), chloramphenicol (30 µg), and kanamycin (30 µg) were used in this study. After incubation, the diameter of the inhibition zone was measured to the nearest whole millimetre. Antibiotic susceptibility profiles of the isolates were determined based on the Clinical and Laboratory Standards Institute (CLSI) guidelines. The value of multiple antibiotic resistance (MAR) index was calculated using the formula, a/b, where 'a' is the number of antibiotics to which the particular isolate was resistant and 'b' is the total number of antibiotics tested (Krumperman, 1983).

3. Results

3.1 Occurrence of Salmonella in raw beef

The occurrence of *Salmonella* in raw beef obtained from the hypermarkets and wet markets are shown in Table 1. The enumeration of *Salmonella* in the raw beef samples through the MPN-plating showed a higher prevalence (64.63%) compared to the MPN-PCR (17.07%) approach. In the case of *Salmonella* concentration in the raw beef samples, the MPN-plating also showed a higher concentration of presumptive *Salmonella* in the samples (ranging from 3 to 4600 MPN/g) than the MPN-PCR method with a range of 3 to 30 MPN/g.

The MPN-PCR results revealed that 9.76% (4/41) of the samples from the hypermarkets and 24.39% (10/41) of the samples from the wet markets were found to harbour *Salmonell*a with the microbial load of 3-30 MPN/g and 3-9.2 MPN/g respectively. The prevalence and concentration of *Salmonella* in the raw beef samples obtained from the wet markets were found to be significantly lower (P<0.05) than those from the hypermarkets.

3.2 Antibiotic susceptibility test

Four confirmed *Salmonell*a isolates were recovered from the samples and tested for antibiotic susceptibility test. Antibiotic susceptibility profiles of *Salmonell*a isolates from raw beef samples are shown in Table 2. All the four (100%) isolates were susceptible to imipenem, gentamicin, kanamycin, and chloramphenicol. About 75% of the isolates were susceptible to ceftriaxone and ciprofloxacin. Half (50%) of the isolates were susceptible to nalidixic acid, sulphamethoxazole/ trimethoprim, tetracycline, ceftazidime, and streptomycin. However, 25% of the isolates showed intermediate resistance to nalidixic acid, ciprofloxacin, ceftazidime, and streptomycin and all the isolates were resistant to cephalothin. In addition, 50% of the isolates were resistant to both of sulphamethoxazole / trimethoprim and tetracycline and 25% resistant to nalidixic acid, ceftriaxone, ceftazidime, and streptomycin. All isolates showed resistance to at least two antibiotics used in this study. Multiple antimicrobial resistance (MAR) index values of the isolates are displayed in Table 3. Isolate A had a MAR index value of less than 0.2. Isolate B, C and D displayed multiple antibiotics resistance with MAR index values of 0.25, 0.25 and 0.33, respectively.

4. Discussion

The study of the prevalence of *Salmonella* in raw beef samples by MPN-plating on XLD agar recorded a higher level of detection rate and microbial concentration (MPN/g) than the MPN-PCR approach. The higher

Table 1. Occurrence of *Salmonella* in raw beef

Location	MPN-Plating		MPN-PCR	
	Prevalence, n(%)	Concentration Range (MPN/g)	Prevalence, n(%)	Concentration Range (MPN/g)
Hypermarket	21/41 (58.54)	3-4600	4/41 (9.76)	3-30
Wet Market	28/41 (68.29)	3-4600	10/41 (24.39)	3-9.2
Overall	53/82 (64.63)	3-4600	14/82 (17.07)	3-30

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Table 2. Antibiotic susceptibility profiles of *Salmonell*a isolates from the raw beef samples

Class of antibiotics	Antibiotics	Resistant (%)	Intermediate (%)	Susceptible (%)
Carbapenem	Imipenem (10 µg)	-	-	4 (100%)
Quinolone	Nalidixic acid (30 µg)	1 (25%)	1 (25%)	2 (50%)
Cephalosporin I	Cephalothin (30 µg)	4 (100%)	-	-
Fluoroquinolone	Ciprofloxacin (5 µg)	-	1 (25%)	3 (75%)
Cephalosporin III	Ceftriaxone (5 µg)	1 (25%)	-	3 (75%)
Folate pathway inhibitor	Sulphamethoxazole/ trimethoprim (25 µg)	2 (50%)	-	2 (50%)
Tetracyclines	Tetracycline (30 µg)	2 (50%)	-	2 (50%)
Cephalosporin III	Ceftazidime (30 µg)	1 (25%)	1 (25%)	2 (50%)
Aminoglycosides	Gentamicin (10 µg)	-	-	4 (100%)
Aminoglycosides	Streptomycin (10 µg)	1 (25%)	1 (25%)	2 (50%)
Phenicols	Chloramphenicol (30 µg)	-	-	4 (100%)
Aminoglycosides	Kanamycin (30 µg)	-	-	4 (100%)

Table 3. Multiple antimicrobial resistance (MAR) index value of *Salmonella* isolates.

Isolate	No of antibiotic resistance	Type of antibiotic resistance	MAR index
A	2	Sulphamethoxazole/ trimethoprim , cephalothin	0.17
B	3	Sulphamethoxazole/ trimethoprim, tetracycline, cephalothin	0.25
C	3	Streptomycin, nalidixic acid, cephalothin	0.25
D	4	Ceftriaxone, tetracycline, ceftazidime, cephalothin	0.33

number of *Salmonella* as determined by MPN-plating may indicate a high number of false-positive presumptive colonies that were morphologically similar as true-positive *Salmonella* colonies on XLD agar. The results obtained from the MPN-PCR were therefore considered more reliable and accurate than MPN-plating in detecting *Salmonella* from the raw beef sample. Similarly, Pui *et al*. (2011) reported that MPN-PCR had higher sensitivity in detecting *Salmonella* from sliced fruits than MPN-plating method. This was further supported by the report of Maks and Fu (2013), in which the PCR approach had significantly better detection of *Salmonella* with fewer false-negative results than the bacteriological medium.

Several studies have also found out that the low specificity of XLD medium created a high number of false positive identification of *Salmonella* species (Rall *et al*., 2005; Hyeon *et al*., 2012; Yhiler *et al*., 2015). Rall *et al*. (2005) stated that XLD allows the growth of selective bacteria with *Salmonella*, *Proteus*, and *Citrobacter* that displayed similar characteristics as negative lactose fermentation and negative hydrogen sulfide (H_2S) production on this classical medium. Thus, the colonies of *Proteus* and *Citrobacter* probably appear and identified as presumptive *Salmonella* colony on XLD medium. In addition, Hyeon *et al*. (2012) and Yhiler *et al*. (2015) concluded that XLD medium had high sensitivity but with low specificity for the detection of *Salmonella*.

The results of the MPN-PCR recorded the overall prevalence of *Salmonella* in the raw beef samples of 17.07%. Comparatively, Thung *et al*. (2017) reported 7.5% of raw beef samples obtained from the retail markets in Selangor Malaysia, positive for *Salmonella* with the microbial load of <3 to 15 MPN/g. Similarly, Chong *et al*. (2017) also reported 10% of beef carcasses collected from the local abattoirs in Selangor, Malaysia as positive for *Salmonella*. Modarressi and Thong (2011) reported that 27.2% of raw beef samples purchased from the retail markets in Kuala Lumpur, Malaysia were contaminated with *Salmonella*. In other countries, the occurrence of *Salmonella* in the raw beef sample collected from the retail markets in Vietnam was reported to be 39.9 to 48.6% (Phan *et al*., 2005; Thai *et al*., 2012).

Moreover, the occurrence of *Salmonella* in the wet market samples was found to be higher than in the hypermarket samples. This finding was similar to the results reported by Shafini *et al*. (2017) in Malaysia. Shafini *et al*. (2017) found out that 20.8%, 14.8% and 11.1% of the beef samples from the wet markets, hypermarkets, and butcher shops respectively were contaminated with *Salmonella*. This corresponds with a finding in China in which Yang *et al*., (2011) reported the prevalence of *Salmonella* on raw poultry with higher prevalence in the wet markets (54.4%) than in large markets (50.3%) and small markets (52.1%). The reason for the higher incidence of *Salmonella* in samples

obtained from the wet markets than those from hypermarket could be due to inadequate food safety practices compared to the food safety measures and sanitation programmes implemented in the hypermarkets. Thung *et al*. (2016) mentioned that hypermarket workers generally practice good personal hygiene and sanitation in the food processing environment than those of the wet markets.

The microbial load of *Salmonella* from the wet market and hypermarket samples were 3-9.2 MPN/g and 3-30 MPN/g, respectively. The concentration of *Salmonella* in the hypermarket samples was found to be slightly higher than that of the samples from the wet market. This finding was in contrast with the study of Thung *et al*. (2016), who reported the microbial load of *Salmonella* in beef samples from the wet market with a higher concentration (<3 to 15 MPN/g) than supermarket samples (<3 to 3.6 MPN/g). The higher *Salmonella* concentration in the hypermarket samples might be due to the longer operating hours than in wet markets, in which the meat exposed to the environment and temperature abuse longer which facilitates bacterial multiplication. Conversely, many wet markets operation hours are usually half a day eventually providing quality fresh produce for the consumers.

The existence of multiple antibiotic resistant bacteria can lead to human infections and probably creates challenges for the treatment of *Salmonella* infections in humans and animals (Thung *et al*., 2016). The use of antibiotics for treatment and as growth promoters in farm animals may promote the emergence and spread of antibiotic-resistant microorganisms to the human population (Khoo *et al*., 2015). Bacterial isolate is classified as multiple antibiotic resistant if the MAR value is higher than 0.2. Three isolates were found with a MAR value of higher than 0.2 and more than 50% (2/4) of *Salmonella* isolates were resistant to cephalothin, sulphamethoxazole/ trimethoprim, and tetracycline in this study. Another study reported that *Salmonella* isolated from the raw beef, chicken meat, and street foods exhibited the highest resistance to tetracycline (73.8%), followed by sulfonamide (63.6%) and streptomycin (57.9%) (Thong and Modarressi, 2011). Minami *et al*. (2010) stated that most of the *Salmonella* isolated showed resistance to sulfisoxazole, tetracycline, and streptomycin. Yoke-Kqueen *et al*. (2008) reported that *Salmonella* isolated from the indigenous vegetables and poultry exhibited multidrug resistance to erythromycin, tetracycline, streptomycin and trimethoprim/sulfamethoxazole.

5. Conclusion

MPN-PCR was more reliable than MPN-plating with regards to the degree of specificity of identification of *Salmonella* species in raw beef samples. Using MPN-plating method, 64.63% of the samples were detected with *Salmonella* with a concentration range of 3-4600 MPN/g in comparison to 17.07% of the samples detected with a concentration range of 3-30 MPN/g via MPN-PCR approach. Raw beef can become a common vehicle for *Salmonella* transmission and if inadequate practice of food hygiene and sanitation, *Salmonella* infection is possible. Multiple antibiotic resistant *Salmonella* should be regarded as an issue and the spread should be viewed as a challenge to combat the bacteria and conserving public health.

Conflict of Interest

The authors declare no conflict of interest.

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