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Antimicrobial resistance of *Listeria monocytogenes* and *Salmonella* Enteritidis isolated from vegetable farms and retail markets in Malaysia

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Abstract

Listeriosis and salmonellosis are the major foodborne illnesses worldwide. Over the last decade, increasing reports about the antibiotic resistance of *Listeria monocytogenes* and *Salmonella* from diverse sources have prompted public health concerns, especially in developing countries with over reliance or misuse of antibiotic drugs in the treatment of humans and animals. In this study, antibiotic susceptibility profiles of 58 *L. monocytogenes* and 12 *Salmonella* Enteritidis strains from vegetable farms and retail markets in Malaysia were testedby the standard disk diffusion method. *Listeria monocytogenes* isolates were found to exhibit 100% resistance to penicillin G. Also, high resistance patterns were observed for meropenem (70.7%) and rifampicin (41.4%). The multiple antibiotic resistance (MAR) index of *L. monocytogenes* isolates ranged from 0.11 to 0.56. Besides, the antibiogram results revealed that multidrug-resistant (MDR) *S.* Enteritidis were detected and all the *S.* Enteritidis isolates demonstrated resistance to at least four antibiotics. Ampicillin, amoxicillin, and trimethoprim failed to inhibit all the *S.* Enteritidis strains. *Salmonella* Enteritidis isolates also displayed high resistance to nalidixic acid (75.0%), trimethoprim-sulfamethoxazole (75.0%), and chloramphenicol (66.7%). Findings in this study indicated that vegetables could be potential sources of multidrug resistance of *L. monocytogenes* and *S.* Enteritidis, which can be a serious issue and a major concern for public health. Thus, there is a great need for surveillance programs in Malaysia to continuously monitor the antibiotic resistance profiles of important pathogens.

Introduction

Ensuring food safety is becoming an important part of consumers' daily lives. The search for healthy eating has favoured the increase consumption of fresh and ready-to-eat vegetables worldwide. Since ready-to-eat vegetables are often consumed raw or minimally processed, and not subjected to any cooking processes that could considerably kill the foodborne pathogens, they may act as potential vehicles for the transmission of pathogens (Elexson *et al*., 2017; New *et al.*, 2017). Over the past decade, *L. monocytogenes* and *Salmonella* have been implicated in many foodborne disease outbreaks related to fresh produce (Beuchat, 2002; Warriner *et al*., 2009; Maffei *et al.*, 2013).

Listeria monocytogenes is one of the important emerging foodborne pathogens as it is ubiquitous in the environment and could cause severe listeriosis (Altuntas *et al*., 2012). Foodborne listeriosis is a severe bacterial infection, with high rates of fatality (20-30%) and hospitalisation (more than 92%), caused by consumption of food contaminated with *L. monocytogenes* (Du *et al*., 2017). Listeriosis is always

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a public health concern as this foodborne infection is the greatest threat to susceptible population groups such as pregnant women, foetuses or newborns, the elderly, and immunocompromised individuals (e.g. immunosuppression, HIV/AIDS) (Sofos and Geornaras, 2010; Todd and Notermans, 2011).

Besides *L. monocytogenes, Salmonella* is another major cause of foodborne illness in humans throughout the world and it can potentially result in economic, morbidity, and mortality loss (Thung *et al*., 2016). Among 2,463 serovars of *Salmonella, S*. Enteritidis and *S.* Typhimurium are the common serovars associated with salmonellosis (Najwa *et al*., 2015). Researchers have estimated that every year there are 9,380,000 enteric infections and 155,000 deaths worldwide caused by *Salmonella* spp. (Majowicz *et al*., 2010; Li *et al*., 2017).

Over the last decade, there is an increasing number of multidrug-resistant *L. monocytogenes* strains were isolated from food samples (Charpentier and Courvalin, 1999; Lyon *et al*., 2008; Yan *et al*., 2010; Tareq *et al.*, 2011). Similar to *L. monocytogenes, Salmonella* species are becoming increasingly resistant to conventional antibiotics, including ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole, and some newer antibiotics such as quinolones and extended-spectrum cephalosporins, making it more complicated and expensive to treat patients with serious infections (Su *et al.*, 2004; Rusul *et al*., 2012).

Since listeriosis and salmonellosis are critical illnesses, a positive outcome for these diseases mainly depends on the early application of suitable antibiotics (Moreno *et al*., 2014). Therefore, antibiotic resistance of these pathogens is a great concern (Marrero-Ortiz *et al*., 2012). Monitoring and reviewing the antimicrobial resistance regularly, especially multidrug resistance of emerging foodborne pathogens, are important aspects of hazard assessment. These generated data can be used to recommend some preventive measures in order to prevent or reduce environmental spread, revise dosing of antibiotics, and inform the suitable medical treatments for the illnesses caused by the pathogens (Du *et al*., 2017).

Bacterial resistance to conventional antibiotic therapies can possibly increase the number of foodborne infections, which lead to the emergence of more virulent pathogenic microorganisms. Thus, there is a crucial need for planning and implementation of a surveillance system to monitor and review the antibiotic susceptibility profiles of pathogens on a regular basis. The objective of this study was to examine the antibiotic resistance profiles of *L. monocytogenes* and *S*. Enteritidis that are isolated from vegetable samples collected from vegetable farms and retail markets in Malaysia.

Materials and Methods

Sample collection

A total of 301 vegetables,including organic and conventional vegetables, and environmental samples such as soils, animal manures, fertilisers, composts, and irrigation water,were collected from wet markets, hypermarkets, farms, and packing houses in Selangor, Kuala Lumpur, Putrajaya, Pahang, and Perak, in the periodof November 2015 to November 2016. All samples were sealed in sterile plastic bags and kept in insulated boxes with ice packs. The samples were transported to the Food Safety and Quality Laboratory, Universiti Putra Malaysia, immediately for microbiological analysis.

Isolation and identification of Listeria *species*

Sample preparation was performed based on the method described by Kuan *et al.* (2013a) and Kuan *et al*. (2013b). Ten grams of each sample was aseptically weighed and mixed with 90 mL of LEB in a sterile stomacher bag and stomached on medium speed for 1-2 min using a stomacher machine (BagMixer® 400P, Interscience, Saint-Nom-la-Bretèche, France). The homogenised sample was incubated at 30°C for 4 h. Then, the selective agents (acriflavine, 10 mg/L; sodium nalidixate, 40 mg/L; cycloheximide 50 mg/L; Sigma, St. Louis, MO) were added into pre-enriched bacteria culture and incubated for another 44 h at 30°C. Thereafter, the enriched bacteria culture was streaked onto PALCAM agar and incubated at 30°C for 48 h. About five to ten presumptive colonies (grey-green colonies surrounded by a black zone) were purified by streaking onto TSAand incubated at 30°C for 48 h. Purified colonies were subjected to PCR assay for confirmation. Genomic DNA extraction and multiplex-PCR assay for the identification of *Listeria* spp. and *L. monocytogenes* were performed according to the procedures described by Kuan *et al.* (2013a) and Kuan *et al.* (2013b).

Isolation and identification of Salmonella *species*

Ten grams of each sample was added to 90 mL of BPW. The mixture was homogenised for 1-2 min using a stomacher machine and incubated at 37°C for 24 h to enrich the target bacteria. Enriched bacteria culture was then streaked onto CHROMagar Salmonella (CHROMagar Microbiology, Paris, France) and incubated at 37°C for 24 h. About five to ten presumptive colonies (mauve-coloured colonies)

were purified by plating onto TSA and incubated at 37°C for 24 h. Boiled-cell method and multiplex-PCR assay for DNA extraction and identification of *Salmonella* spp., *S.* Enteritidis, and *S.* Typhimurium were carried out base on the procedures described by Pui *et al.* (2011) and Thung *et al.* (2016).

Antimicrobial susceptibility testing

In this study, a total of 58 *L. monocytogenes* strains and 12 *S.* Enteritidis strains confirmed by PCR were subjected to antibiotic susceptibility test. The distribution of the 58 *L. monocytogenes* and 12 *S.* Enteritidis isolates by types of samples, location, and time of collection were summarised in Table 1. All Isolates were screened for susceptibility using the Kirby-Bauer disk diffusion method according to the guidelines described by Bauer *et al.* (1966) andClinical and Laboratory Standards Institute(CLSI) (2016).

A total of nine and thirteen antibiotics were selected to determine their susceptibility to *L. monocytogenes* and *S.* Enteritidis, respectively. Selection of antibiotics was according to their importance, inhibitory effect, and frequent usage in clinical and agricultural practices. Five antibiotics were shared among *L. monocytogenes* and *S.* Enteritidis isolates, and they were: ampicillin (10 µg), gentamicin (10 µg), trimethoprim-sulfamethoxazole (1.25/ 23.75 µg), tetracycline (30 µg), and ciprofloxacin (5 µg). At the same time, erythromycin (15 µg), penicillin G (10 units), meropenem (10 µg), and rifampicin (5 µg) were used to test on susceptibility of *L. monocytogenes* whereas amoxicillin (10 µg), ceftriaxone (30 µg), nalidixic acid (30 µg), trimethoprim (5 µg), streptomycin (10 µg), chloramphenicol (30 µg), amoxicillin/clavulanic acid (30 µg), and ceftazidime (30 µg) were tested on *S.* Enteritidis isolates. All antibiotic discs were placed on Mueller-Hinton agar (MH agar; Merck, Darmstadt, Germany) using a disc dispenser and incubated at 37°C for 24 h for *S.* Enteritidis and 24-48 h for *L. monocytogenes*. *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as used quality control strains throughout the study.

Antibiotic susceptibility profile of a bacterial isolate was identified by measuring the size of growth inhibition zone to the nearest millimetre. The inhibition zones were interpreted as sensitive, intermediate susceptibility and resistant, according to the breakpoints recommended by the CLSI (2016) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2017) guidelines. As currently, there are no established guidelines for *Listeria* antibiotic susceptibility testing (Byrnea *et al*., 2016; Du *et al.*, 2017), except for antibiotic ampicillin (10 µg) and meropenem (10 µg), which their inhibition zones were referred to the breakpoints for *L. monocytogenes* in EUCAST (2017) guidelines. Thus, the antibiotic susceptibility profile of *L. monocytogenes* isolates was determined by compared the diameters of the inhibition zones to the breakpoints for *Staphylococcus* spp. (CLSI 2016).

Multiple antibiotic resistance (MAR) index

Antibiotic resistance pattern of each bacterial isolate was determined by calculating the MAR index, as described by Krumperman (1983):

MAR index = a/b

"a" = Number of antibiotics to which the particular isolate was resistant;

"b" = total number of antibiotics tested

Results

Table 2 and 3 show the antimicrobial profiles and MAR index of *L. monocytogenes* isolates against nine antibiotics. All the *L. monocytogenes* isolates were found to be resistant to penicillin G. Also, *L. monocytogenes* strains showed high resistance to meropenem (70.7%) and rifampicin (41.4%). Ampicillin, gentamicin, and trimethoprim-sulfamethoxazole were effective in restraining the growth of *L. monocytogenes* with the percentage susceptibility of 100%, 91.4% and 84.5%, respectively.

Table 4 summarises the antibiotic susceptibility profiles of 12 *S.* Enteritidis isolated from vegetable samples. Ampicillin, amoxicillin, and trimethoprim failed to inhibit the growth of all isolates of *S.* Enteritidis. In addition, high levels of resistance were observed for nalidixic acid (75.0%), trimethoprim-sulfamethoxazole (75.0%), and chloramphenicol (66.7%). In contrast, ceftazidime, gentamicin, and tetracycline were found to be 100% effective against *S.* Enteritidis isolates. As shown in Table 5, multidrug-resistant (MDR) of *S.* Enteritidis were detected with the highest MAR index value of 0.62. In this study, all the *S.* Enteritidis isolates exhibited resistance to at least four different antibiotics.

Discussion

Antimicrobial susceptibility testing (AST) is an in vitro method commonly used to determine the susceptibility of a pathogenic microorganism to an antimicrobial agent, hence, predicts the effectiveness of an antibiotic therapy (Govan, 2006). The

Table 1. The distribution of 58 *L. monocytogenes* and 12 *S.* Enteritidis isolates by type of samples, location, and time of collection

Bacterial strains	Type of samples	Location	Time of collection	Total number of isolates
L.monocytogenes	Carrot	Hypermarket in Selangor	March 2016	7
	Calamondin	Hypermarket in Selangor	March 2016	2
	Cucumber	Hypermarket in Selangor	March 2016	2
	Winged bean	Hypermarket in Selangor	March 2016	16
	White radish	Hypermarket in Selangor	April 2016	16
	Cabbage	Hypermarket in Selangor	April 2016	15
*S.*Enteritidis	Carrot	Hypermarket in Selangor	April 2016	9
	Eggplant	Conventional vegetable farm in Perak	October 2016	3

Table 2. Antibiotic susceptibility profiles of 58 *L. monocytogenes* strains isolated from vegetable samples tested by disc diffusion method

Antibiotic	Antimicrobial profile of *L. monocytogenes*		
	Susceptible (%)	Intermediate (%)	Resistant (%)
Ampicillin, (10 µg)	58 (100)	-	-
Penicillin G (10 Unit)	-	-	58 (100)
Erythromycin (15 µg)	1 (1.7)	57 (98.3)	-
Rifampicin (5 µg)	8 (13.8)	26 (44.8)	24 (41.4)
Gentamicin (10 µg)	53 (91.4)	4 (6.9)	1 (1.7)
Tetracycline (30 µg)	17 (29.3)	37 (63.8)	4 (6.9)
Ciprofloxacin (5 µg)	5 (8.6)	50 (86.2)	3 (5.2)
Trimethoprim-sulfamethoxazole (1.25/ 23.75 µg)	49 (84.5)	6 (10.3)	3 (5.2)
Meropenem (10 µg)	17 (29.3)	-	41 (70.7)

emergence of antimicrobial resistance in pathogens from foods, environments, humans, and animals has led to increased number of surveillance programs to monitor the antibiotic resistance profiles of important pathogens. In this study, antimicrobial susceptibility test was performed using the standard disc diffusion method and the antibiogram results were interpreted based on the updated breakpoints provided by CLSI (2016) and EUCAST (2017). Jorgensen and Ferraro (2009) pointed that use of outdated or erroneous information from the international guidelines could lead to wrong judgements or faulty conclusions.

Listeria monocytogenes and *S.* Enteritidis were the most common pathogenic microorganisms detected and isolated in this study as compared to *Listeria* spp., *Salmonella* spp., and *S.* Typhimurium. In this study, ampicillin, gentamicin, and trimethoprim-sulfamethoxazole were found to be effective against *L. monocytogenes* with the high susceptibility of 100%, 91.4% and 84.5%, respectively. The high susceptibility of these antibiotics observed in this study is also comparable to the current clinical practices, in which ampicillin alone or in combination with gentamicin is used as the first-line drugs for the treatment of severe listeriosis (Hof, 2004; Altuntas *et al.*, 2012). Besides, a combination of two antibiotics, sulfamethoxazole and trimethoprim also have been used as a second-choice drug in treating human listeriosis, especially for patients who are allergic to penicillin (Hof, 2004; Altuntas *et al.*, 2012).

It is worth noting that all the *L. monocytogenes* isolates were resistant to penicillin G, commonly used as the primary therapy in treating severe listeriosis. This finding is in line with previous studies that most of the *L. monocytogenes* strains isolated from food, seafood, and human were found to be resistant to penicillin (Issa *et al.*, 2011; Abdollahzadeh *et al.,* 2016). The emergence of penicillin resistance strains is of particular importance and calls for attention, since it may cause failure in antibiotic treatments. Indiscriminate use or overuse of penicillin as first-line drugs may be the main reason that contributed to reducing the susceptibility of this antibiotic. Many studies have reported the susceptibility of *L. monocytogenes* to tetracycline and ciprofloxacin (Shen *et al*., 2013; Chen *et al.*, 2014; Wu *et al.*, 2015; Li *et al.*, 2016; Du *et al*., 2017). However, 63.8% and 86.2% of *L. monocytogenes* isolates in

Table 3. Antibiotic resistance profiles and MAR index of *L. monocytogenes* strains isolated from vegetable samples

MAR Index	Antibiotic resistance profile[a]	Source and isolate's code[b]	Percentage of isolate (%)
0.56	PRDTECIPMEM	C2	1.7
	PRDCIPSXTMEM	C3	1.7
0.44	PRDTEMEM	C1, 40	3.4
	PRDSXTMEM	C6	1.7
	PRDCNMEM	R37	1.7
0.33	PRDMEM	C4, C5, C7, D8, R28,R33,R34, R35, R36, R38, R39, R41, R42, G45	24.1
	PCIPMEM	G32	1.7
0.22	PMEM	D9, B10, W12, W14, W20, W22, W23, W25, W26, W27, R29, R30, R43, G49, G50, G52, G53, G54, G55, G58	34.5
	PTE	W13	1.7
	PSXT	W15	1.7
	PRD	W16, W21, 51, 57	6.9
0.11	P	B11, W17, W18, W19, W24, R31, G44, G46, G47, G48, G56	19.0

[a]AMP - Ampicillin; P - Penicillin G; E - Erythromycin; RD - Rifampicin; CN - Gentamicin; TE - Tetracycline; CIP - Ciprofloxacin; SXT - Trimethoprim-Sulfamethoxazole; MEM - Meropenem

[b]C - Carrot; D - Calamondin; B - Cucumber; W - Winged bean; R - White radish; G - Cabbage

Table 4. Antibiotic susceptibility profiles of 12 *S.* Enteritidis strains isolated from vegetable samples tested by disc diffusion method

Antibiotics	Antimicrobial profile of *S.* Enteritidis		
	Susceptible (%)	Intermediate (%)	Resistant (%)
Ampicillin, (10 µg)	-	-	12 (100)
Amoxicillin (30 µg)	-	-	12 (100)
Amoxicillin/ Clavulanic acid (30 µg)	2 (16.7)	9 (75.0)	1 (8.3)
Ceftriaxone (30 µg)	1 (8.3)	6 (50.0)	4 (33.3)
Ceftazidime (30 µg)	12 (100)	-	-
Gentamicin (10 µg)	12 (100)	-	-
Streptomycin (10 µg)	4 (33.3)	8 (66.7)	-
Tetracycline (30 µg)	12 (100)	-	-
Ciprofloxacin (5 µg)	6 (50.0)	6 (50.0)	-
Nalidixic acid (30 µg)	-	3 (25.0)	9 (75.0)
Trimethoprim-sulfamethoxazole (1.25/ 23.75 µg)	-	3 (25.0)	9 (75.0)
Trimethoprim (5 µg)	-	-	12 (100.0)
Chloramphenicol (30 µg)	-	4 (33.3)	8 (66.7)

this study were found to have intermediate resistance towards tetracycline and ciprofloxacin, respectively, indicating that *L. monocytogenes* is acquiring resistance to these two antibiotics.

Erythromycin, rifampicin, and meropenem have been reported to be effective in treating confirmed cases of listeriosis (Charpentier and Courvallin, 1999; Matano *et al.*, 2010; Altuntas *et al.*, 2012). Surprisingly, low susceptibilities to these three antibiotics were observed in this study, which showed 1.7%, 13.8%, and 29.3% of susceptibility levels to erythromycin, rifampicin, and meropenem, respectively. Overall, *L. monocytogenes* isolates in this study demonstrated MAR index ranging from 0.11 to 0.56. It was found that 81% of isolates exhibited resistance to at least two antibiotics. Generally, bacterial strains with MAR index higher than 0.2 are considered to originate from high-risk sources of contamination or environments that are always exposed to antibiotic drugs (Gwendelynne *et al.*, 2005; Singh *et al.*, 2010).

Previous studies have found that *Salmonella* displayed multidrug-resistant patterns in recent years (Adley *et al.*, 2011; Abakpa *et al.*, 2015, Thung *et al.*, 2016). The increase in antimicrobial resistance among the *Salmonella* isolates worldwide is mainly

Table 5.Antibiotic resistance profiles and MAR index of *S.* Enteritidis strains isolated from vegetable samples

MAR Index	Antibiotic resistance profiles[a]	Sources and isolate's codes[b]	Percentage of isolate (%)
0.62	AMPAMLAMCCRONASXTWC	C8	8.3
0.54	AMPAMLCRONASXTWC	C4, C6, C9	25.0
0.46	AMPAMLCRONAWC	C1	8.3
	AMPAMLNASXTWC	C3, E10	16.7
0.38	AMPAMLNASXTW	C7	8.3
0.31	AMPAMLWC	C2	8.3
	AMPAMLNAW	C5	8.3
	AMPAMLSXTW	E11, E12	16.7

[a]AMP - Ampicillin; AML - Amoxicillin; AMC - Amoxicillin/Clavulanic acid; CRO - Ceftriaxone; CAZ - Ceftazidime; CN - Gentamicin; S - Streptomycin; TE - Tetracycline; CIP - Ciprofloxacin; NA - Nalidixic acid; SXT - Trimethoprim-Sulfamethoxazole; W - Trimethoprim; C - Chloramphenicol
[b]C - Carrot; E - Eggplant

due to the excessive use of antimicrobial drugs for the empiric treatment of febrile syndromes and used as animal growth promoters (Bukitwetan *et al*., 2007). In this study, multidrug resistance was detected in all isolates of *S.* Enteritidis. One of the *S.* Enteritidis strains was observed with the highest MAR index value of 0.62, which was found to be resistant to eight antibiotics out of a total 13 antibiotics. *Salmonella* Enteritidis isolates in the present study exhibited resistance to at least four different antibiotics.

In this study, ampicillin, amoxicillin, and trimethoprim failed to inhibit the growth of all isolates of *S.* Enteritidis. In addition, high levels of resistance were observed for nalidixic acid (75.0%), trimethoprim-sulfamethoxazole (75.0%), and chloramphenicol (66.7%). These findings are in agreement with the results from other studies in which *Salmonella* strains displayed high rates of resistance to ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole (Su *et al*., 2004; Mijovic *et al*., 2012; Najwa *et al*., 2015; Thung *et al*., 2016). High-level ampicillin resistance in *Salmonella* is alarming since this antibiotic is one of the traditional first-line antibiotic medications (de Oliveira *et al*., 2006). In contrast, ceftazidime, gentamicin, and tetracycline were found to be 100% effective in inhibiting the growth of *S.* Enteritidis isolates. Thung *et al.* (2016) also reported that all the *S.* Enteritidis and *S.* Typhimurium isolates (n = 11) were 100% sensitive to gentamicin and tetracycline while 72.7% to ceftazidime. Results in our study showed that about 75.0%, 66.7%, 50%, and 50% of *S.* Enteritidis strains demonstrated intermediate resistance to amoxicillin/clavulanic acid, streptomycin, ceftriaxone, and ciprofloxacin, respectively. This phenomenon can become a very frightening issue as this indicated that *Salmonella* is slowly developing resistance and making the bacterial infection much more difficult to treat with current antibiotics.

Antimicrobial multiple resistance could lead to approximately 25,000 deaths worldwide annually (Du *et al*., 2017). Due to the emergence and spread of resistant bacterial strains as shown in this study towards antimicrobial drugs which are currently deemed as critically important in human medicine, it is crucial to implement surveillance system to screen, evaluate, and investigate the susceptibility of globally important pathogens towards particular antibiotics, and hence provide the necessary foundation for effective mitigation strategies (de Oliveira *et al.*, 2012; Balouiri *et al.*, 2016).

Conclusion

The detection of multidrug-resistant *L. monocytogenes* and *Salmonella* strains in this study deserves a public attention. Our findings revealed that vegetables can serve as a reservoir for harbouring multidrug-resistant foodborne pathogens, which can pose a significant impact on public health. Given the increasing number of antibiotic resistance in *Salmonella* and *L. monocytogenes* strains being detected worldwide, it has become a formidable public health challenge. Thus, continuous monitoring

programs at the national level, which focusing on the surveillance of antibiotic resistance are indispensable for empiric antimicrobial therapy in treating bacterial infections as well as to prevent the spread of multidrug-resistant bacteria.

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