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 tested by 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.

Keywords

Antibiotic susceptibility
Disk diffusion test
*Listeria monocytogenes*
*Salmonella Enteritidis*
Fresh produce

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 period of 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®, 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 TSA and 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 based 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) and Clinical 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 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 (Byrne 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 comparing 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):

\[
\text{MAR index} = \frac{a}{b}
\]

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

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Type of samples</th>
<th>Location</th>
<th>Time of collection</th>
<th>Total number of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. monocytogenes</em></td>
<td>Carrot</td>
<td>Hypermarket in Selangor</td>
<td>March 2016</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Calamondin</td>
<td>Hypermarket in Selangor</td>
<td>March 2016</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Cucumber</td>
<td>Hypermarket in Selangor</td>
<td>March 2016</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Winged bean</td>
<td>Hypermarket in Selangor</td>
<td>March 2016</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>White radish</td>
<td>Hypermarket in Selangor</td>
<td>April 2016</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Cabbage</td>
<td>Hypermarket in Selangor</td>
<td>April 2016</td>
<td>15</td>
</tr>
<tr>
<td><em>S. Enteritidis</em></td>
<td>Carrot</td>
<td>Hypermarket in Selangor</td>
<td>April 2016</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Eggplant</td>
<td>Conventional vegetable farm in Perek</td>
<td>October 2016</td>
<td>3</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Antibiotic profile of <em>L. monocytogenes</em></th>
<th>Susceptible (%)</th>
<th>Intermediate (%)</th>
<th>Resistant (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin (10 μg)</td>
<td>58 (100)</td>
<td>-</td>
<td>-</td>
<td>58 (100)</td>
</tr>
<tr>
<td>Penicillin G (10 Unit)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Erythromycin (15 μg)</td>
<td>1 (1.7)</td>
<td>57 (98.3)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rifampicin (5 μg)</td>
<td>8 (13.8)</td>
<td>26 (44.8)</td>
<td>24 (41.4)</td>
<td>25 (42.1)</td>
</tr>
<tr>
<td>Gentamicin (10 μg)</td>
<td>53 (91.4)</td>
<td>4 (6.9)</td>
<td>1 (1.7)</td>
<td>-</td>
</tr>
<tr>
<td>Tetracycline (30 μg)</td>
<td>17 (29.3)</td>
<td>37 (63.8)</td>
<td>6 (10.9)</td>
<td>-</td>
</tr>
<tr>
<td>Ciprofloxacin (5 μg)</td>
<td>5 (8.6)</td>
<td>50 (86.2)</td>
<td>3 (5.2)</td>
<td>2 (3.5)</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole (1.25/ 23.75 μg)</td>
<td>49 (84.5)</td>
<td>41 (70.7)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
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...
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. Amoxicillin, ampicillin, 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., 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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