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Antibiogram Pattern among Cultures of *Listeria monocytogenes* Isolated from Frozen Burger Patties in Malaysia

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ABSTRACT

Forty-one isolates of *Listeria monocytogenes*, which were obtained from raw burger patties, were tested for their susceptibility against eleven antibiotics by using standard disc diffusion method. In particular, 31.7% of the isolates were found to be not resistant to any of the antibiotic tested while the rest showed resistance to at least one antibiotic. The result showed that resistance to tetracycline was the most common (46.3%), followed by erythromycin (36.6%), amikacin (31.7%), and sulfamethoxazole-trimethoprim (17.1%). All the isolates of *Listeria monocytogenes* were sensitive towards imipenem and gentamicin. The findings of the present study revealed the presence of multidrug-resistant *Listeria monocytogenes* isolates in the processed meat products and hence suggested the emergence of antibiotic resistance in bacterial strains in the food chain.

Keywords: *Listeria monocytogenes*, antibiotic, susceptibility, resistance, dendrogram pattern, standard disc diffusion method

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INTRODUCTION

Listeria monocytogenes is transmitted to human through contaminated food. Ingestion of this bacterium may cause severe adverse health effects to a group of well-defined high risk people such as pregnant woman, neonates and elderly. Researchers defined those infected individuals with altered or deficient immune system, or

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contracted with non-invasive febrile gastroenteritidis as particularly at higher risk for *listeria* infection (Dalton *et al*., 1997; Heitmann *et al*., 1997; Aureli *et al*., 2000). However, mild to moderate symptoms will also be manifested in healthy adults when the ingested dose is high, i.e. approximately 10-100 million CFU (Farber *et al*., 1996). The non-specific flu-like symptom is always complicated with other illnesses, causing it be probably under-diagnosed and eventually leading to fatality case.

Thus, early diagnosis is important so that appropriate antibiotic treatment can be applied to cure *listeria* infection before the occurrence of more serious consequences. Jones and MacGowan (1995) asserted that *L. monocytogenes* is generally susceptible to all antibiotics. The selection of the antibiotic for listeriosis therapy was further narrowed down by Rota *et al*. (1996) and Teuber (1999), who later specified some antibiotics that are commonly used, including ampicillin, penicillin, trimethoprim, tetracycline, erythromycin, and gentamicin.

Despite the fact that there are many reports published on the susceptibility of *L. monocytogenes* against antibiotics, Charpentier *et al*. (1999) pointed out that the first antibiotic-resistant strains of *L. monocytogenes* were reported as early as 1988. In this particular case, tetracycline resistance was the first encountered antibiotic resistance in *L. monocytogenes* (Poyart-Salmeron *et al*., 1990). Since then, Charpentier and Courvalin (1999) have reported the antibiotic resistance of particular isolates from sporadic clinical cases, food, or environment towards antibiotics. The researchers further added that the emergence of antibiotic resistant bacterial strains had been accelerated by the selective pressure caused by over-prescription of drugs in clinical settings and heavy use as growth promoters in livestock husbandry. Meanwhile, the emergence of antibiotic-resistant *L. monocytogenes* strains implicates the possibility of clinical treatment failure for listeriosis in future.

Antimicrobial susceptibility testing has emerged as one of the effective tools to provide *in vivo* prediction on the success or failure of an antibiotic therapy (Govan, 2006). Besides, antimicrobial susceptibility testing also determines the relatedness of a group of isolated bacterial strains based on their antibiotic resistant pattern. Numerous researchers have used this useful epidemiological marker because it is simple to perform and less time-consuming (Aureli *et al*., 2003; Yucel *et al*., 2005; Arslan & Ozdemir, 2008; Harakeh *et al*., 2009).

The objective of this study was to determine the susceptibility of *L. monocytogenes* isolates against eight groups of antibiotic, and cluster them according to their resistant patterns.

MATERIALS AND METHODS

Isolation of L. monocytogenes

Forty-one isolates of *L. monocytogenes* were obtained from retail burger patties. These isolates were collected from each positive palcam agar plate (MERCK, Germany), in which typical presumptive *L. monocytogenes*

colonies were subcultured onto tryptic soy agar (TSA; MERCK, Germany) and confirmed using PCR assay (Wong *et al*., 2011). The number of isolates collected from each sample was determined by the level of *L. monocytogenes* contamination (Chasseignaux *et al*., 2001). At low level, only one isolate could be obtained, whereas at high levels, up to four isolates were collected. In this study, fifteen strains and eighteen strains of *L. monocytogenes* were obtained from beef and chicken patties purchased from supermarket, respectively, while eight other strains were obtained from vegetarian burger patties purchased from a retail shop.

Antimicrobial Susceptibility Testing (AST)

This was performed using Standard Disc Diffusion method according to NCCLS standard reference procedure (National Committee for Clinical Laboratory Standards, 1993). For this purpose, a total of eleven antibiotics were used: ampicillin (10 µg); penicillin-G (10 U); imipenem (10 µg); vancomycin (30 µg); amikacin (30 µg); gentamicin (10 µg); tetracycline (30 µg); erythromycin (15 µg); chloramphenicol (30 µg); rifampicin (5 µg); sulfamethoxazole-trimethoprim (1.25/23.75 µg). Meanwhile, the antimicrobial impregnated discs were purchased from Oxoid (England).

All the isolates were revived in tryptic soy broth (TSB; Merck, Germany) containing 0.6 % (w/v) yeast extract at 37°C for 24 hours (Yucel *et al*., 2005). The inoculums were swabbed using sterile swabs onto Mueller-Hinton agar (MERCK, Germany) to form a uniform lawn of *L. monocytogenes* on the surface of agar. It is important to note that *Listeria monocytogenes* ATCC 19155 and *Staphylococcus aureus* ATCC 25923 were used as reference strains in this study. The antimicrobial impregnated discs were then dispensed onto the agar using Antibiotic Disc Dispenser. The plates were incubated at 37°C for 48 hours.

To determine the antibiogram pattern of an isolate, the diameter of the inhibition zone was measured to the nearest millimetre. Each isolate was classified as resistant (R), intermediate (I) or susceptible (S), to the antibiotics, using NCCLS guidelines (2004). Intermediate-resistant isolates were classified together with resistant isolates for further interpretation of the data (Wayne, 2006).

Multiple Antibiotic Resistance (MAR) Indexing

Each isolate was assigned an MAR index, as defined by Krumperman (1983):

$$\text{MAR index} = \frac{a}{b}$$

a = Number of antibiotics to which the particular isolate was resistant;
b = Number of antibiotics to which the particular isolate was exposed.

Antibiotic Resistance Clustering Analysis

The associations of the resistant patterns among all the tested isolates were analyzed and computed using the software BioNumerics Version 4.5 (Applied Maths, Belgium). Meanwhile, Pearson correlation coefficient and unweighted average linkage

(UPGMA) were employed to construct a dendrogram for clustering analysis. Susceptible results were coded as '0' and resistant results were coded as '1'.

Statistical Analysis

A statistical analysis was performed using SPSS version 16.0. Friedman test was used to determine whether there is significant difference in the percentages of the antimicrobial resistance levels among the different antibiotics used, followed by a post hoc Nemenyi test.

RESULTS AND DISCUSSION

Results for the susceptibility rates of *L. monocytogenes* isolates against eleven antimicrobial agents are shown in Table 1 and Table 2. All the isolates were found to be sensitive towards both imipenem and gentamicin. More than 50% of the isolates tested showed susceptibility towards all the antibiotics. Meanwhile, the antibiotics other than tetracycline, erythromycin, and amikacin were found to have exhibited effective inhibition on the growth of more than 80% of *L. monocytogenes* isolates.

Resistance pattern of forty-one isolates of *L. monocytogenes* towards eight classes of antibiotic is illustrated in Fig.1. More isolates were observed to be significantly resistant to tetracycline (46.3%) and macrolide (36.6%) than to the rest of the antibiotic classes tested in this study ($P<0.05$). The isolates of *L. monocytogenes* were moderately resistant to both sulfamethoxazole-trimethoprim (SMZ-TMP) and aminoglycoside. Surprisingly, there were small percentages of isolates that were resistant towards ß-lactams (2.4%), glycopeptides (2.4%) and rifamycins (9.8%) which are often used in the treatments of the listeriosis manifestation.

Table 3 shows the antibiotic resistant patterns resulted from all strains, together with the percentage of the isolates within the same pattern. It is worthy to note that 31.7% (13/41) of *L. monocytogenes* isolates were affected by all types of antibiotics

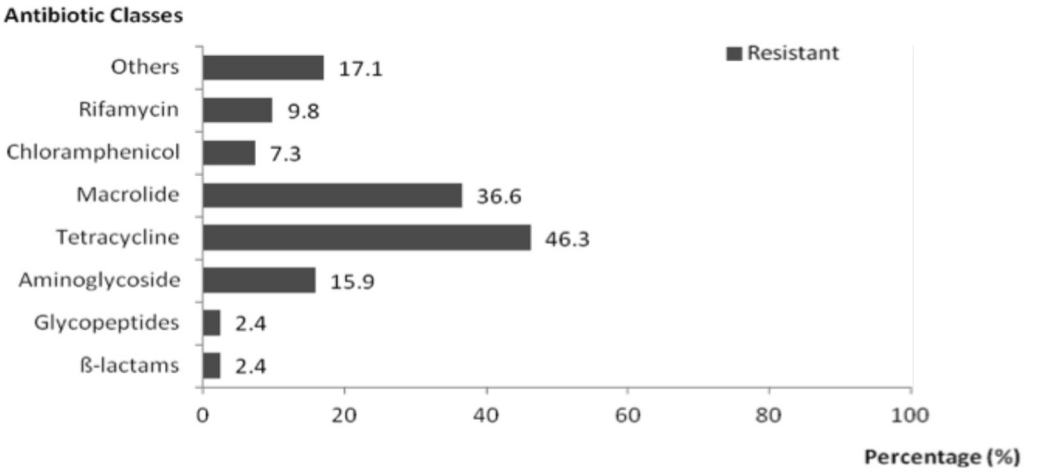

Fig.1: The percentage of *Listeria monocytogenes* isolates resistant to different classes of antibiotic

TABLE 1
Resistant/susceptibility pattern of *Listeria monocytogenes* isolates towards different antimicrobial agents.

Antibiotic classes	Antimicrobial agents (Concentration)	Activity (Prevalence, %)	
		Resistant	Susceptible
ß-lactams	Ampicillin (10 µg)	2 (4.9)	39 (95.1)
ß-lactams	Penicillin (10 U)	1 (2.4)	40 (97.6)
ß-lactams	Imipenem (10 µg)	0 (0)	41 (100)
Glycopeptides	Vancomycin (30 µg)	1 (2.4)	40 (97.6)
Aminoglycoside	Gentamicin (10 µg)	0 (0)	41 (100)
Aminoglycoside	Amikacin (30 µg)	13 (31.7)	28 (68.3)
Tetracycline	Tetracycline (30 µg)	19 (46.3)	22 (53.7)
Macrolides	Erythromycin (15 µg)	15 (36.6)	26 (63.4)
Chloramphenicol	Chloramphenicol (30 µg)	3 (7.3)	38 (92.7)
Rifamycin	Rifampicin (5 µg)	4 (9.8)	37 (90.2)
Others	SMZ-TMP (1.25 + 23.75 µg)	7 (17.1)	34 (82.9)

TABLE 2
The percentage of resistant strains from different sources (%; number of isolate)

Antibiotic	Beef patties	Chicken patties	Vegetarian patties
Ampicillin	50.0 (1)	50.0 (1)	0
Penicillin	100 (1)	0	0
Vancomycin	100 (1)	0	0
Amikacin	61.5 (8)	0	38.5 (5)
Tetracycline	63.1 (12)	5.3 (1)	31.6 (6)
Erythromycin	60.0 (9)	13.3 (2)	26.7 (4)
Chloramphenicol	66.7 (2)	33.3 (1)	0
Rifampicin	100 (4)	0	0
SMZ-TMP	42.8 (3)	42.8 (3)	14.3 (1)

tested in this study. These isolates were obtained from chicken burger patties (12/41) and vegetarian burger patty (1/41). Eight (19.5%) out of 41 *L. monocytogenes* isolates showed resistance to single antibiotic, while 48.7% of the *L. monocytogenes* isolates demonstrated multiple antibiotic resistances (i.e. resistance to at least two antibiotics).

A dendrogram obtained from the clustering analysis is shown in Fig.2, to compare the resistant patterns among *L. monocytogenes* isolated from different sources. From the dendrogram, all the isolates can be clustered with a cut off value at 70% similarity. Meanwhile, thirty-five out of 41 *L. monocytogenes* isolates were differentiated into five discriminatory clusters, namely, C1, C2, C3, C4, and

TABLE 3
Multiple Antibiotic Resistance (MAR) index and antibiotic resistant pattern of *Listeria monocytogenes* isolated from burger patties.

MAR Index	*Antibiotic Resistant Pattern	Isolate number	Percentage of isolate (%)
0.45	AkERdSTe	13	2.4
0.36	AkCES	6	7.3
	AkEPTe	7	
	AkESTe	19	
0.27	AkERd	5	19.5
	AkETe	1, 4, 17	
	AkEVa	2	
	AmpRdTe	11	
	CSTe	9	
	ERdTe	8	
0.18	AkE	22	19.5
	AkTe	12, 16, 18	
	AmpC	25	
	ETe	10, 20, 27	
0.09	Te	3, 14, 15, 21	19.5
	S	33, 35, 41	
	E	38	
0	-	23, 24, 26, 28, 29, 30, 31, 32, 34, 36, 37, 39, 40	31.7

Ak – Amikacin; Amp – Ampicillin; C – Chloramphenicol; E – Erythromycin; P – Penicillin; Rd – Rifampicin; S – Sulfamethoxazole-trimethoprim; Te – Tetracycline; Va – Vancomycin.

C5. The remaining six isolates were left uncategorized into either group. However, these six single isolates were found to possess similarity with clusters C2 - C5 at 71.2%. The isolates from various sources seemed to cluster together into four clusters with only one exception, i.e. cluster C4, which was only occupied by the isolates from the same source (i.e. chicken burger patties). The finding indicated that the *L. monocytogenes* isolates derived from beef, chicken and vegetarian burger patties were highly correlated to each other, as shown in the dendrogram in Fig.2. This may indicate that the *L. monocytogenes* strains isolated from frozen burger patties taken from the supermarkets and retail shop have been exposed to a similar contamination source along the production chain of such product.

Antibiotic or antimicrobial agents are defined as chemical compounds which are either synthesized or derived from natural sources and influence the growth of bacteria. In clinical perspective, it is essential for

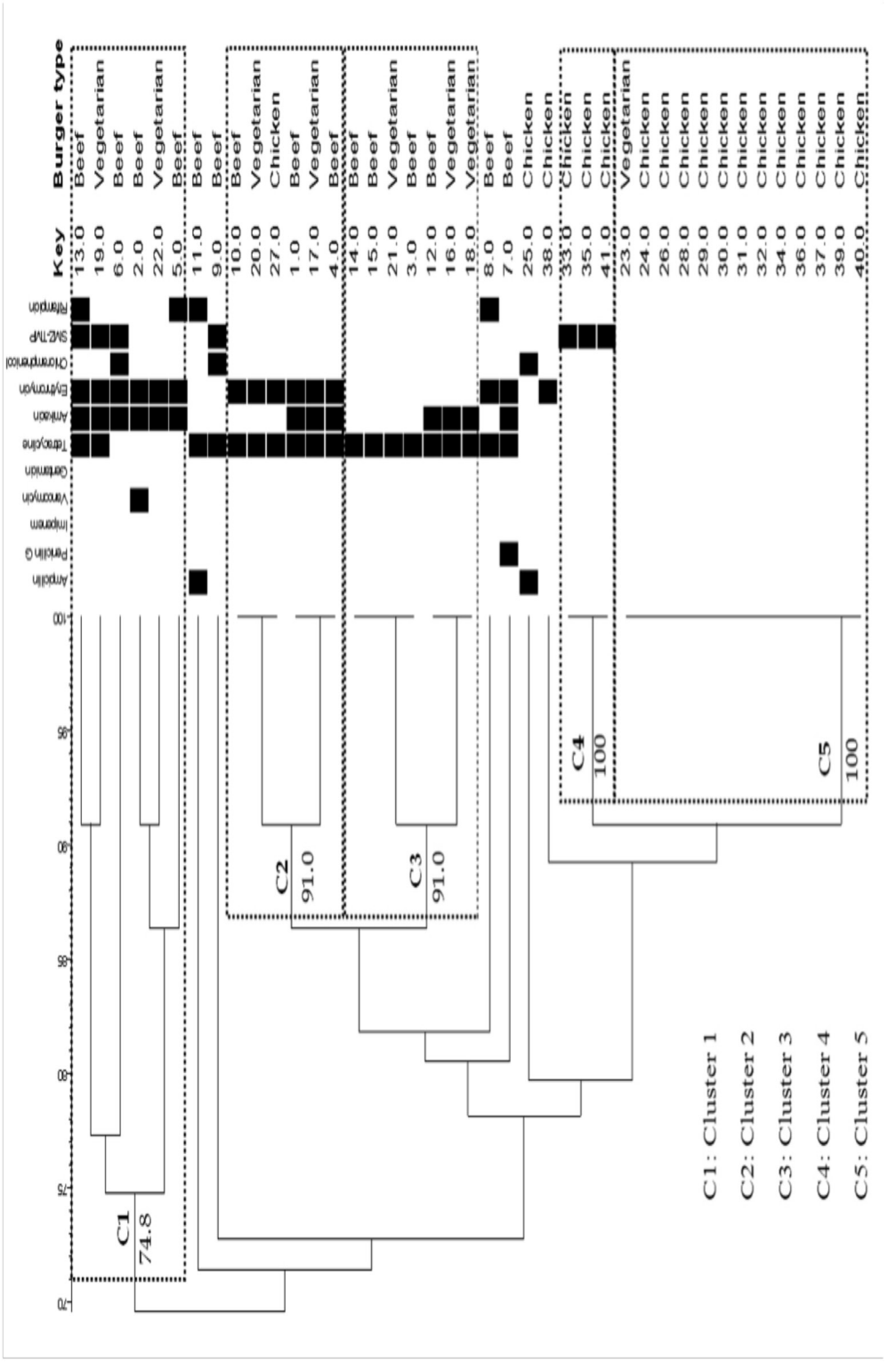

Fig.2: Dendrogram showing the resistance profile using Pearson/UPGMA clustering

treatment of bacteria-causing infectious disease since its discovery. However, due to emergence of bacterial resistant strains, many of the available antibiotics are no longer useful. Antibiotic resistance of bacterial strains is in the realm of public health issues that are of all peoples' concern. All in all, the resulting data from the present study have suggested that the overall incidence of the antibiotic resistance in *L. monocytogenes* is still relatively low. This also reveals that most of the antibiotics used are powerful agents against the tested strains.

From this study, all the isolates were found to be sensitive to at least two antibiotics, namely gentamicin and imipenem. This phenomenon was observed to be uniform among the isolates although they were obtained from different sources and locations, as supported by a study which also reported a similar finding (Conter *et al*., 2009). Besides, *L. monocytogenes* cultures were highly susceptible towards ampicillin and penicillin. In the normal case of listeriosis, the treatment of choice is ampicillin or penicillin G in combination with an aminoglycoside, which is usually gentamicin (Espaze & Reynaud, 1988; Franco Abuin *et al*., 1994; Jones & MacGowan, 1995). High efficacies of these antibiotics against *L. monocytogenes* strains in this *in vivo* result may suggest successful antibiotic treatment for patients. Thus, these antibiotics remain applicable in clinical settings.

SMZ-TMP is the antibiotic used as a second choice therapy for listeriosis, especially for patients who are allergic to ß-lactams (Boisivon *et al*., 1990; MacGowan *et al*., 1990; Lorber, 1997). However, it was found to be ineffective to 17.1% of *L. monocytogenes* isolates in this study. This finding supports other results reported elsewhere (dated back in last five years), whereby the percentages of resistance to SMZ-TMP ranged from 1.6% to 66% (Yucel *et al*., 2005; Arslan & Ozdemir, 2008; Harakeh *et al*., 2009; Conter *et al*., 2009). The emergence of SMZ-TMP resistance strains is of particular importance and it requires more attention since this antimicrobial drug is a successful alternative treatment for listeriosis in human beings.

It is important to note that tetracycline and erythromycin are the most common antibiotics which have shown reduced sensitivity against *L. monocytogenes* isolates, with a prevalence of 46.3% and 36.6%, respectively. This finding is comparatively higher than other published findings. Only 2.4% of *L. monocytogenes* has been reported to exhibit intermediate-resistance towards erythromycin (Conter *et al*., 2009). Similarly, Aureli *et al*. (2003) and Conter *et al*. (2009) reported low prevalence (0–0.8%) of tetracycline resistance of food borne *L. monocytogenes* isolated from food in Italy. Nonetheless, Walsh *et al*. (2001) substantiated that *L. monocytogenes* isolated from various retail foods in Northern Ireland was most commonly resistant to tetracycline. Hence, it is possible to suggest that the isolates from different geographic location

may pose different levels of tetracycline resistance, as noted in the present and other studies. In addition, Chai (2007) speculated that the comparatively lower prevalence of erythromycin and tetracycline resistance in some countries might be due to controlled usage of antimicrobial drugs in the livestock industry and in hospitals. In fact, Denmark, United Kingdom, United States of America and a few other advanced countries have banned the application of tetracycline in agricultural practices (Chai, 2007).

In this study, 48.7% of *L. monocytogenes* isolates demonstrated multiple antibiotic resistances. These isolates were resistant to at least two of the eleven antibiotics tested. Any bacterial strain exhibiting MAR index values lower than 0.2 is deemed to be originated from animals, in which the antibiotics are seldom or never used (lower risk). Meanwhile, the isolates having MAR index values higher than 0.2 are regarded as originating from higher risk sources of contamination such as from animal farms which are greatly exposed to antibiotics. Twelve out of forty-one isolates of *L. monocytogenes* had the MAR index greater than 0.2 (0.27 – 0.45) and therefore, it might be evident that their origin would have been from a high risk source of contamination. Hence, it was not surprising to find 83.3% of these high MAR index isolates originated from the beef patties.

Nowadays, many drugs, antibiotics, and hormones are applied in the livestock industry with the aims to raise cows that can produce more milk and to maximize the return profits from their meat. Based on this observation, it can therefore be assumed that the application of antimicrobial drugs in the poultry farm is much lesser than that in the cow's farm. Recently, Vinesh (2010) reported the usage of herb-based supplement by a local poultry rearing industry to replace antibiotic. The herbs are deemed to boost up chicken's immune system, revitalize their reproductive organs, and improve their digestive system.

Cluster analysis is useful for clustering a set of bacterial isolates corresponding to the dynamic of resistance (Chai, 2007). It is of particular significance in showing epidemiological relatedness and association among isolates with different variables and outputs. In this study, the isolates of *L. monocytogenes* from different sources and locations were classified into five clusters. Nonetheless, there was no significant pattern observed between these clusters. Hence, this might suggest that the isolates could have originated from a similar contamination source. The acquisition of antibiotic resistance genes in *L. monocytogenes* might have occured at the early stage before they were processed into respective products. It would appear that the spread of antibiotic resistance genes might have occurred among *L. monocytogenes* hosted in animals (cow and chicken) in farm and vegetations.

The issue of bacterial resistance to antimicrobial drugs is not only limited to the food industry, animal farms, nor clinical settings, but it also appears to be a global problem. It gives impacts to both industrialized and resource-poor countries since bacterial resistance implicates the

difficulty to treat both community and hospital-acquired infections; this leads to increase the rate of morbidity and mortality, and eventually results in great economic loss to a country. However, there is scarcity of studies which anticipate the cost of antibiotic resistance, particularly in terms of increased morbidity, mortality or cost to hospitals or the society (Liss & Batchelor, 1987; Andersson & Hughes, 2010).

CONCLUSION

The results of this study suggest that the food isolates of *L. monocytogenes* are mostly susceptible to commonly used antibiotics in veterinary and human listeriosis therapy. However, manifestation of resistance towards some antibiotics could be addressed as this might be the sign where *L. monocytogenes* gradually became antibiotic resistant by acquiring potential antibiotic resistant genes from environmental background gram-positive bacteria. To the best of the authors' knowledge, this is the first study carried out on antimicrobial susceptibility testing on *L. monocytogenes* strains isolated from processed meat in Malaysia. A continuous programme focusing on antimicrobial resistance of *L. monocytogenes* is of necessity to assure the effectiveness of listeriosis treatment. Besides, it will also provide insights into the problems of overuse and/or misuse of antimicrobial agents in human and veterinary medicine.

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