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Detection, isolation and antimicrobial testing of *Listeria monocytogenes* in chicken from wet markets in Terengganu, Malaysia

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This study was conducted to determine the prevalence of *Listeria monocytogenes* in chicken from wet markets and its antibiotic susceptibility. A total of 48 chicken parts samples were collected from three districts in Terengganu which are Besut, Kuala Terengganu and Setiu. Samples were enriched with Listeria Enrichment Broth (LEB) and incubated at 30ºC for 24 h. DNA extraction was done onto the enriched samples by boiled- cell method. Next, the detection of *L. monocytogenes* was performed using real- time PCR. Isolation of *L. monocytogenes* was done using selective PALCAM agar. From 48 chicken meat tested, 16.7% of the meats were detected to harbour *L. monocytogenes*. Six *L. monocytogenes* isolates were successfully recovered from the chicken samples. The highest resistance of the isolates was observed towards vancomycin (100%) and most susceptible to enrofloxacin (12.5%). All the isolates were found to have multiple resistance towards antibiotics since the Multiple Antibiotic Resistance (MAR) index was more than 0.2. In conclusion, *L. monocytogenes* was found in the chickens from wet markets and it can pose a risk of food poisoning from undercooked and contaminated chickens**.**

Keywords: *L. monocytogenes*, real- time PCR, DNA extraction, antibiotic resistance, wet market

INTRODUCTION

L. monocytogenes is an emerging food borne pathogen that can be transmitted through food (Saha et al. 2015). Listeriosis which is caused by *L. monocytogenes* can cause mortality. The listeriosis severity can range from mild gastroenteritis to severe disease conditions such as septicemia, encephalitis, meningitis, abortions, stillbirth and can even cause mortality (Zhu et al*.* 2017). Immunocompromised individuals, pregnant women, newborns and elders are more susceptible to listeriosis (WHO, 2019). The symptoms are varied depending on the health status of the infected person. For example, for immunocompromised individuals, symptoms can include fever, muscle aches, headache, stiff neck, confusion, loss of balance and convulsions. Meanwhile for pregnant women, they typically experience fever, chills and headaches. Healthy individuals usually get sick with mild symptoms and can usually heal over time without the need for an antibiotic prescription (WHO, 2018).

The published information of *L. monocytogenes* from meat, milk and dairy products from all over the world is very scattered and unsystematic, both in veterinary and public health sectors (Khan et al. 2013). As compared to prevalence studies on beef, pork and chicken meat, the highest number of *L. monocytogenes* was found in chicken for about 15.5% (Ochiai et al. 2010). However, different findings from other studies where beef had the highest number of *L. monocytogenes,* compare to other types of meat which is 8.33% and 34.8% (Mashak et al. 2015; Wu et al. 2015).

Malaysian has the highest poultry consumers in the world with 35.3 kg consumption per- capita in 2011. From 100% of the total livestock production in Malaysia during 2010, 53.2% of it came from poultry production (Mohd et al. 2015). In European countries, over 100 million pounds of antibiotics are used in the animal feed per year as estimated by FDA. For example, antibiotics are needed to promote growth of poultry and prevent it from enteric diseases (Poultry Hub, 2018). In the last decades, *L. monocytogenes* has become resistant to many types of antibiotics. Risk of infection for the population need to be prevented by the continuous study on the incidence of listeriosis and its emerging resistances for the effectiveness of antibiotics treatment on listeriosis (Pesavento et al. 2010).

A present study aimed to determine the prevalence of *L. monocytogenes* in chicken from wet markets in Terengganu and also antibiogram profiles of *L. monocytogenes* isolates.

MATERIALS AND METHODS

Sampling of chicken sample

A total of 48 chicken part (thigh, breast, wing, drumstick) from six (6) different location of wet markets were collected from three Terengganu districts. All the samples were transported to the laboratory in an ice box and analyzed within 24 h during sampling.

Sample enrichment and isolation of *L. monocytogenes*

A 10 g of chicken sample was aseptically weighed and added to 90 ml of Buffered Listeria Enrichment Broth (BLEB) (Merck, Germany) in a sterile stomacher bag and homogenized using stomacher machine (Seward, UK) for 1 min at 250 rpm. For the enrichment step, the homogenized solution was incubated for 24 h at 30ºC. The enriched sample was streaked on PALCAM selective agar plates (Oxoid, CM0877) and it was incubated at 30ºC for 48 h. After incubation, the presumptive colonies formed on the agar was picked about 2 to 3 colonies and they were re-streaked on PALCAM agar for the purification step. The plates were incubated once again at 30ºC for 24 h and the pure colony obtained was confirmed using real- time PCR method.

DNA extraction and detection using real- time PCR

DNA extraction was done by boiling cell method in which 1 ml of enriched sample was centrifuged at 10,000 *xg* to pellet the cells. The supernatant was discarded and the pellet was re-suspended with sterile TE Buffer in a 1.5 ml micro centrifuge tube. The suspension was boiled at 100ºC for 10 min using a digital dry bath (Corning, Japan). The mixture immediately cooled at -20ºC for 10 min before undergone centrifugation for 10 min at 10,000 *xg*. The supernatant contains DNA was used as a DNA template. The PCR amplification was performed in 15 µl reaction mixtures containing 7.5 µl SYBR Green,0.45 µl forward primer (5'-CCT AAG ACG CCA ATC GAA 3'), 0.45 µl reverse primer (5'-AAG CGC TTG CAA CTG CTC3'), 5.6 µl nuclease- free water and 1 µl DNA template. All the PCR reagents were purchased from Fermentas, Lithuania. Thermal cycling was carried out in real-time PCR machine (Corbet RotorGene TM 6000, Sydney) by using the thermocycler condition for 40 cycles: initial denaturation at 95°C for 10 min, denaturation at 95°C for 20 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s.

Antimicrobial testing

The disc diffusion method described by Napisah et al (2011) with slight modification was used to measure the antimicrobial resistance on Mueller-Hinton agar (MHA). The disc contained appropriate concentrations of different antibiotics which include: vancomycin (30mcg), penicillin G (5iu), kanamycin (30mcg), streptomycin (25mcg), rifampicin (5mcg), tetracycline (30mcg), erythromycin (15mcg), ceftazidime (10mcg), ciprofloxacin (5mcg), clindamycin (2mcg), ampicillin (10mcg), amikacin (30mcg), enrofloxacin (5mcg), and norfloxacin (5mcg). Using a sterile cotton swab, the *L. monocytogenes* isolates were swabbed evenly on MHA. The plates were then incubated at 30ºC for 24 h. After incubation, the zones of inhibition surrounding each disc were observed.

Multiple antibiotic resistance (MAR) indexes

Each resistant isolate was calculated for MAR index using the formula:

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

Where, a: number of antibiotics which isolate was resistant to

b: number of antibiotics tested on isolate

THE RESULTS

A total of 48 chicken part samples were examined for the prevalence of *L. monocytogenes* by using plating method and real-time PCR assay. The prevalence of *L. monocytogenes* was eight positives out of 48 (16.7%) samples using PCR method and six positives out of 48 (12.5%) samples using the plating method.

Table 1: Prevalence of *L. monocytogenes* isolates in chickens from the wet market based on molecular PCR and plating methods.

Parts of chicken	District	N	Molecular PCR n (%)	Conventional Plating n (%)
Wing	Besut	6	1 (8.3)	0
	Kuala Terengganu	4	0	0
	Setiu	2	0	0
Breast	Besut	6	0	0
	Kuala Terengganu	4	0	0
	Setiu	2	0	0
Drumstick	Besut	6	2 (16.7)	1 (8.3)
	Kuala Terengganu	4	0	0
	Setiu	2	1 (8.3)	1 (8.3)
Thigh	Besut	6	1 (8.3)	1 (8.3)
	Kuala Terengganu	4	2 (16.7)	2 (16.7)
	Setiu	2	1 (8.3)	1 (8.3)
	Total	48	8 (16.7)	6 (12.5)

The prevalence of *L. monocytogenes* in chicken samples is tabulated in Table 1. Based on the data obtained in Table 1, the study revealed that the presence of *L. monocytogenes* in chicken parts was highest in the thigh (33.3%) followed by drumstick (25.0%), wing (8.3%), and breast (0%).

Table 2 shows the result of antimicrobial susceptibility test using disk diffusion assay. *L. monocytogenes* isolates showed 100% resistance to Vancomycin, followed by Erythromycin, Ampicillin and Tetracycline (75%), Rifampicin (62.5%), Clindamycin, Streptomycin and Ceftazidime (50%), Penicillin G and Ciprofloxacin (37.5%), Norfloxacin and Kanamycin (25%), and lastly Enrofloxacin and Amikacin (12.5%). Table 3 summarizes MAR index of *L. monocytogenes.* All *L. monocytogenes* isolates have shown MAR index of more than 0.2. The highest MAR index was 0.89 and the isolates were from B6-thigh parts while the lowest MAR index was 0.29 from SU-thigh and PP- thigh parts.

Table 2: Antimicrobial Susceptibility Test of *Listeria monocytogenes* isolated from chicken from wet markets

Antibiotics	Disk content	N	Resistant, n (%)	Susceptible, n (%)
Penicillin G	5 iu	8	3 (37.5)	5 (62.5)
Ciprofloxacin	5 mcg	8	3 (37.5)	5 (62.5)
Norfloxacin	5 mcg	8	2 (25.0)	6 (75.0)
Vancomycin	30 mcg	8	8 (100.0)	0 (0)
Enrofloxacin	5 mcg	8	1 (12.5)	7 (87.5)
Erythromycin	15 mcg	8	6 (75.0)	2 (25.0)
Kanamycin	30 mcg	8	2 (25.0)	6 (75.0)
Clindamycin	2 mcg	8	4 (50.0)	4 (50.0)
Rifampicin	5 mcg	8	5 (62.5)	3 (37.5)
Streptomycin	25 mcg	8	4 (50.0)	4 (50.0)
Ceftazidime	10 mcg	8	4 (50.0)	4 (50.0)
Amikacin	30 mcg	8	1 (12.5)	7 (87.5)
Ampicillin	10 mcg	8	6 (75.0)	2 (25.0)
Tetracycline	30 mcg	8	6 (75.0)	2 (25.0)

Table 3: Antimicrobial resistance profile for *L. monocytogenes* isolates obtained from chicken in the wet markets (n=8)

MAR index	Antibiogram	Isolates	No. of isolates (%)
0.89	Va, P, K, S, RD, TE, E, CAZ, DA, AMP, ENR, NOR	B6-Thigh	1 (12.5)
0.64	Va, S, RD, TE, E, CIP, DA, AMP, NOR	KB-Drumstick	1 (12.5)
0.5	Va, RD, TE, E, CIP, DA, AMP Va, S, RD, E, CAZ, CIP, AMP	KB-Thigh KR-Wing	2 (25.0)
0.43	Va, P, RD, TE, AMP, AK Va, P, TE, E, CAZ, DA	KR-Drumstick SU-Drumstick	2 (25.0)
0.29	Va, S, RD, TE Va, K, CAZ, AMP	SU-Thigh PP-Thigh	2 (25.0)

TE Tetracycline, P Penicillin G, CIP Ciprofloxacin, NOR Norfloxacin, Va Vancomycin, ENR Enrofloxacin, E Erythromycin, K Kanamycin, DA Clindamycin, RD Rifampicin, S Streptomycin, CAZ Ceftazidime, AK Amikacin, AMP Ampicillin

DISCUSSION

The highest prevalence of *L. monocytogenes* was found in thigh parts and it was in agreement with the previous study which reported *L. monocytogenes* in the breast was 8.64% while in the thigh being 44.19% (Schafer et al. 2017). They concluded that the high contamination from thigh parts might be due to the different cutting process where the cutter used for thigh was more cross- contaminated compared to a breast cutter. The thigh cutter is more frequently used to cut bones compared to the breast which consists of lean meat and few tiny bones (Schafer et al. 2017). Besides, the position of thigh and drumstick which is the nearest to the cloaca might be the reason why they have the highest prevalence of *L. monocytogenes.* Feces can contaminate the carcass meat (Kalender, 2003). It was found that ≤25% of litter samples were positive for *L. monocytogenes* which proved that poultry could shed *L. monocytogenes* in fecal material which will eventually contaminate the environment (Rothrock et al. 2017).

The low prevalence of *L. monocytogenes* in chicken samples (Table 1) was in line with previous studies on South East Asia region where *L. monocytogenes* were isolated from chicken offal and chicken meat, ranges from 8% to 26.39% (Goh et al. 2012; Kuan et al. 2013; Indrawattana et al. 2013; Sugiri et al. 2014; Islam et al. 2016; Mahantesh et al. 2017; Ahmed et al. 2017; Oh et al. 2018).

Poultry, slaughtered poultry and its product were a common reservoir for foodborne pathogen which includes *L. monocytogenes* (Kuan et al. 2013; Oliveira et al. 2018; Hertanto et al. 2018). Hertanto et al. (2018) reported live poultry is 'sterile' from bacteria but during the slaughtering process, the contamination sources from its alimentary tracts, water, packaging, utensils and food handlers occurred. Besides, chicken meat has a high water activity of more than 0.98 and pH ranges from 6.26 to 6.30 which is almost neutral thus become favorable conditions for bacteria growth (Hertanto et al. 2018). To reduce the multiplication of spoilage microorganism and pathogenic bacteria on fresh foods, the foods need to be kept at temperature lower or minimal 5ºC (Yaacob and Anuntagool, 2019). From previous studies, the source of *Listeria* contamination in raw poultry products was identified to come from the surrounding environment in poultry processing plants. The plants consisted of various equipment such as scalding tanks, slaughtering and defeathering machines which provided surfaces for the attachment of *Listeria* (Sasaki et al. 2014; Regenstein and Singh, 2018).

The psychrotrophic characteristic of *L. monocytogenes* helps it to continue to survive and proliferate even under frozen temperature for long periods (Tang et al. 2017; Olaimat et al. 2018). Besides, the nature of *L. monocytogenes* which enables it to grow at a temperature above than 45ºC, in a wide range of pH from 4.0 to 9.6 and minimum water activity of 9.0 ensure the survival of *L. monocytogenes* under extreme conditions (Mohd Noor and Tang, 2019). *L. monocytogenes* can overcome physical, chemical and biological stresses during food processing by regulating the normal functions of its bacterial cell to stay alive (Olaimat et al. 2018).

From previous studies, chicken samples from supermarket had shown higher prevalence (25.71% and 43.8%) compared to wet market (14.29% and 14.6%) due to different holding time and processing methods of chickens before being on sale (Goh et al. 2012; Tang et al. 2018). Chopping board, mincing machine and cleaning cloths might be the sources of contamination during chicken processing chain (Mahmood et al. 2003). As mentioned by Oliveira et al. (2018) packed chicken cuts had higher *L. monocytogenes* contamination compared to the chicken carcass, 11.3% over 6.7% due to cross-contaminated with the packaging, equipment, utensils and environment.

The holding time of chickens from supermarkets was longer because they were stored in a cold room first before being displayed on ice while wet markets commonly sold fresh chicken slaughtered on the same day. Yet, to preserve the freshness of chicken, it was kept on ice before going on sale. Increased holding time also caused the number of *L. monocytogenes* to increase too (Kuan et al. 2013). Sudin and Tang (2018), reported the main cause of cross-contamination food occurred at supermarkets was caused by mishandling and poor hygiene. A previous study also proved that the prevalence of *L. monocytogenes* was much higher in frozen poultry meat and poultry products (21.67%) compare to fresh poultry meat (11.25%) (Mahmood et al. 2003).

For antimicrobial testing, the findings are not correlated with previous studies by Sakaridis et al. (2011) whereby only a few of *L. monocytogenes* isolated from chicken were resistant to tetracycline (12.72%) and all of them were susceptible to the antimicrobials prescribed to treat human listeriosis. The isolates were also susceptible to ampicillin, ciprofloxacin, enrofloxacin, gentamicin, kanamycin, penicillin, streptomycin and vancomycin (Sakaridis et al. 2011). Penicillin and ampicillin are the most popular drugs used to treat patient with listeriosis as they have beta- lactam ring in their molecular structure (Ruiz- Bolivar et al. 2011). This beta-lactam structure will inhibit the synthesis of bacterial cell wall peptidoglycan therefore, most Gram-positive bacteria including *L. monocytogenes* are naturally sensitive to this β-lactams antibiotics (Sanlibaba et al. 2018). However, from antibiotic susceptibility data tested on *L. monocytogenes* isolated from RTE foods which include cooked chicken product (24%), all of the isolates posed resistant towards β- lactams antibiotics (Sanlibaba et al. 2018).

Some other common antibiotics used for listeriosis include vancomycin, erythromycin, tetracycline and chloramphenicol (Ruiz- Bolivar et al. 2011). However, from Table 2, these antibiotics were not effective against *L. monocytogenes* isolates due to high resistance. Previous studies reported high resistance of *L. monocytogenes* against vancomycin which is from 50- 74.8% (Ieren et al. 2013; Obaidat et al. 2015; Terzi et al. 2015). The resistance result of ciprofloxacin in this study is 37.5% which is quite similar to the previous study; 35.3% (Sanlibaba et al. 2018). Considering tetracycline and ciprofloxacin are not among the listeriosis treatment options and also tetracycline is not suggested to treat children and pregnant women, thus the resistance of *L. monocytogenes* isolates towards these antibiotics is noncrucial (Sanlibaba et al. 2018 and Wilson et al. 2018).

Previous studies stated that *L. monocytogenes* strains from chicken were 92.9% sensitive to streptomycin while 100% sensitivity is shown by rifampicin (Altuntas et al. 2012). In this study, high rifampicin and streptomycin resistance was observed. Alonso- Hernando et al. (2012) did a comparison on *L. monocytogenes* isolates resistance towards enrofloxacin between the years 1996 and 2003 in which the resistance was increased from 23.3 to 68.0% while the present study showed enrofloxacin resistance at 12.5%. Resistance of 75% showed against ampicillin was also not in agreement with previous studies which reported 6.9% and 10.25% respectively (Sugiri et al. 2014; Dan et al. 2015). High erythromycin (75%) resistance which was found in this study was not in agreement with previous antimicrobial studies tested on *L. monocytogenes* isolated from chicken which were 15.3% and 6.9% (Fallah et al. 2012; Sugiri et al. 2014). Based on a documentation from Health Action International Asia Pacific (HAIAP, 2013), erythromycin is widely used for human health and in poultry farms in Malaysia. To prevent antibiotic resistance, WHO had enlisted erythromycin and other drugs under Critically Important Antimicrobials so that the uses of these bacteria in the veterinary sector must be under strict supervision (HAIAP, 2013).

From above comparisons with other *L. monocytogenes* isolated from chicken, we can conclude that *L. monocytogenes* isolated from Terengganu; part of east- coast Malaysia posed higher resistance compared to previous studies except for enrofloxacin that might be caused by excessive use of antimicrobials in chicken feed to decrease the risk of epidemics spread within husbandry which may lead to the selection for antibiotic resistant microorganism. The lower resistance posed by enrofloxacin; a type of fluoroquinolone is probably due to the recommendation of World Organisation for Human Health (OIE) for lesser use of this type of antibiotic to reduce the risk of acquiring fluoroquinolone-resistant *Campylobacter* in poultry (CDC, 2019; Mohamed et al. 2019). The supplemented animal feed can also produce chickens with marketable weight over a short period to increase the profit obtained by the farmers (CDC, 2015; Manyi-loh et al. 2018). For instance, according to the data by OECD, 148 mg of antimicrobials are used to produce 1 kg of chicken compared to beef; 45 mg and pork; 172 mg (Hasali et al. 2018). Asia recorded 31.1% from total of 45 countries still using antibiotics as growth promoters (Gulland, 2019). Although 66.6% of antibiotics were registered for legal use in livestock, some of them were under strict supervision by WHO due to the critical uses in human health such as ampicillin and erythromycin (Hasali et al. 2018).

As aforementioned, due to *L. monocytogenes* being intrinsically resistant to various environmental changes, but over time, the bacteria have evolved to become highly adaptable not just towards the environment but also antibiotics whether by mutation or genetic alteration (Lungu et al. 2011; Olaimat et al. 2018). According to the previous study, MAR index of *L. monocytogenes* isolated from poultry ranging from 0.29 to 0.93 is quite similar to this study (Tang et al. 2018). It was mentioned that the source of isolates had MAR index higher than 0.2 coming from high-risk sources such as humans and farm animals which were regularly exposed to antibiotics (Elexson et al. 2014).

The emergence of antibiotic resistance may cause harm towards an infected patient in the future as antibiotics are the only certified therapy to treat listeriosis as no vaccine has existed to prevent *Listeria* infection at the first place to date (Newman, 2017; Olaimat et al. 2018). Antibiotic-resistance causes minor infections which becomes difficult to treat thus requires additional treatment. Besides, the duration of hospital stay also increase as well as the risk of death (Hasali et al. 2018).

CONCLUSION

The results of this study provide information about the contamination level of *L. monocytogenes* in chicken samples from wet markets. The presence of *L. monocytogenes* in chicken might pose a health risk to consumers especially when it is under-cooked or cross contaminated. Besides, the presence of multidrug resistant *L. monocytogenes* isolates found in this study suggested that controlling the use of antimicrobials is important in reducing the emergence of drug resistant *Listeria* strains.

CONFLICT OF INTEREST

The authors declare no conflict of interest during the research was conducted.

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AUTHOR CONTRIBUTIONS

NMN performed the experiments and wrote the manuscript, JYHT and NAB designed the experiments and reviewed the manuscript. All authors read and approved the final version.

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