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Short communication

Detection, Isolation and Antimicrobial Testing of Listeria monocytogenes in Chicken from Supermarket

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Abstract

Listeria monocytogenes play a vital role in causing an infrequent and sometimes deadly foodborne disease known as Listeriosis. In this study, the presence of *L. monocytogenes* in chicken at supermarket and the antimicrobial susceptibility of the pathogen were determined. Detection of *L. monocytogenes* in chickens from supermarket was carried out using real time PCR and isolation of *L. monocytogenes* by plating onto PALCAM agar. The *L. monocytogenes* isolates were tested against 14 antibiotics for resistance determination using disc diffusion method. From 56 chicken samples tested, 35.7 % of the samples were detected contained the pathogen using real time PCR. Only 15 samples (26.8%) were positive for *L. monocytogenes* isolates. The isolates showed high resistance towards Penicillin G (100%), Ampicillin (93%), Rifampicin (93%), Ceftazidime (87%), Erythromycin (87%), and Tetracycline (80%). All the isolates were found to have the Multiple Antibiotic Resistance (MAR) index more than 0.2 which indicates multiple resistance towards antibiotics. In conclusion, *L. monocytogenes* was found in the chicken at supermarket and the isolates were found to be highly resistance towards multiple antibiotics. Thus, chicken meat must be properly cooked before consumption to prevent foodborne infection.

Keywords: *Listeria monocytogenes, real time PCR, antibiotic resistance, chicken, supermarket.*

1. Introduction

Based on previous research study had summarized that invasive listeriosis is categorized into three types which are bacteremia, neurolisteriosis, and maternal–neonatal infection [1]. Then, from the studies done by few researchers in 2012, they had found several high risks host for bacteremia and neurolisteriosis such as old people, infant and cellular immune defects and immunosuppressive therapies were taken into the considerations [1]. Listeriosis by *Listeria monocytogenes* is a life-threatening infection that happened due to food is contaminated with virulent strains of *L. monocytogenes*. This can be proven with data submitted by World Health Organization in 2002, they had stated that *L. monocytogenes* is the foodborne pathogen responsible for the majority of deaths in the States which second-ranked after non-typhoid *Salmonella* spp. This bacteria become a major source of transmission in contaminated food which it has been reported that the presence of *L. monocytogenes* in many foods as raw and/or pasteurized milk, cheeses, chicken meat, fish, and processed meat products [2, 4].

In Malaysia, as for chicken meat, it is an example of poultry product which is well known for its consumption by consumers. Therefore, the biological protection from *L. monocytogenes* in chicken is important because it may cause heavy worrisome to the public because the majority of Malaysian population consume chicken products. The per capita chicken meat consumption has increased from 38.59 kg in year 2007 to 50.32 kg in year 2016 [3]. *L. monocytogenes* capability to grow at low temperature make it an important pathogen in refrigerated chicken meat [2]. Its growth in chicken meat increase the risk of listeriosis through consumption of undercooked chicken meat [2].

Generally, the poultry may come in distinct forms such as fresh-cut chicken and deli-meat and it is easy to be found either at the retail supermarket in food frozen section. Basically, deli meats have been reported as being the major spread of listeriosis infection in the United States which the hazard goes similarly to RTE product [4]. For the retail supermarket, ready-to-eat meat or minimally processed meat product are usually stored as raw-cut at the food frozen section. Thus, higher detection of *Listeria monocytogenes* can be found especially on the surface of fresh-cut poultry like chicken. They can grow well on the surface because usually in a supermarket, the refrigeration temperature to store fresh-cut poultry product is favorable for the growth of *Listeria monocytogenes* which is around 4° C and below. *L. monocytogenes* also can grow across a relatively wide temperature range, as many were identified at temperature between 2°C to 45°C, meaning that the safety of food is not always guaranteed from microbial colonization by *L. monocytogenes* even at refrigeration condition [4].

The goal of the present study was to determine the presence of *L. monocytogenes* in chicken parts sold at supermarket in Terengganu, Malaysia and to study antibiotics resistance profile of the isolates.

2. Methodology

2.1. Sampling of chicken from supermarket

Total of 56 samples of raw chicken parts which include chicken breast, thigh, drumstick and wing were collected from three districts in Terengganu, Malaysia namely Besut, Setiu and Kuala Terengganu. The samples were purchased from seven local supermarkets. The samples were handled in separate sterile plastic bags to prevent spilling and cross contamination.

2.2. Sample enrichment

Amount of 10 g for each sample was aseptically cutted to become small in size and measured. Then, the measured sample was added together with 90 mL of Bufferred *Listeria* Enrichment Broth (BLEB)(Merck, Germany) in a sterile stomacher bag and it was homogenized for 1 min at 250 rpm using a stomacher machine (Stomacher® 400 Circulator, Seward, UK**)**. The homogenized sample was then incubated at 30°C for 24 h for enrichment step.

2.3. Isolation of *L. monocytogenes*

One loop of the enriched samples was streaked onto PALCAM selective agar plates (Oxoid, CM0877) before the plates were incubated at 30°C for 24 h. After incubation, the presumptive colonies that produced gray-green sunken with black precipitate had been shown on PALCAM plate (Oxoid, CM0877) which show the presence of *Listeria monocytogenes* colonies (Oxoid, CM0877). Basically, about 2 to 3 suspected colonies were picked to be purified on PALCAM agar plate and incubated at 30°C for 24 h. Lastly, a single colony from the grown plate was picked and proceed to PCR analysis to obtain the positive pure colony of the samples.

2.4. DNA extraction

About 1 mL portion of each enriched turbid culture broth was transferred into Eppendorf tube. After that, the centrifugation was conducted at 10,000 ×*g* for 10 min to pellet the cells. The supernatant was discarded. The pellet cells were resuspended in 500 µL of sterile TE buffer and boiled for 10 min at 100°C using digital dry bath (Thermo Scientific, USA). Then, the tube were cooled at −20°C for 10 min before it was re-centrifuged at 10,000 ×*g* for another 10 min at the ambient temperature. The supernatant produced was then used as DNA template for detection of *L. monocytogenes* [5]. Lastly, before proceed to real time PCR, the supernatant was transferred into new microcentrifuge tube and stored at 4°C.

2.5. Real time PCR detection of *L. monocytogenes*

Real time PCR was done in a 15 µL of reaction mixture that obtained from PCR Master Mix (Fermentas, Thermo Fisher Scientific, USA) contained 7.5 µL of SYBR Premix Ex Taq and 5.6 µL of nuclease free water. It was added with 0.45 µL of each primers which the reverse primer (R: 5'AAGCGCTTGCAACTGCTC 3') and forward (F: 5' CCTAAGACGCCAATCGAA 3'), and 1 µL of template DNA. The PCR reaction was conducted using Rotor-Gene 6000 thermal cycler (Corbett Research Ltd, UK). Then, the amplification process started according to the following thermal cycling protocol: with initial denaturation at 95°C for 20 s, followed by 40 cycles at 60°C for 30 s and 72°C for 30 s. Following PCR amplification, the melting curve was constructed. The fluorescence signals obtained from it was monitored to confirm amplification specificity during analyzing time.

2.6. Antimicrobial testing of bacterial strains

Antimicrobial susceptibility testing of *Listeria* was conducted using the disc diffusion method. For the preparation of medium, Mueller-Hinton Agar (MHA) plates were used in duplicates (Oxoid, Hampshire, UK). The isolated *L. monocytogenes* strains was transferred directly into Tryptic Soy Broth (TSB) (Merck, Germany) and incubated at 24 h to obtain an enriched broth. Next, by using cotton swab technique, the standardized cell suspension was be inoculated onto the entire surface of a dried Mueller-Hinton Agar (MHA, Oxoid) plate. Then, the antibiotic discs (Oxoid) that consist of fourteen antibiotic agents had been tested which were Penicillin G (10 IU), Kanamycin (30 µg), Tetracycline (30 µg), Erythromycin (15 µg), Vancomycin (30 µg), Clindamycin (2 µg), Ciprofloxacin (5 µg), Rifampicin (5 µg), Streptomycin (25 µg), Norfloxacin (5 µg), Ceftazidime (10 µg), Enrofloxacin (5 µg), Amikacin (30 µg) and also Ampicillin (10 µg). The discs had been placed onto the surface of MH agar (Oxoid) using a disk dispenser (Oxoid) and the plates were incubated at 30°C for 24 h. Finally, inhibition zone was measured and compared to the standards set by Clinical and Laboratory Standards Institure (CLSI) [6].

2.7. Multiple antibiotic resistance (MAR) index

Each isolate was assigned with an MAR index to determine the multi-resistant *L. monocytogenes* strains:

MAR index= a / b

where 'a' is the number of antibiotics to which the particular isolate was resistant and 'b' is the number of antibiotics to which the particular isolate was exposed [7].

3. Results and Discussion

3.1. Prevalence of *L. monocytogenes* in chicken samples

L. monocytogenes was found present in chicken parts sold at supermarket located in Besut, Setiu and Kuala Terengganu with percentage less than 50%. Table 1 summarized the prevalence of *L. monocytogenes* in 56 chicken parts tested. The prevalence of *L. monocytogenes* ranges from 32.3 to 43.8% based on real time PCR detection.

Table 1: Prevalence of *L. monocytogenes* in chicken parts from supermarkets using real time PCR and plating methods.

Location	Chicken Parts	N	real time PCR n (%)	Plating n (%)
Besut	Breast	8	2 (25.0)	2 (25)
	Thigh	8	2 (25.0)	1 (14.3)
	Drumstick	8	3 (37.5)	2 (25)
	Wing	8	3 (37.5)	2 (25)
	Total	*32*	*10 (32.3)*	*7 (22.6)*
Setiu	Breast	2	1 (50.0)	1 (50.0)
	Thigh	2	1 (50.0)	0
	Drumstick	2	0	0
	Wing	2	1 (50.0)	0
	Total	*8*	*3 (37.5)*	*1 (12.5)*
Kuala Terengganu	Breast	4	2 (50.0)	2 (50.0)
	Thigh	4	1 (25.0)	1 (25.0)
	Drumstick	4	2 (50.0)	2 (50.0)
	Wing	4	2 (50.0)	2 (50.0)
	Total	*16*	*7 (43.8)*	*7 (43.8)*

From 56 chicken samples, only 15 (26.8%) *L. monocytogenes* isolates were successfully recovered from conventional plating method.

The prevalence of *L. monocytogenes* (35.7%) was in agreement with the findings from previous report by Uyttendaele et al. [8], in

which from the total of 225 chicken cuts samples, 105 of them were found to be positive *L. monocytogenes* isolates which had the rate of occurrence of 46.7% [8].

Typically, small and older birds can cause only little or no prevalence of this pathogen in the sample because usually, the older birds have lower *L. monocytogenes* colonization compared to the younger birds and it harder to be colonized compared to *Salmonella* spp. [9]. The findings obtained was lower compared to the previous study by which it had been reported in Estonia that raw chicken legs obtained from retail market in Estonia indicated a high incidence of 70% of contamination with *L. monocytogenes* strains. Besides, the occurrence of the pathogen in broiler legs of Estonian origin were at high level of 88% and 89%, respectively which it is acquired from market selling only products of the mainly Estonian poultry meat [10].

Meanwhile, in the other report conducted in 2005, the detection of *Listeria monocytogenes* that had contaminated the samples of frozen breast, wing, and leg, were ultimately 100% (15 of 15), 93.33% (14 of 15), and 60% (9 of 15) respectively [11]. Even so, the incidence of the pathogen does exist in the samples despite different part being tested. This corresponds to the previous study that showed that the prevalence of *L. monocytogenes* in the each chicken sample carried slight difference due to the variation in water content and nutrient level of chicken parts which it may lead to the occurrence of the pathogen in the different chicken part. Nonetheless, the chicken obtained whether in supermarket or wet market, the prevalence of the pathogen in the samples carried little or no difference. The finding can be proven by previous study by Arumugaswamy et al. [12] reported that the prevalence of *Listeria* between the supermarket and wet market in chicken samples did not differ significantly by which roughly 60% of raw chicken portions were contaminated with *L. monocytogenes* [5].

It had been stated that although the occurrence of *L. monocytogenes* was low in the poultry particularly in raw chicken, the prevalence is however depends on the conditions of environment it was lived in [10]. Typically, this pathogen is preferred to grow at refrigeration temperature below 4°C which it is suits the surrounding temperature of the cold area for frozen products in the supermarket. Thus, it had caused the higher rates of multiplication of *L. monocytogenes* strains on the frozen products especially on the surface of raw chicken cuts as it is easier to be cross-contaminated with the other foods that harbor more bacteria. Spoilage in poultry occurred more frequently on the surface because of high humidity storage environment which susceptible to aerobic bacteria and the poultry skin support the growth of microorganisms better than muscle tissue. Besides that, the capability of the pathogen to strive in the cold chain is the result from the cross protection against osmotic stress such as salt stress and low pH of the food [13]. Apart from that, the cross contamination that had occurred between the chicken with the chopping board as a result of improper hygienic practice by food handler in the supermarket which had probably transmitted more pathogen into the chicken. Additionally, other than inadequate hygiene during cutting, chilling, packaging, the way the frozen chicken was transported using ice in ice box probably helped the remaining pathogens to survive longer in the sample because through appropriate storage below 4°C and the way how the sample is being transferred from one place to another may also influenced the rate of contamination of the bacteria inside the chicken carcasses [14].

3.2. Antimicrobial resistance test among the L. monocytogenes isolates

For the antimicrobial testing, 14 types of antibiotics were tested against 15 isolates using disk-diffusion method. Based on the result, it was found 100% of the strains were completely resistant to Penicilin G as shown in Table 2. High resistance were found towards ampicillin (93.3%) and tetracycline (80%). The high resistance was reported to be due indiscriminate use or overuse of penicillin as treatment of prophylaxis in human and animal farming [3].

Table 2: Antibiotics resistance of L. monocytogenes isolates from chicken from supermarkets.

Antibiotics	N	Resistant n (%)	Susceptible n (%)
Ampicillin	15	14 (93.3)	1 (16.7)
Ceftazidime	15	13 (86.7)	2 (13.3)
Amikacin	15	4 (26.7)	11 (73.3)
Kanamycin	15	9 (60.0)	6 (40.0)
Streptomycin	15	7 (46.7)	8 (53.3)
Tetracycline	15	12 (80.0)	3 (20.0)
Ciprofloxacin	15	5 (33.3)	10 (66.7)
Norfloxacin	15	3 (20.0)	12 (80.0)
Penicillin G	15	15 (100.0)	0 (0.0)
Clindamycin	15	10 (66.7)	5 (33.3)
Rifampicin	15	14 (93.3)	1 (16.7)
Vancomycin	15	6 (40.0)	9 (60.0)
Erythromycin	15	13 (86.7)	2 (13.3)
Enrofloxacin	15	9 (60.0)	6 (40.0)

Despite of that, the present study on tetracycline was not correlated with the previous study done by Charpentier.,et al [15] where they reported the *L. monocytogenes* strains were highly sensitive to the antibiotic. However, higher resistance developed towards tetracycline was observed as time passes. The heavy use of antibiotics in hospitals for treatment as well as antibiotics added into the chicken feed as growth promoters in the production of animal foods has been blamed for this resistancy [16]. As a consequence, the chickens which continuously fed with the same feed will harbors more resistance bacteria. Proper handling and husbandry is important to reduce the resistance [17].

Table 3 summarized the antibiotic resistance profile and the MAR index of *L. monocytogenes* isolates in which multiple resistance to antibiotics were discovered. It was found that at least 13.3% (2/15), 33.3% (5/15) and 6.67% (1/15) isolates were resistant to 9, 10 and 11 antibiotics, respectively. The highest MAR index was 0.93 measured from isolates that showing resistance to 13 antibiotics known as K, S, Rd, Te, E, Caz, Va, P, Da, Amp, Ak, Enr and Nor.

The MAR index shown that all of the isolates (100%) taken from raw supermarket chicken displayed higher MAR value more 0.2. The finding was similar to the previous report in which all the isolates exhibited MAR index greater than 0.2 were originated either from chicken or meat products [7].

Basically, the multi resistance pattern become a particular concern to public especially to infected patient as the significance of these antibiotics in becoming the first choice therapy of listeriosis can be ruined. Moreover, the probability of ineffective treatment using selected antibiotics will be increase. Notably, the antimicrobial resistance of *L. monocytogenes* toward many drugs are eventually increased over time as a result of the transfer of antibiotic resistance genes from Gram positive bacteria such as from *Listeria* spp. The capability of the Gram-positive organisms or mutational events in chromosomal genes to transfer their plasmids and transposons into this pathogen genes will cause the newly acquired resistance able to shield the bacteria from being damaged during antibiotic treatment [7]. Hence, a continuous supervision of evolving antimicrobial resistance of this pathogen is important to make sure an effective treatment of Listeria infection in human [18].

Table 3: MAR Index and antibiogram of *L. monocytogenes* isolates.

MAR Index	Antibiogram	Isolates	(%)
0.29	RdEPDa	PTW1	1 (6.7)
0.36	KRdCazPAmp RdECazPAmp	PTD SBW	2 (13.3)
0.43	RdTeECazPAmp	SMD	1 (6.7)
0.50	TeECazPCipDaAmp	PTW2	1 (6.7)
0.64	KSRdECazPDaAmpEnr	SUB	2 (13.3)

	KSRdTeCazVaPAmpEnr	MYB	
0.71	KSRdTeECazPAmpAkEnr KRdTeECazVaPDaAmpEnr RdTeECazVaPDaAmpEnrNor KSRdTeECazPDaAmpAk RdTeECazPCipDaAmpEnrNor	PTB1 PTB2 PTT SBB MYW	5 (33.3)
0.79	KSRdTeEVaPCipDaAmpEnr	SBD	1 (6.7)
0.86	KSRdTeECazVaPDaAmpAkEnr	MYT	1 (6.7)
0.93	KSRdTeECazVaPDaAmpAkEnrNor	MYD	1 (6.7)

Amp, Ampicillin; Caz, Ceftazidime; Ak, Amikacin; K, Kanamycin; S, Streptomycin; Te, Tetracycline; Cip, Ciprofloxacin; Nor, Norfloxacin; P, Penicillin G; Da, Clindamycin; Rd, Rifampicin; Va, Vancomycin; E, Erythromycin; Enr, Enrofloxacin

4. Conclusion

L. monocytogenes was present in the chicken samples sold at supermarket. Real-Time PCR is a sensitive and robust method for the detection of the pathogen in raw chicken where it is proven to give more rapid detection compared to conventional method. Multiple antibiotic resistance isolates found in this study suggest the need of proper hygienic practices in poultry processing or at home kitchen during preparation in order to minimize the potential risk of infection.

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