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DETECTION OF *LISTERIA* SPP. AND *LISTERIA MONOCYTOGENES* IN VEGETABLES BY LOOP-MEDIATED ISOTHERMAL AMPLIFCATION (LAMP) AND MULTIPLEX POLYMERASE CHAIN REACTION (PCR)

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ABSTRACT

This paper aimed to study the prevalence and antibiotic resistance pattern among *Listeria monocytogenes* in raw vegetables sourced from commercial farms and local farms in Terengganu. Thirteen types of vegetables investigated for the presence of *L. monocytogenes* using multiplex PCR and LAMP methods. Isolation of *L. monocytogenes* was done according to FDA-BAM standard protocol. Seven *Listeria* isolates were subjected to antibiotic test against 10 types of antibiotics. *L. monocytogenes* was found in 3.8% and 7.3% of vegetables sourced from commercial farms and local farms respectively. Both multiplex PCR and LAMP methods capable of detecting *L. monocytogenes*, but LAMP method was simpler with visual observation of the completed amplification. The isolates were highly resistance towards penicillin G (86%) and vancomycin (71%) and susceptible towards most of the antibiotics tested.

Keywords: *L. monocytogenes*; prevalence; antibiotics resistance; LAMP; multiplex PCR.

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1. INTRODUCTION

1.1. *Listeria monocytogenes*

The genus of Listeria consisted of *L. monocytogenes, Listeria grayi, Listeria inocua, Listeria ivanovii, Listeria seeligeri* and *Listeria welshimeri* [1]. Currently, only strains of *L. monocytogenes* are pathogenic to humans and animals for causing listeriosis [2]. *Listeria monocytogenes* is known to contaminate milk and milk products such as cheese because of the complex nature of these products [3]. However, *Listeria* outbreak in vegetables has also been reported which linked to the consumption of coleslaw. In this outbreak, it is believed that the source of cabbage was from the fields where both raw and composted manure from the flock of sheep [4]. Pregnant women, newborns, immune suppressed patients and the elderly (> 65 years old) are at the highest risk of listeriosis. The disease has a long incubation period and causes abortions, premature births, meningitis, septicemia and has a high (20-30%) mortality rate [5-6].

1.2. Foodborne Pathogens in Vegetables

Health awareness among consumers had led to the increase in the intake of vegetables due to their nutritional benefits such reducing risk of certain cancer, heart disease and diabetes [7-8]. Malaysia per capita consumption of vegetables in Malaysia increased from 7.25 kg in 1982 to 40.58 kg in 2001 [9]. Besides providing health benefits, studies had found fresh produce can be an important vehicle for foodborne pathogens [10-15]. This is due to the presence of pathogen in vegetables and the increase in the consumption of ready-to-eat or minimally cooked vegetables will also increases the risk of foodborne illness [16]. Foodborne illness outbreaks caused by bacteria, viruses and parasites in fresh produce were reported to be on the increasing trend [17-19]. Several important pathogens were found to contaminate raw or minimally processed vegetables which include *Staphylococcus*, *Shigella*, *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Campylobacter jejuni* [11, 20-21].

1.3. Objectives of Study

In recent years, *L. monocytogenes* has caught attention for causing several outbreaks related to fresh produce in United States [19, 22] and *L. monocytogenes* ubiquituous occurrence in soil, manure and water [23] increase the possibility of vegetables to be contaminated. Previous study reported significant presence of *L. monocytogenes* in vegetables in Selangor which source from commercial farms [24]. It is timely to study on *Listeria monocytogenes* occurrence in vegetables since there has been limited data available in Malaysia. The objectives of this study are determining the occurrence of *L. monocytogenes* in vegetables sourced from

commercial farms and those grown by local farmers. Antibiotics sensitivity profiles of *L. monocytogenes* isolates recovered from the vegetables were also investigated.

2. EXPERIMENTAL

2.1. Sample Collection

A total of 327 vegetables samples were bought from wet markets located in different districts of Terengganu state, namely Besut, Setiu, Kuala Terengganu, Dungun dan Kemaman. The types of raw vegetables were summarized in Table 1. Lettuce, broccoli, cabbage and parsley were grown in commercial farms while other types of vegetables were grown locally in small scale farms or backyards. All vegetables selected in this study can be eaten raw or after minimal processed by blanching.

Table 1. Vegetable collected from retail outlets in Besut, Setiu, Kuala Terengganu, Dungun and Kemaman

Scienctific Name	Local Name	English Name	N
Lactuca sativa	Daun salad	Lettuce[a]	25
Brassica oleracea var. *italica*	Brokoli	Broccoli[a]	20
Brassica oleracea var *alba*	Kobis	Cabbage[a]	25
Petroselinum crispum	Daun pasli	Parsley[a]	15
Poligonum minus	Kesum	Vietnamese coriander[b]	19
Ardisia crispa	Mata ayam	Village ardisia[b]	10
Cosmos caudatus	Ulam raja	Wild cosmos[b]	24
Ipomoea aquatica	Kangkung	Water spinach[b]	35
Centella asiatica	Pegaga	Indian pennywort[b]	46
Oenanthe javanica	Selom	Japanese parsley[b]	38
Syzygium polyanthum	Daun salam	Bay leaf[b]	11
Barringtonia racemosa	Daun putat	Common putat[b]	15
Anacardium occidentale	Daun gajus	Cashew leaf[b]	44
		Total	327

[a]Vegetables were sourced from commercial farms

[b]Vegetables were sourced from local farms

2.2. Sample Enrichment

Enrichment of vegetable samples was performed according to the procedure described in FDA-BAM Standard for detection of Listeria with a slight modification [42]. Vegetable

sample was cut into small pieces and a 25 g portion was added into stomacher bag containing 225 ml of Buffered Listeria Enrichment Broth (BLEB, Merck, Darmstadt, Germany). The sample was homogenised by stomacher for 60 s and incubated at 30°C for 4 h as a pre-enrichment step. BLEB supplements which contain acriflavin, sodium nalidixate and cycloheximide were added to the pre-enriched sample with final concentration of 10 mg/l, 40 mg/l and 50 mg/l respectively.

2.3. DNA Preparation

L. monocytogenes DNA from vegetables samples will be extracted using boiled-cell method as described in [24] with slight modification. A portion of 500 µL enriched sample was centrifuged at 10 000*xg* to pellet the cells. The supernatant was discarded and the pellet will be re-suspended with 500 µL of sterile deionised water. The tube containing the re-suspended cells pellet was boiled at 100 ˚C for 10 min using digital dry bath (Corning, Japan). The boiled mixture was cooled at -20˚C for 10 min before undergone centrifugation for 10 min at 10 000 *xg*. The supernatant containing DNA was used for *L. monocytogenes* detection by multiplex PCR or LAMP.

2.4. Multiplex PCR Detection of *Listeria* spp and *Listeria monocytogenes*

Table 2 showed specific primers for *L. monocytogenes* detection using multiplex polymerase chain reaction (PCR). The primers used were based on published nucleotide sequence of the 16s rDNA gene for genus detection where it is the most stable gene to analysed and targeted *hlyA* virulence gene for species detection [43].

Table 2. Sequences of oligonucleotide primers used to target specific genes in *Listeria* spp. and *L. monocytogenes* and their respective amplicons sizes

Target Gene	Primer	Sequence (5'-3')	Amplicon (bp)	References
16S rDNA	L1	CAG CMG CCG CGG TAA TWC	938 bp	[43]
	U1	CTC CAT AAA GGT GAC CCT		
hlyA	LM1	CCT AAG ACG CCA ATC GAA	702 bp	
	LM2	AAG CGC CCG CAA CTG CTC		

PCR reagents from Promega Madison, USA GoTaq Flexi were used throughout this study. The PCR reaction mixture include 5µL of 5x Green GoTaq Flexi buffer, 2µL of 25mM $MgCl_2$, 0.5µL of 10mM dNTP, 0.4µL of GoTaq DNA polymerase, 2µL of DNA template, 11.1µL of sterile distilled water and 1µL of each primers LM1 and LM2, 1µL of each primers U1 and L1.

Multiplex PCR was performed with the Veriti 96-Well Thermal Cycler (Applied Biosystems, Singapore). The PCR steps include initial pre-denaturation at 95ºC for 5 mins, 35 cycles of denaturation at 95ºC for 45 s, annealing at 55ºC for 1 min and extension at 72ºC for 1 min 30s, followed by a final extension at 72ºC for 5 mins.

Electrophoresis of the PCR products were ran through 1.0% of an agarose gel using 100V for 30 mins. A 100 bp DNA-molecular ladder (Promega Madison, USA) was included for each electrophoresis gel run. The result was visualized using the Fujifilm LAS-3000 Imager (Fujifilm, Japan).

2.5. Loop Mediated Isothermal Amplification (LAMP)

LAMP detection was performed with the *Listeria monocytogenes* Loopamp Kit (Eiken Chemical Co. Ltd., Tokyo, Japan) on the vegetable samples. Reaction mixture consist of 20 µl 2× reaction mix which include 6 sets of primers (1.6 mM FIP, 1.6 mM BIP, 0.8 mM LF, 0.8 mM LB, 0.2 mM F3 and 0.2 mM B3), 1 µl of *Bst* DNA polymerase (8 U), 1 µl Loopamp Fluorescent Detection Reagent (FD) and 2 µl DNA template. The reactions mixture was incubated at 63°C for 1 h and then at 80°C for 10 min to terminate the amplification. Mixture without DNA template was used as a negative control. Visual detection of positive sample from LAMP amplification tubes were done as described in [44].

2.6. Isolation and Identification of *Listeria monocytogenes*

A portion of 100 µl from enriched samples was plated onto PALCAM agar and incubated for 48 h at 30 C. At least five presumptive colonies (black with grey zone) were picked and subcultured onto Tryptone Soy Yeast Extract Agar and subjected to confirmatory tests using colony PCR method.

2.7. Antimicrobial Susceptibility Test

Antibiotic resistance of *L. monocytogenes* isolates were tested against Amikacin (30 µg), Ampicillin (10 µg), Tetracycline (30 µg), Penicillin G (10 IU), Ciprofloxacin (5 µg), Enrofloxacin (5 µg), Norfloxacin (10µg), Vancomycin (5µg), Gentamicin (10µg) and Erythromycin (15µG) using disc diffusion method on Muller Hinton (MH) agar. The plates were incubated at 30 ˚C for 24 h. The diameter of clear zone will be measured and analysed according to Clinical and Laboratory Standards Institute [45] to determine the susceptibility of the isolates.

3. RESULTS AND DISCUSSION

This study examined 13 types of vegetables totalling 327 raw vegetable samples for *Listeria*

spp. and *L. monocytogenes* using multiplex PCR and LAMP methods. Table 3 summarized the detection of *Listeria* spp. and *L. monocytogenes* in vegetables from Besut, Kuala Terengganu, Setiu, Dungun and Kemaman. In short, percentages of *Listeria* spp. in vegetables from different district were found to be at 21.5% (Besut), 10.3% (Kuala Terengganu), 41.5% (Setiu), 31.4% (Dungun) and 39.06% (Kemaman) and percentages of *L. monocytogenes* in vegetables was at 6.3% (Besut), 3.4% (Kuala Terengganu), 14.6% (Setiu), 2.3% (Dungun) and 7.8% (Kemaman). *Listeria* spp. were found in most vegetables except bay leaf in Besut; cabbage, broccoli, indian pennywort, parsley and cashew leaf in Kuala Terengganu; indian pennywort in Setiu; water spinach in Dungun; and cabbage, village ardisia and cashew leaf in Kemaman. *Listeria* spp. was found in 16.3% of vegetables sourced from commercial farms and 28.3% of vegetables sourced from local small scale farms. *L. monocytogenes* was found in 3.8% of vegetables sourced from commercial farms and 7.3% of vegetables sourced from local small scale farms.

Current findings showed lower percentage of *Listeria* spp. (26.6%) and *L. monocytogenes* (7.0%) than the previous study sampled from supermarkets as reported by [24] in which 29.0% of *Listeria* spp. and 24.1% *L. monocytogenes* were found in the vegetables. The different ways for vegetables storage and display in wet markets and supermarkets are thought to be the contributing factor for these findings. Wet markets stored and displayed vegetables at ambient temperature while supermarket stored and kept vegetables at low temperature. *Listeria* is an psychrotrophic microorganism that survives and grows at low temperature [25-26]. Storage of vegetables at low temperature was thought to reduce spoilage and extend the shelf life of vegetables [16]. This might be true for mesophilic spoilage microorganisms but the presence of psycrothrophic pathogen such as *Listeria* might posed risk of foodborne illness from consumption of raw or minimally processed vegetables.

Table 3. Number of *Listeria* spp. and *L. monocytogenes* in vegetables detected using multiplex PCR and LAMP methods. *L. monocytogenes* isolates were recovered from the vegetable samples according FDA-BAM standard for detection of *Listeria*

Location	Vegetables	N	Multiplex PCR		LAMP	Isolates
			***Listeria* spp.**	***L. monocytogenes***	***L. monocytogenes***	***L. monocytogenes***
			n	**n**	**n**	**n**
Besut	Lettuce[a]	15	3	2	2	1
	Cabbage[a]	15	5	2	2	1
	Broccoli[a]	15	2	0	0	0
	Parsley[a]	10	2	0	0	0
	Indian pennywort[b]	25	3	0	0	0
	Cashew leaf[b]	15	2	0	0	0
	Village ardisia[b]	5	2	1	1	0
	Vietnamese coriander[b]	15	4	1	1	0
	Water spinach[b]	12	4	2	2	0
	Japanese parsley[b]	10	3	0	0	0
	Bay leaf[b]	11	0	0	0	0
	Common putat[b]	10	4	2	2	1
	Total	158	34	10	10	3
Kuala Terengganu	Lettuce[a]	5	1	1	1	1
	Cabbage[a]	5	0	0	0	0
	Broccoli[a]	5	0	0	0	0

	Parsley[a]	5	0	0	0	0
	Indian pennywort[b]	5	0	0	0	0
	Cashew leaf[b]	4	0	0	0	0
	Total	29	1	1	1	1
Setiu	Japanese parsley[b]	10	5	2	2	1
	Water spinach[b]	12	7	2	2	0
	Wild cosmos[b]	6	2	1	1	0
	Indian pennywort[b]	5	0	1	1	0
	Cashew leaf[b]	8	2	0	0	0
	Total	41	16	6	6	1
Dungun	Japanese parsley[b]	8	5	1	1	0
	Water spinach[b]	11	0	0	0	0
	Wild cosmos[b]	8	3	0	0	0
	Cashew leaf[b]	8	2	0	0	0
	Total	35	10	1	1	0
Kemaman	Cabbage[a]	5	0	0	0	0
	Japanese parsley[b]	10	5	3	3	2
	Indian pennywort[b]	21	10	2	2	0
	Wild cosmos[b]	10	4	0	0	0
	Village ardisia[b]	5	0	0	0	0
	Common putat[b]	5	3	0	0	0

	Cashew leaf[b]	8	0	0	0	0
	Total	64	22	5	5	2
	Grand Total	327	87	23	23	7

[a]vegetables were sourced from commercial farms

[b]vegetables were sourced from local farms

The presence of *L. monocytogenes* in both commercial and local farms might be due to contamination from soil, manure or water used for irrigation [23]. Studies have found leafy vegetables were easily contaminated with soil and microorganisms because of their larger surface area [15, 27-28]. Leafy vegetables were found to harbour more microorganisms than non-leafy vegetables [29].

The amplified PCR products from current study were shown in Fig. 1. This study successfully detected the presence of *Listeria* spp. based on 16S rRNA gene and *L. monocytogenes* based on *hlyA* gene. Polymerase chain reaction (PCR) is an established nucleic acid amplification technique for detecting pathogenic microorganisms [30]. Simultaneous amplification of more than one locus can be done by using several sets of specific primers into single PCR assay which also known as multiplex PCR (mPCR) [31]. Multiplexing PCR has been a useful technique to identify pathogen of interest within a genus or different pathogens in a single assay [31]. This study utilized the flexibility of PCR assay to detect *L. monocytogenes* within the *Listeria* genus in a single assay. There are six closely related species within genus *Listeria*, namely *Listeria monocytogenes, L. ivanovii, L. innocua, L. welshimeri, L. seeligeri* and *L. grayi* [32-34]. *L. monocytogenes* is an important human pathogen among the six species which cause serious illness in infants, pregnant women, elderly and immunocompromised individuals [33]. Though multiplex PCR is very useful tool for pathogen detection, typical PCR cycle will take from 1.5 to 2.5 hours to complete and the need for gel electrophoresis for 1.5 hours before being view using gel documentation system to record the result. The requirement for these three systems had highlighted the need for simple, rapid and visual observation of result become necessary.

Introduction of novel method known as Loop-mediated isothermal amplification (LAMP) in year 2000 [35] has gained wide interest of this rapid and simple method in pathogen detection [36-37]. LAMP assay requires only an hour to complete and uses single temperature at 63^{o}C instead of multiple cycle of denaturation, annealing and elongation as found in PCR method. This study showed same result on both PCR and LAMP methods. Fig. 2 showed positive and negative reaction of LAMP method. Significant colour changes helped in differentiating positive samples from the negative samples by visual observation without the need of gel

documentation system or UV transilluminator to view the end product. Such method is important for a small and less equip laboratory setting or during field work.

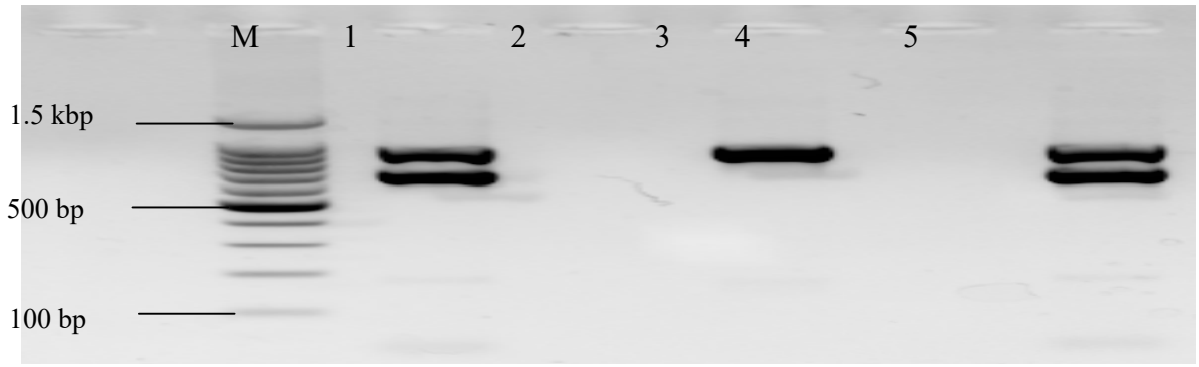

Fig.1. Representatives electrophoresis gel picture of multiplex PCR detection of *Listeria* spp. (16s rRNA: 938 bp) and *L. monocytogenes* (*hly*A gene: 701 bp). Lane M: DNA Marker; Lane 1: Positive control of *L. monocytogenes* ATCC 13932; Lane 2: Negative control; Lane 3: Positive detection of *Listeria* spp. in vegetable samples; Lane 5: Positive detection of *Listeria* spp. and *L. monocytogenes* in vegetable sample; Lane 4: Negative detection of *Listeria* spp. and *L. monocytogenes* in vegetable samples

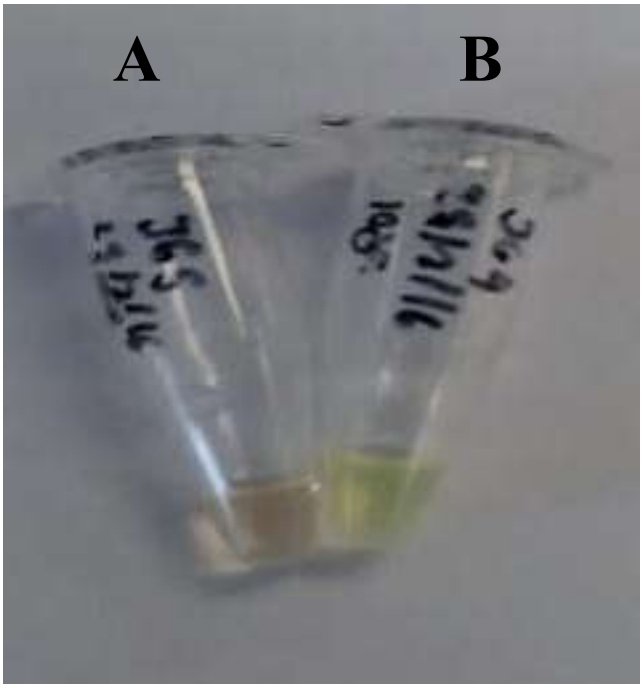

Fig.2. Representatives picture of LAMP detection of *L. monocytogenes*. A) Negative detection of *L. monocytogenes* in vegetables samples; B) Positive detection of *L. monocytogenes* in vegetable samples

Infection by *L. monocytogenes* in human can cause serious complications which include meningitis, encephalitis, septicemia and abortion in pregnant women [38]. Thus, early intervention by administration of antibiotics would be necessary for better outcome in the case of listeriosis. Current study (Table 4) found *L. monocytogenes* isolates were highly susceptible towards amikacin (100%), enrofloxacin (100%), gentamicin (100%), ciprofloxacin (85%), norfloxacin (85%), erythromycin (57%) and tetracycline (57%). About 86% and 71% of the isolates were resistant towards penicillin G and vancomycin, respectively. This finding was in line with previous reports that *Listeria* was susceptible to most antibiotics [38-39]. The study also reported 71.4% of isolates resistance towards penicillin and the possible explanation was that penicillin is the drug of choice and widely used in listeriosis treatment [40-41]. In addition, antimicrobial agents are very unlikely to be used in vegetables farming than in animal farming.

Table 4. Antimicrobial susceptibility test result for *Listeria monocytogenes* isolates from vegetables

Antimicrobial Agent	Disk Content	N	Number of Samples	
			Resistant	Susceptible
			n	n
Amikacin	30 µg	7	0	7
Ampicillin	10 µg	7	4	3
Ciprofloxacin	5 µg	7	1	6
Enrofloxacin	5 µg	7	0	7
Erythromycin	15 µg	7	3	4
Gentamicin	10 µg	7	0	7
Norfloxacin	10 µg	7	1	6
Penicillin G	10 IU	7	6	1
Tetracycline	30 µg	7	3	4
Vancomycin	5 µg	7	5	2

4. CONCLUSION

Current study showed *Listeria monocytogenes* was present at low percentage in vegetables sampled at wet markets which stored and display vegetables at ambient temperature. This condition limits the growth of psychrotrophic pathogen such as *L. monocytogenes*. Both multiplex PCR and LAMP successfully detected *L. monocytogenes* in vegetables and LAMP method showed ease of use by visual observation of positive detection. *Listeria monocytogenes* isolates were susceptible towards most antibiotics except for penicillin G and vancomycin. Proper decontamination of vegetables before consumption is very important in order to prevent listeriosis from the consumption of minimally processed or ready-to-eat vegetables.

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