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Evaluation of *Listeria* spp. and *Listeria monocytogenes* in selected vegetable farms

(Penilaian ***Listeri***a spp. dan ***Listeria monocytogenes*** di kebun sayur terpilih)

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Abstract

The aim of the study was to examine the prevalence of *Listeria* spp. and *Listeria monocytogenes* in soil, poultry manure, irrigation water, and freshly harvested vegetables from three vegetable farms in Cameron Highlands. A total of 177 samples including environmental and vegetable samples were collected. Among the environmental samples (n = 94), poultry manure was found to have a higher prevalence of *Listeria* spp. and *L. monocytogenes* at 77.8% and 61.1% respectively using the MPN-PCR analysis procedure. Soil samples were also found to harbour *Listeria* spp. and *L. monocytogenes* at 47.6% and 38.1% respectively. Irrigation water sampled from the farms were found to be free from *Listeria* spp. Both *Listeria* spp. and *L. monocytogenes* were also detected in 24% and 12% of swabs done at the three farms respectively. Among the freshly harvested vegetables, *Listeria* spp. was detected in cabbages (30%), cucumber (20%), yardlong beans (10%) and carrots (10%) whereas *L. monocytogenes* was detected in cabbages (10%), yardlong beans (10%) and carrots (10%) using the MPN-PCR analysis procedure. The present results signify that *Listeria* spp. poses a potential risk for raw vegetable consumption in Malaysia. The study also provides baseline data on *Listeria* spp. contamination at farm level.

Introduction

Listeria monocytogenes is a widely distributed food pathogen that can cause listeriosis which is a serious invasive human illness with mortality rates on average approaching 30% (Vazquez-Boland et al. 2001). Pre-harvest contamination of vegetables with *L. monocytogenes* can provide a source for human listeriosis infections as seen from the 1981 outbreak of listeriosis in Nova Scotia. Here, 42 human cases were linked with the consumption of coleslaw that had been produced from cabbage fertilized with sheep manure obtained from a farm with a history of ovine listeriosis (Schelch et al. 1983).

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Members of the genus *Listeria*, including *L. monocytogenes*, occur widely in the agricultural environment. *Listeria* spp. have been isolated from soil (Weiss and Seeliger 1975), water and sewage (Watkins and Sleath 1981), manure (Fenlon 1986) and farm environments (Ueno et al. 1996).

Contamination of vegetables by *L. monocytogenes* at the pre-harvest stage may occur through agricultural practices such as irrigation with contaminated water, fertilization with contaminated manure, contamination from the soil, air, animals, unsanitized transportation vehicles and human handling (Beuchat 1996).

The methods originally developed for detection of *Listeria* spp. and *L. monocytogenes* from foods have been successfully adapted for isolation of *L. monocytogenes* from environmental samples (Weidmann et al. 1994; Aravanitidiou et al. 1997). The most commonly used enrichment procedure for samples from natural environments includes selective enrichment in Listeria Enrichment Broth (LEB) with varying concentrations of selective agents, followed by plating on selective and differential media such as Oxford or Lithium Chloride Phenylethanol Moxalactam (LPM) Plating Agar (Husu et al. 1990; Wiedmann et al. 1994).

Application of molecular methods has improved the ability to probe and understand the ecology of *Listeria* in the natural environment. Similar to commonly used 16S rDNA-based approaches to characterize bacterial population structures, *Listeria* and *L. monocytogenes*-specific polymerase chain reaction (PCR) primers have been used to detect and characterize *Listeria* isolates and their diversity directly, including through construction and sequencing of clonal libraries (Walker and Morgan 1993; Cai et al. 2002).

Cameron Highlands, which is located in the state of Pahang, is the primary vegetable farming area in Malaysia. With a land area of 71,000 ha and a population of 30,000, the state is divided into eight sub-districts with approximately 2,200 farms of various sizes. A large portion of the domestic vegetable supply is grown here and subsequently distributed throughout the country as well as to neighbouring countries.

The aim of this study was to determine the prevalence and concentration of *Listeria* spp. and *L. monocytogenes* in environmental samples and freshly harvested vegetable samples obtained from vegetable farms, packing houses and the loading bays located at Cameron Highlands, Pahang. The results of this study would provide important baseline data on *Listeria* spp. and *L. monocytogenes* contamination at the farm level.

Materials and methods

Bacterial strains and preparation of inocula

Listeria monocytogenes strain ATCC 19115 was used in this study to test the detection limit of the four analytical methods and as a positive control in PCR assays. Pure cultures of *L. monocytogenes* were grown at 37 °C for 24 h in tryptone soy broth with yeast extract (TSBYE) (Oxoid, United Kingdom) and the DNA was extracted to obtain the positive control. The cultures, with final concentrations adjusted to about 3 x10^8 cfu/ml using McFarland's nephelometric tube No.1, served as inocula for spiking sterilized environmental samples in order to test the limit of detection of the analytical methods.

Determination of detection limit of analytical methods

The inocula was serially diluted in Tryptone Salt (Oxoid,United Kingdom) and spiked into sterilized samples of soil, animal manure and distilled water before being detected using the direct plating, enrichment plating, direct PCR and MPN-PCR methods (Chai et al. 2009).

Sample collection

Sampling was carried out at three vegetable farms in Cameron Highlands, Pahang. The farms were chosen at random and were located at three of the eight sub-districts, i.e.

Tanah Rata, Brinchang and Lembah Bertam. The farms were of medium size (4,000 to 6,000 m^2) and produced various crops from time to time. Farm A practised organic farming whereas Farms B and C practised traditional farming.

The samples collected for analysis from the farms were soil and poultry manure, irrigation water from the reservoir, tap and irrigation pipes, swabs from transportation vehicles and baskets and freshly cut vegetables. Soil and animal manure were collected from Farms B and C since Farm A practised organic farming. The samples collected from the packing houses were swabs from vats, food sorting equipment as well as freshly harvested vegetables. The samples collected from the loading bays were freshly harvested vegetables that were about to be transported downhill to markets throughout the country as well as neighbouring countries.

Soil samples were collected aseptically from different planting sites at the farms at random. The samples were carefully placed in sterile plastic sampling tubes (about 200 g). Water was aseptically collected from different points of the main reservoir, distribution tanks, taps and irrigation pipes. Water samples were placed into sterile tubes. Poultry manure was collected aseptically from different planting sites at the farms. The samples were carefully placed in sterile plastic sampling tubes (about 200 g).

Swabs were taken from baskets, transportation vehicles, knives, food sorting equipment and vats. The swabs were placed in sterile plastic tubes. Fresh vegetable samples were aseptically obtained from different locations and placed in sterile plastic bags. Sample collection was conducted at random and was dependent on the vegetables available at the time of the visit. All samples were transported to the laboratory and analysis initiated within 24 h of collection.

Sample preparation

Sample preparation was done according to the method described by Hitchins (2003). Twenty-five grams (or 25 ml) of sample (soil, poultry manure, freshly cut vegetables and swab samples) were weighed in a sterile stomacher bag, homogenised with 225 ml of Buffered Listeria Enrichment Broth (BLEB, Merck, Darmstadt, Germany) for 60 s. The bag was incubated at 30 °C for four hours as a pre-enrichment step. Then acriflavin (final concentration:10 mg/ml); sodium nalixidate (final concentration :40 mg/ml) and cycloheximide (final concentration :50 mg/ml) (Sigma, St.Louis, USA) were added as selective agents. Tenfold and hundredfold dilutions of the stomacher fluid were prepared with BLEB.

Culturing method

Direct-plating and enrichment plating were carried out to determine the prevalence of *Listeria* spp. in the various samples (Chai et al. 2009). Polymyxin Acriflavin Lithium-chloride Ceftazidime Esculin Mannitol (PALCAM) agar was used as the selective plating media for both methods. For direct plating, 0.1 ml of each of the serial dilutions (from 10^{-1} to 10^{-3}) was directly plated onto PALCAM agar and incubated for another 44 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.

For enrichment plating, the serial dilutions were incubated for another 44 h at 30 °C (Hitchins 2003). Subsequently, 0.1 ml of each of the serial dilutions (from 10^{-1} to 10^{-3}) was plated onto PALCAM agar and incubated for another 48 h at 30 °C. Then the plates were checked for presumptive *Listeria* colonies. At least five presumptive colonies (black with grey zone) were picked and subcultured onto Tryptone Soy Yeast Extract Agar and subjected to confirmatory tests.

Direct-PCR detection for confirmation

Direct-PCR confirmation method was only used for environmental samples such as soil, animal manure and irrigation water from farms and was modified from the method by Chai et al. (2009). Direct extraction of DNA samples and identification of *Listeria* spp. and *L. monocytogenes* were done using the polymerase chain reaction (PCR) technique.

For soil and poultry manure samples, 10 g of sample was weighed in a 50 ml Falcon tube, mixed with sterile distilled water up to a final volume of 40 ml and incubated in a shaker incubator at 30 °C for 60 min to release any microorganism from the samples. Then the mixture was centrifuged at 600 x g for 15 min and the supernatant filtered through a sterile cheese cloth to separate coarse particles in the supernatant. The supernatant was discarded and 400 µl of sterile distilled water was added. The mixture was then transferred to a sterile 1.5 ml microcentrifuge tube and boiled for 10 min. Then the sample was cooled at –20 °C for 5 min before it was centrifuged at 12000 x g for 10 min. The supernatant was transferred to a new 1.5 ml centrifuge tube and 800 µl of 95 % ethanol was added. The tube was inverted several times and left on ice for 5 min prior to centrifugation at 12000 x g for 10 min. The supernatant was discarded and the pellet washed with one ml of 97% ethanol. It was centrifuged again at 12000 x g for 10 min and the pellet was dried under laminar air flow. The DNA was resuspended with 200 µl of sterile distilled water and kept for PCR analysis.

For water samples, 50 ml was poured into a 50 ml Falcon tube and centrifuged at 600 x g for 15 min to enable sedimentation of sand and coarse particles present in the water. The supernatant was transferred to a new tube and subjected to centrifugation at 12000 x g for 30 min. The supernatant was discarded and the pellet was resuspended with 400 µl of sterile distilled water. The mixture was transferred to a sterile 1.5 ml centrifuge tube and boiled for 10 min. Then the sample was cooled at –20 °C for 5 min before it was centrifuged at 15000 x g for 10 min. The supernatant was transferred to a new 1.5 ml centrifuge tube and 800 µl of 95 % ethanol was added. The tube was inverted several times and left on ice for 5 min prior to centrifugation at 12000 x g for 10 min. The supernatant was discarded and the pellet washed with one ml of 97 % ethanol. It was centrifuged again at 12000 x g for 10 min and the pellet was dried under laminar air flow. The DNA was resuspended with 200 µl of sterile distilled water and kept for PCR analysis.

Most Probable Number-Polymerase Chain Reaction (MPN-PCR)

For MPN analysis (Hitchins 2003), 0.1 ml of the serial dilutions (from 10^{-1} to 10^{-3}) was inoculated into three tubes, each containing 0.9 ml of BLEB (Merck). The tubes were incubated for another 44 h at 30 °C and checked for turbidity

DNA was extracted from the turbid MPN tubes. DNA extraction was carried out using a boiled-cell method (Chai et al. 2009) where 500 µl of the broth underwent centrifugation at 15000 x g for 10 min to pellet the microorganism. The pellet was resuspended in 500 µl of sterile, distilled water and boiled for 10 min. After boiling, the sample was cooled at –20 °C for 5 min before it was centrifuged at 15000 x g for 10 min. The supernatant was kept for PCR analysis.

Polymerase Chain Reaction (PCR)

DNA from boiled-lysate was subjected to multiplex PCR to detect *Listeria* spp. and *L. monocytogenes*. The primer pairs for the detection of *Listeria* spp. (genus specific) were designed to amplify a 938bp region in the 16S rRNA gene. The primer pairs used were 5'CTC CAT AAA GGT GAT CCT 3' and 5' CAG CAG CCG CGG TAA TAC 3'. The primer pairs for the detection of *L. monocytogenes* were used to specifically amplify a 701-bp region in the hly gene. The primer pairs used were 5' CCT AAG ACG

CCA ATC GAA 3' and 5' AAG CGC TTG CAA CTG CTC 3'. The oligonucleotide primers used in this study were obtained from Invitrogen. DNA extracted from the BLEB cultures of *L. monocytogenes* ATCC 19155 was used as the positive control in every PCR assay.

PCR amplification was performed in 25 µl of reaction mixture containing 1.5 µl 10 x PCR buffer, 0.2 µl 10mM deoxynuclease triphosphate mix; 0.5 µl of each primer, 15 µl sterile distilled water, 0.3 µl Taq DNA polymerase, 1.5 µl $MgCl_2$ and 2 µl of DNA template solution (Rawool et al. 2007). All items used in PCR were purchased from Promega Research Instruments,USA.

Reaction mixtures were heated at 94 °C for 5 min in the initial denaturation step. PCR was then performed. The reaction was terminated after a 7-min extension period at 72 °C. All thermal cycling reactions were performed with a Thermo Hybrid PxE Thermal Cycler (Thermo Electron Corporation, Franklin, MA, USA).

For visualization of PCR products, 10 µl of PCR products were run on 1.0% agarose gel at 100V for 28 min. The gel was then stained with ethidium bromide and viewed under ultra-violet light. A DNA-molecular ladder (100 bp) (Vivantis Technologies) was included in each gel.

Statistical analysis

SPSS software (version 16.0) was utilised to analyze the data to determine if there was any significant difference between the prevalence of *Listeria* spp. and *L. monocytogenes* among the sampling locations and types of environmental samples and vegetables. The level of significance was set at $p < 0.05$. Whenever there was a significant difference, post-hoc comparison was done using Tukey's Honestly Significant Difference (HSD) to identify the groups that are significantly different.

Results and discussion

A total of 177 samples including environmental and vegetable samples were collected from three farms in Cameron Highlands. *Figure 1* shows the representative electrophoretic gel pictures of *Listeria* spp. and *L. monocytogenes* by PCR while *Table 1* shows the limit of detection for the four methods of analysis.

The prevalence of *Listeria* spp. and *L. monocytogenes* in environmental samples in vegetable farms as assessed by four types of enumerative assays are shown in *Tables 2* and *4* respectively while a summary of the prevalence based on MPN-PCR analysis is given in *Table 8*. Among the environmental samples (n = 94), poultry manure had the highest prevalence of *Listeria* spp. and *L. monocytogenes* at 77.8% and 61.1% respectively using the MPN-PCR analysis procedure (*Table 8*). Soil samples were also found to harbour *Listeria* spp. and *L. monocytogenes* at 47.6%

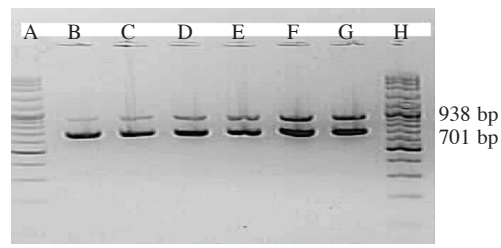

Figure 1. Representative amplification of the 16S rRNA and LLO genes for identification of ***Listeria*** *spp. (genus) and* ***Listeria monocytogenes*** *respectively. Lanes A and H show the 100-bp DNA ladder. Lanes F and G show the PCR amplicons specific for* ***Listeria*** *spp. at 938 bp and 701 bp in the reference strain whereas lanes B-E show DNA from MPN broth*

Table 1. Limit of detection for test strain in environmental samples using four analytical methods

Type of analysis	Soil and animal manure (CFU/g)	Water (CFU/ml)
Direct plating	10^2	10^6
Enrichment plating	10	10^6
Direct PCR	10^3	10^4
MPN-PCR	10	10^2

Table 2. Prevalence (%) of *Listeria* spp. in environmental samples in vegetable farms assessed using four types of enumerative assays

Sample type	Sampling location	Direct-plating		Enrichment plating		Direct PCR		MPN-PCR	
		No.	%	No.	%	No.	%	No.	%
Soil	Farm B	(2/9)	22.2[a]	(1/9)	11.1[a]	(2/9)	22.2[a]	(4/9)	44.4[a]
Soil	Farm C	(3/12)	25.0[a]	(2/12)	16.7[a]	(4/12)	33.3[a]	(6/12)	50.0[a]
Animal manure	Farm B	(4/11)	36.4[b]	(3/11)	27.2[b]	(5/11)	45.5[b]	(8/11)	72.7[b]
Animal manure	Farm C	(3/7)	42.9[b]	(2/7)	28.6[b]	(4/7)	57.1[b]	(6/7)	85.7[b]
Irrigation water from reservoir	Farm A	(0/1)	0.0[c]	(0/1)	0.0[c]	(0/1)	0.0[c]	(0/1)	0.0[c]
Irrigation water from reservoir	Farm B	(0/1)	0.0[c]	(0/1)	0.0[c]	(0/1)	0.0[c]	(0/1)	0.0[c]
Irrigation water from reservoir	Farm C	(0/1)	0.0[c]	(0/1)	0.0[c]	(0/1)	0.0[c]	(0/1)	0.0[c]
Irrigation water from tank	Farm A	(0/3)	0.0[c]	(0/3)	0.0[c]	(0/3)	0.0[c]	(0/3)	0.0[c]
Irrigation water from tank	Farm B	(0/1)	0.0[c]	(0/1)	0.0[c]	(0/1)	0.0[c]	(0/1)	0.0[c]
Irrigation water from tank	Farm C	(0/2)	0.0[c]	(0/2)	0.0[c]	(0/2)	0.0[c]	(0/2)	0.0[c]
Irrigation water from pipes	Farm A	(0/2)	0.0[c]	(0/2)	0.0[c]	(0/2)	0.0[c]	(0/2)	0.0[c]
Irrigation water from pipes	Farm B	(0/1)	0.0[c]	(0/1)	0.0[c]	(0/1)	0.0[c]	(0/1)	0.0[c]
Irrigation water from pipes	Farm C	(0/3)	0.0[c]	(0/3)	0.0[c]	(0/3)	0.0[c]	(0/3)	0.0[c]
Environmental swabs	Farm A	(0/8)	0.0[c]	(0/8)	0.0[c]	ND	ND	(2/8)	25.0[c]
Environmental swabs	Farm B	(0/7)	0.0[c]	(0/7)	0.0[c]	ND	ND	(2/7)	28.6[c]
Environmental swabs	Farm C	(0/10)	0.0[c]	(0/10)	0.0[c]	ND	ND	(2/10)	20.0[c]
Total		(12/79)	15.2	(8/79)	10.1	(15/54)	27.8	(30/79)	38.0

ND = Not done

a, b,c in column denote significant differences at $p < 0.05$ using HSD test

Table 3. Prevalence (%) of *Listeria* spp. in freshly harvested vegetables in vegetable farms assessed using four types of enumerative assays

Sample type	Sampling location	Direct-plating		Enrichment plating		Direct PCR		MPN-PCR	
		No.	%	No.	%	No.	%	No.	%
Carrot	Farm A	(0/3)	0.0	(0/3)	0.0	ND	ND	(0/3)	0.0
Yardlong bean	Farm A	(0/3)	0.0	(0/3)	0.0	ND	ND	(1/3)	33.3
Cabbage	Farm A	(0/3)	0.0	(0/3)	0.0	ND	ND	(1/3)	33.3
Tomato	Farm A	(0/4)	0.0	(0/4)	0.0	ND	ND	(0/4)	0.0
Cucumber	Farm A	(0/3)	0.0	(0/3)	0.0	ND	ND	(0/3)	0.0
Carrot	Farm B	(1/4)	25.0	(0/4)	0.0	ND	ND	(1/4)	25.0
Yardlong bean	Farm B	(0/3)	0.0	(0/3)	0.0	ND	ND	(0/3)	0.0
Cabbage	Farm B	(0/3)	0.0	(0/3)	0.0	ND	ND	(1/3)	33.3
Tomato	Farm B	(0/3)	0.0	(0/3)	0.0	ND	ND	(0/3)	0.0
Cucumber	Farm B	(0/4)	0.0	(1/4)	25.0	ND	ND	(1/4)	25.0
Carrot	Farm C	(0/3)	0.0	(0/3)	0.0	ND	ND	(0/3)	0.0
Yardlong bean	Farm C	(1/4)	25.0	(0/4)	0.0	ND	ND	(1/4)	25.0
Cabbage	Farm C	(0/4)	0.0	(1/4)	25.0	ND	ND	(1/4)	25.0
Tomato	Farm C	(0/3)	0.0	(0/3)	0.0	ND	ND	(0/3)	0.0
Cucumber	Farm C	(1/3)	33.3	(0/3)	0.0	ND	ND	(1/3)	33.3
Total		(3/50)	6.0	(2/50)	4.0	–	–	(8/50)	16.0

ND = Not done

Table 4. Prevalence (%) of *Listeria monocytogenes* in environmental samples in vegetable farms assessed using four types of enumerative assays

Sample type	Sampling location	Direct-plating		Enrichment plating		Direct PCR		MPN-PCR	
		No.	%	No.	%	No.	%	No.	%
Soil	Farm B	(1/9)	11.1[a]	(1/9)	11.1[a]	(1/9)	11.1[a]	(3/9)	33.3[a]
Soil	Farm C	(3/12)	25.0[a]	(2/12)	16.7[a]	(3/12)	25.0[a]	(5/12)	41.6[a]
Animal manure	Farm B	(4/11)	36.4[b]	(3/11)	27.2[b]	(4/11)	36.4[b]	(7/11)	63.6[b]
Animal manure	Farm C	(2/7)	28.6[b]	(2/7)	28.6[b]	(3/7)	42.8[b]	(4/7)	57.1[b]
Irrigation water from reservoir	Farm A	(0/1)	0.0[c]	(0/1)	0.0[c]	(0/1)	0.0[c]	(0/1)	0.0[c]
Irrigation water from reservoir	Farm B	(0/1)	0.0[c]	(0/1)	0.0[c]	(0/1)	0.0[c]	(0/1)	0.0[c]
Irrigation water from reservoir	Farm C	(0/1)	0.0[c]	(0/1)	0.0[c]	(0/1)	0.0[c]	(0/1)	0.0[c]
Irrigation water from tank	Farm A	(0/3)	0.0[c]	(0/3)	0.0[c]	(0/3)	0.0[c]	(0/3)	0.0[c]
Irrigation water from tank	Farm B	(0/1)	0.0[c]	(0/1)	0.0[c]	(0/1)	0.0[c]	(0/1)	0.0[c]
Irrigation water from tank	Farm C	(0/2)	0.0[c]	(0/2)	0.0[c]	(0/2)	0.0[c]	(0/2)	0.0[c]
Irrigation water from pipes	Farm A	(0/2)	0.0[c]	(0/2)	0.0[c]	(0/2)	0.0[c]	(0/2)	0.0[c]
Irrigation water from pipes	Farm B	(0/1)	0.0[c]	(0/1)	0.0[c]	(0/1)	0.0[c]	(0/1)	0.0[c]
Irrigation water from pipes	Farm C	(0/3)	0.0[c]	(0/3)	0.0[c]	(0/3)	0.0[c]	(0/3)	0.0[c]
Environmental swabs	Farm A	(0/8)	0.0[c]	(0/8)	0.0[c]	ND	ND	(0/8)	0.0[c]
Environmental swabs	Farm B	(0/7)	0.0[c]	(0/7)	0.0[c]	ND	ND	(1/7)	14.2[c]
Environmental swabs	Farm C	(0/10)	0.0[c]	(0/10)	0.0[c]	ND	ND	(2/10)	20.0[c]
Total		(10/79)	12.6	(7/79)	8.8	(11/54)	20.3	(22/79)	27.8

ND = Not done

a, b, c in column denote significant differences at $p < 0.05$ using HSD test

Table 5. Prevalence (%) of *Listeria monocytogenes* in freshly harvested vegetables in vegetable farms assessed using four types of enumerative assays

Sample type	Sampling location	Direct-plating		Enrichment plating		Direct PCR		MPN-PCR	
		No.	%	No.	%	No.	%	No.	%
Carrot	Farm A	(0/3)	0.0	(0/3)	0.0	ND	ND	(0/3)	0.0
Yardlong bean	Farm A	(0/3)	0.0	(0/3)	0.0	ND	ND	(0/3)	0.0
Cabbage	Farm A	(0/3)	0.0	(0/3)	0.0	ND	ND	(0/3)	0.0
Tomato	Farm A	(0/4)	0.0	(0/4)	0.0	ND	ND	(0/4)	0.0
Cucumber	Farm A	(0/3)	0.0	(0/3)	0.0	ND	ND	(0/3)	0.0
Carrot	Farm B	(0/4)	0.0	(0/4)	0.0	ND	ND	(1/4)	25.0
Yardlong bean	Farm B	(0/3)	0.0	(0/3)	0.0	ND	ND	(0/3)	0.0
Cabbage	Farm B	(0/3)	0.0	(0/3)	0.0	ND	ND	(1/3)	33.3
Tomato	Farm B	(0/3)	0.0	(0/3)	0.0	ND	ND	(0/3)	0.0
Cucumber	Farm B	(0/4)	0.0	(0/4)	0.0	ND	ND	(0/4)	0.0
Carrot	Farm C	(0/3)	0.0	(0/3)	0.0	ND	ND	(0/3)	0.0
Yardlong bean	Farm C	(0/4)	0.0	(0/4)	0.0	ND	ND	(1/4)	25.0
Cabbage	Farm C	(0/4)	0.0	(0/4)	0.0	ND	ND	(0/4)	0.0
Tomato	Farm C	(0/3)	0.0	(0/3)	0.0	ND	ND	(0/3)	0.0
Cucumber	Farm C	(0/3)	0.0	(0/3)	0.0	ND	ND	(0/3)	0.0
Total		(0/50)	0.0	(0/50)	0.0	–	–	(3/50)	6.0

ND = Not done

Table 6. Prevalence (%) of *Listeria* spp. in environmental and vegetable samples from packing bay and loading bay assessed using four types of enumerative assays

Sample type	Sampling location	Direct-plating		Enrichment plating		Direct PCR		MPN-PCR	
		No.	%	No.	%	No.	%	No.	%
Environmental swabs	Packing House A	(1/10)	10.0	(0/10)	0.0	ND	ND	(3/10)	30.0
Environmental swabs	Packing House B	(0/5)	0.0	(1/5)	20.0	ND	ND	(1/5)	20.0
Carrot	Packing House A	(0/3)	0.0	(0/3)	0.0	ND	ND	(0/3)	0.0
Tomato	Packing House A	(0/3)	0.0	(0/3)	0.0	ND	ND	(0/3)	0.0
Cabbage	Packing House A	(1/3)	33.3	(0/3)	0.0	ND	ND	(1/3)	33.3
Carrot	Packing House B	(0/3)	0.0	(0/3)	0.0	ND	ND	(0/3)	0.0
Tomato	Packing House B	(0/3)	0.0	(0/3)	0.0	ND	ND	(0/3)	0.0
Cabbage	Packing House B	(0/3)	0.0	(0/3)	0.0	ND	ND	(0/3)	0.0
Carrot	Loading Bay	(0/3)	0.0	(0/3)	0.0	ND	ND	(0/3)	0.0
Tomato	Loading Bay	(0/3)	0.0	(0/3)	0.0	ND	ND	(0/3)	0.0
Cabbage	Loading Bay	(1/3)	33.3	(1/3)	33.3	ND	ND	(1/3)	33.3
Yardlong beans	Loading Bay	(0/3)	0.0	(0/3)	0.0	ND	ND	(0/3)	0.0
Sweet potato	Loading Bay	(0/3)	0.0	(0/3)	0.0	ND	ND	(0/3)	0.0
Total		(3/48)	6.3	(2/48)	4.2	–	–	(6/48)	12.5

ND = Not done

and 38.1% respectively. Irrigation water sampled from all points at the three farms was free from the pathogens. *Listeria* spp. and *L. monocytogenes* were also detected in 24% and 12% of swabs done at the three farms respectively. There was a significant difference in the microbial load of soil samples and poultry manure; soil and environmental swabs; soil and irrigation water; poultry manure and irrigation water; poultry manure and environmental swabs at the 0.05 level of significance when the data were analysed statistically.

The prevalence of *Listeria* spp. and *L. monocytogenes* in freshly harvested vegetable samples obtained from the vegetable farms are shown in *Tables 3* and *5* respectively. *Listeria* spp. was detected in cabbages (30%), cucumber (20%), yardlong beans (10%) and carrots (10%) whereas *L. monocytogenes* was detected in cabbages (10%), yardlong beans (10%) and carrots (10%) using the MPN-PCR analysis precedure (*Table 8*). *Table 6* shows the prevalence of *Listeria* spp. in environmental and vegetable samples from the packing houses and loading bays while *Table 7* shows the microbial load of *Listeria* spp. and *L. monocytogenes* enumerated using the MPN-PCR technique.

There was no significant difference between the microbial load of the vegetables and type of vegetable or sampling location when analyzed statistically at a 0.05 level of significance. *Listeria* spp could be detected in 26.7% of swabs carried out at packing houses. *Listeria* spp. was only detected in cabbages (33.3%) sampled from the packing house and loading bay *(Table 6)*.

The microbial load for *Listeria* spp in animal manure and soil enumerated using MPN-PCR was found to range from <3 to 1100 MPN/g whereas for *L. monocytogenes*, it was found to range between <3 and 150 for soil and between <3 and 460 for manure. Both *Listeria* spp and *L. monocytogenes* were detected at low levels in the vegetables and environmental swabs *(Table 7)*.

In terms of analytical efficiency, the MPN-PCR method was more sensitive compared to the other three methods whereas enrichment plating had the least sensitivity.

The present study demonstrated that *Listeria* spp. and *L. monocytogenes* are present at the vegetable farms. Among

Table 7. Microbial load of *Listeria* spp. and *L. monocytogenes* in environmental and vegetable samples enumerated using MPN-PCR assay

Sample type	Sampling location	*Listeria* spp. (MPN/g)			*L. monocytogenes* (MPN/g)		
		Min	Med	Max	Min	Med	Max
Soil	Farms	<3	9.4	1100.0	<3	7.4	150
Manure	Farms	<3	210	1100.0	<3	21.0	460
Irrigation water	Farms	<3	<3	<3	<3	<3	<3
Environmental swabs	Farms	<3	<3	6.7	<3	<3	3.0
Carrot	Farms	<3	<3	6.2	<3	<3	3.0
Yardlong bean	Farms	<3	<3	9.2	<3	<3	3.6
Cabbage	Farms	<3	<3	6.2	<3	<3	3.0
Tomato	Farms	<3	<3	<3	<3	<3	<3
Cucumber	Farms	<3	<3	7.4	<3	<3	<3
Carrot	Packing houses	<3	<3	<3	<3	<3	<3
Tomato	Packing houses	<3	<3	<3	<3	<3	<3
Cabbage	Packing houses	<3	<3	7.2	<3	<3	3
Carrot	Loading bay	<3	<3	<3	<3	<3	<3
Tomato	Loading bay	<3	<3	<3	<3	<3	<3
Cabbage	Loading bay	<3	<3	3.0	<3	<3	3.0
Yardlong bean	Loading bay	<3	<3	<3	<3	<3	<3
Sweet potato	Loading bay	<3	<3	<3	<3	<3	<3

Table 8. Summary of prevalence of contamination of various samples based on MPN-PCR analysis

Type of sample	Prevalence of *Listeria* spp. contamination			Prevalence of *Listeria monocytogenes* contamination		
	Number of samples taken	Number of positive samples	Prevalence (%)	Number of samples taken	Number of positive samples	Prevalence (%)
Animal manure	18	14	77.8	18	11	61.1
Soil	21	10	47.6	21	8	38.1
Swabs	25	6	24	25	3	12
Cabbages	10	3	30	10	1	10
Cucumber	10	2	20	10	0	0
Yardlong beans	10	1	10	10	1	10
Carrots	10	1	10	10	1	10

the environmental samples collected, soil and poultry manure were found to harbour *Listeria* spp. and *L. monocytogenes*.

There have been many reports on the detection of this pathogen in the soil in temperate countries (Weiss and Seeliger 1975; Fenlon et al. 1986) and our results appear to agree with these findings. The presence of the pathogen in manure is also not surprising. Watkins and Sleath (1981) reported *L. monocytogenes* levels at 18,000 CFU/l in trade effluents associated with animal and sewage sludge after treatments, and suggested that biological oxidation may not be an effective method for eliminating viable *Listeria* in sewage.

Water used for irrigation purposes is also a potential source of *L. monocytogenes* contamination and the pathogen has been detected in surface water and groundwater (Watkins and Sleath 1981; Djikstra 1982). However, in this study all irrigation water samples collected were free from *Listeria* spp. Nonetheless, the possibility of water as

a potential source of contamination cannot be excluded as the lowest detection limit among the four methods applied in this study was 10^2 cells/ml.

Therefore, the method used in this study would be unable to detect water samples with less than 100 cells/ml. Thus the detection of *Listeria* spp in water may require a more sensitive method.

Vegetables from the farm were found to be contaminated with *Listeria* spp. and *L. monocytogenes* at a prevalence of 16% and 6% respectively using MPN-PCR. The vegetables that were found to contain *Listeria* spp. and *L. monocytogenes* were cabbages, carrots, cucumber and yardlong beans. The microbial load was generally found to be low with the maximum count being 1100 MPN/g. *Listeria monocytogenes* has previously been isolated from produce such as cabbage, corn, lettuce, sprouts, cucumber, parsley and potatoes (Wong et al. 1990; Beuchat 1991; Odumeru et al. 1997).

The present study found considerable difference in the prevalence of *Listeria* assayed using different analytical methods. Many studies on *L. monocytogenes* contamination in produce have found little or no incidence of the bacteria in the samples (Kaneko et al. 1999; Pingulkar et al. 2001). Obvious difficulties arise when the food sample contains small populations of *L. monocytogenes* in combination with large numbers of other indigenous microoganisms. Outbreaks of foodborne listeriosis coupled with high mortality rates have underscored the need for faster and more efficient methods to detect small numbers of *Listeria* in a wide range of foods.

The advent of polymerase chain reaction has made detection of pathogenic microorganisms easier since it targets virulence genes that are unique to the pathogen resulting in high specificity, sensitivity, rapid turnover and ease of automation. In this study, the MPN-PCR method was more effective in detecting the pathogen compared to other conventional methods as it combines an enrichment culture step and the PCR method. The molecular method would detect non-viable cells as well as viable cells whereas the culture method would only detect cells capable of proliferation. The culture method has a drawback in that it is not possible to distinguish between *L. monocytogenes* and other *Listeria* spp. on PALCAM agar. The standard method requires five colonies to be picked up for confirmation. If the *L. monocytogenes* is considerably outnumbered by other *Listeria* spp., then the chances of a positive result are considerably reduced.

This study was designed to generate baseline information on the possible routes of *Listeria* spp and *L. monocytogenes* entry into vegetables at the pre-harvest stage in Malaysia. To obtain representative data, sampling was conducted at Cameron Highlands, Pahang which is the primary farming area in the country that contributes to a large portion of the domestic vegetable supply. Sampling was carried out at three farms that were selected at random. Environmental samples and freshly harvested vegetable samples were also taken at random where possible but there were some constraints since it was only possible to take vegetables that were at the correct stage of maturity at the selected farms at the time of the visit. Nevertheless, the findings in the present study provide baseline data for future risk assessments and the results show the potential risk posed by *L. monocytogenes* in raw vegetables.

Conclusion

It was found that MPN-PCR assay was more effective in detecting *Listeria* spp. and *L. monocytogenes*. Among the vegetables taken from the various farms, cabbages, cucumber and yardlong beans had the highest prevalence of *Listeria* spp. There was no significant difference in the prevalence or microbial load between the farms. The findings of this study highlights the potential of soil and poultry manure as sources of contamination of raw vegetables.

Further research should be carried out to study the role played by various factors along the production chain from pre-harvest, harvesting, processing to distribution.

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Abstrak

Tujuan kajian ini adalah untuk menyiasat kehadiran *Listeria* spp. dan *L. monocytogenes* di dalam tanah, baja ayam itik, air pengairan dan sayur-sayuran yang baru dituai daripada tiga kebun sayur di Cameron Highlands. Sebanyak 177 sampel termasuk sampel daripada persekitaran dan sampel sayur-sayuran diambil. Antara sampel persekitaran (n = 94), baja ayam itik didapati mengandungi kehadiran *Listeria* spp. dan *L. monocytogenes* yang tinggi masing-masing pada tahap 77.8% dan 61.1% menggunakan kaedah analisis MPN-PCR. Sampel tanah juga didapati mengandungi *Listeria* spp. dan *L. monocytogenes* masing-masing pada tahap 47.6% dan 38.1%. Sampel air pengairan di dalam kebun didapati bebas daripada *Listeria* spp. Di ketiga-tiga kebun, *Listeria* spp. dan *L. monocytogenes* masing-masing dikesan pada 24% dan 12% calitan yang dilakukan. Antara sayur-sayuran yang baru dituai, *Listeria* spp. dikesan di dalam kubis (30%), timun (20%), kacang panjang (10%) dan lobak merah (10%), sementara *L. monocytogenes* dikesan di dalam kubis (10%), kacang panjang (10%) dan lobak merah (10%) menggunakan kaedah analisis MPN-PCR. Keputusan ini menunjukkan bahawa *Listeria* spp. merupakan faktor risiko bagi sayur-sayuran yang dimakan mentah di Malaysia. Kajian ini juga menyumbang data asas mengenai pencemaran *Listeria* spp. di kebun sayur.

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