ORIGINAL ARTICLE

Incidence of *Listeria* spp. in Vegetables in Kuala Lumpur

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Summary

From April 1992 to September 1992, 280 samples of 10 different fresh vegetables, bought from four different market outlets in Kuala Lumpur were examined for the presence of *Listeria* spp. Most of the market produce were locally grown with the exception of carrots. The isolation procedure was based on the Food & Drug Administration method (modified) used for the detection of *Listeria* spp. Isolation media used were Listeria Selective medium and LiCl- phenylethanol-Moxalactam agars. The identification of isolates was by means of conventional biochemical tests and API Listeria identification system. Five out of the 280 samples showed Listeria contamination, *Listeria monocytogenes* was isolated in lettuce, sengkuang (*Pachyrrhizus erosus*) and selom *Oenanthe javanica*) and *Listeria innocua* was isolated from sengkuang (*Pachyrrhizus erosus*) and pegaga (*Hydrocotyle asiatica*).

Key Words: Listeria monocytogenes, Incidence, Local vegetables, Root crops

Introduction

Listeria monocytogenes is a recently recognised food borne pathogen which has been of great concern to the food industry. The WHO Informal Working Group on Food Listeriosis has identified four major food commodities implicated in food borne listeriosis i.e. milk and dairy products, meat especially raw meat products, vegetables and salads and seafoods[1].

Outbreaks of listeriosis in humans due to the consumption of contaminated foods such as cheese, pasteurised milk and coleslaw in the past decade had received much attention. Investigations into these outbreaks had shown evidence that coleslaw and salads were the vehicles of transmission[2,3]. Raw vegetables such as cabbage, celery, lettuce and tomatoes used in their preparations were implicated.

Listeriosis is a rare illness and clinical features which range from mild influenza-like illness to meningitis and meningoencephalitis are encountered[4]. Symptoms experienced by patients include fever, vomiting, diarrhoea, muscle aches and abdominal pain[5]. The pathogenesis of *L. monocytogenes* in humans remains unclear and exposure to the organism does not always result in disease. Elderly patients, pregnant women, newborns and individuals with poor cell-mediated immunity are primarily the main victims of this infection[6].

In Malaysia, there has been no reported cases of food borne listeriosis and this absence may be due to nonrecognition of the condition. A local study showed that between 19 - 50% of our retail beef and poultry meat products sold in Malaysian wet markets and supermarkets were contaminated with *L. monocytogenes* [7].

The purpose of this study is to determine the incidence of *L. monocytogenes* in fresh vegetables available at the local markets and to compare various media used for its isolation.

Materials and Methods

A total of 280 samples of vegetable produce from three wet markets and one supermarket were examined using the Food and Drug Administration modified method of isolation for *Listeria* spp[8].

Fresh cabbage, lettuce, carrots, cucumber, sengkuang (yam bean)[9], tomatoes, spring onions, celery, pegaga (Indian pennywort) and selom (*Oenanthe javanica*) were obtained from the market and were processed within an hour after purchase. Without any further rinsing and cleaning, outer portions of the vegetables were cut and weighed[10]. Twenty-five gms of each vegetable were added to 225 ml of Oxoid Listeria Selective Enrichment Broth (CM 862) containing a selective supplement (SR 141) and homogenised in a stomacher for one minute, and incubated at 30°C.

After 24, 48 hours and seven days' incubation, the enrichment broths were streaked onto Oxoid Listeria Selective Medium (Oxford formulation) CM 856, prepared by adding Oxoid Listeria selective supplement SR 140 (LSM), Difco LiCl - phenylethanol - Moxalactam Agar with Oxoid MOX supplement SR 157 (LPM) and 10% Ox-Blood Agar (BA). (Note: Oxoid MOX supplement containing 7.5 mg Moxalactam and 5.0 mg Colistin was added to every 500 ml LPM agar base). Another 1 ml of the enrichment broth (EB) was diluted with 9 ml of 0.5% KOH and was similarly streaked onto another set of LSM, LPM and BA respectively.

All plates were incubated at 30°C and examined at 24 hrs and 48 hrs. The LPM were further incubated for another three days and examined.

Two strains of *L. monocytogenes* obtained from the IMR Bacteriology Division, Freeze-drying Unit were used as positive controls and a negative control was an uninoculated Broth.

Suspicious colonies from the three culture media which showed typical colonial morphology were picked.

From the LSM, colonies measuring 1.5 - 2 mm in diameter, discrete, slightly raised, smooth colonies with black zones surrounding them were picked. The LPM plates were scanned with a oblique transmitted light dissecting scope, and small colonies approximately 1 mm that appeared bluish in colour with the crushed glass appearance were identified. Shining discrete colonies showing beta hemolysis on Blood Agar plates were also examined. Initial tests such as Gram's stain, catalase test, motility test (Hanging drop at 25 - 30°C) and production of beta hemolysis were initially done for all suspicious isolates.

Isolates that were gram positive short regular rods, catalase positive, beta hemolytic and showed tumbling motility were then tested for growth at 35°C, oxygen requirement (OF), hydrogen sulphide production, acid production from glucose, Methyl red and Voges Proskauer reactions, indole production, citrate utilisation and urease activity as given by Seeliger *et al*[11]. In addition to these tests, other confirmatory biochemical characteristics for *Listeria* spp. like nitrate reduction, growth at 4°C and growth in 10% NaCl, sodium hippurate hydrolysis, tellurite reduction, carbohydrate fermentation of mannitol, maltose, lactose, sucrose, aesculin hydrolysis, "umbrella motility" in semi-solid agar, oxidase test and gentamicin susceptibility[12] were also observed. Species differentiation of *L. monocytogenes* from other *Listeria* spp. was based on CAMP test, fermentation patterns with rhamnose, xylose, alpha-methyl-D-mannoside and mannitol[12]. The API Listeria system for the identification of *Listeria* spp. was also employed.

The growth of *Listeria* as well as microbial contaminants was scored as NG - no growth; Few = 1-10 colonies; 1+ = 11-50 colonies; 2+ = 50-100 colonies; 3+ = >100 colonies. These estimations were recorded on all solid media after 24 hours' and 48 hours' incubation with/without the KOH treatment during the different enrichment times.

Results

Out of the 10 varieties of vegetables tested, *Listeria* spp. was isolated from four types (Table I). The vegetables were yam bean (sengkuang), *Oethanthe javanica* (selom), Indian pennywort (pegaga) and lettuce. Sengkuang, a root crop appears to have a slightly higher incidence of

Table I
Isolation of *Listeria* from vegetables tested on LSM, LPM and BA

Vegetable	No. of samples	No. of positives (%)	
		L. Monocytogenes	*Other Listeria*
Yam bean (sengkuang)	28	1 (3.6%)	1 (3.6%)
Oenanthe javanica (selom)	28	1 (3.6%)	0
Lettuce	28	1 (3.6%)	0
Indian pennywort	28	nil	1 (3.6%)

Table II
Semiquantitative growth of contaminants on LSM, LPM and BA after 24 hours' and 48 hours' incubation with/without KOH treatment

Without KOH treatment

Medium	24 hrs' incubation		48 hrs' incubation	
	0-10 col	≥11 col	0-10 col	≥11 col
LSM (840)	432 (51%)	408 (49%)	378 (45%)	462 (55%)
LPM (780*)	671 (86%)	109 (14%)	457 (59%)	323 (41%)
BA (820**)	3 (<1.0%)	817 (>99.0%)	3 (<1.0%)	817 (>99.0%)

With KOH treatment

Medium	24 hrs' incubation		48 hrs' incubation	
	0-10 col	≥11 col	0-10 col	≥11 col
LSM (840)	641 (76%)	199 (24%)	578 (69%)	262 (31%)
LPM (780*)	737 (94%)	43 (06%)	638 (81%)	142 (19%)
BA (820**)	15 (2.0%)	805 (98.0%)	13 (2.0%)	807 (98.0%)

*Note: * LPM was not introduced to the first two batches of samples of vegetables owing to the delayed delivery of medium*
*** Two subcultures of BA were not done owing to contamination of BA during the specific days of subcultures.*

Listeria contamination compared with other vegetables. No *Listeria* spp. were isolated in cabbage, cucumbers, celery, tomatoes, spring onions and carrots.

Only two species of *Listeria*, namely, *L. monocytogenes* and *L. innocua* were isolated from the 280 vegetables samples analysed. *L. monocytogenes* was recovered in two of the 70 samples (2.8%) taken from the one supermarket outlet and one isolate of *L. monocytogenes* was found in 210 samples (0.5%) from the three wet markets. The overall isolation rate of *L. monocytogenes* is 1.1%.

From the five test samples positive from *Listeria* spp., one sample was positive for *Listeria* spp. after 48 hours' of enrichment and the rest took seven days' of enrichment before they yielded *Listeria* growth. The results were not affected by KOH treatment.

The API Listeria System used for the identification of *Listeria* spp. matched the Numerical profile of 6510 for *L. monocytogenes* and 7510 for *L. innocua* in the Profile List.

Table II shows the semiquantitative scoring of growth of contaminants, where Blood Agar was found to be least useful in isolation of *Listeria*. LSM was moderately selective and LPM the most selective. This inhibitory effect was further improved with KOH treatment.

Discussion

The incidence of *Listeria* contamination (1.1%) in the local vegetable produce is relatively lower than that reported in other countries. A literature review[13] cited an incidence of *Listeria* contamination of 18% in vegetables with a 5% incidence of *L. monocytogenes*. Heisick *et al*[10] reported 9.5% (95/1000) incidence of *Listeria* contamination in her 1000 vegetables samples analysed with a 5% incidence of *L. monocytogenes* (50/1000). Chao of Taiwan[14] detected a 12.2% incidence of *L. monocytogenes* in the 49 samples of domestic vegetables investigated. Out study gave a lower prevalence.

Possible reasons for this lower recovery may be attributed to the freshness of the vegetables and climatic factors. The amount of soil contact has also been reported to affect the recovery in freshly grown vegetables. Heisick and her co-workers observed that root crops[10] like radishes and potatoes demonstrated significant amount of *Listeria* contamination compared to other crops like broccoli, cauliflower and tomatoes. This lower contamination in these vegetables may be due to the crops having less contact with the soil in which they were grown. Locally grown vegetables like cucumber, spring onions, celery and tomatoes similarly, too, did not demonstrate the presence of *Listeria* spp.

The 28 samples of each of the 10 vegetables were taken randomly during the six months' period. It may be postulated here that the *Listeria* spp. may be environmentally stressed and together with the ambient tropical temperatures, its proliferation may be inhibited. Environmental conditions like temperature and humidity changes, differences in soil pH and the amount of sunlight exposure which *Listeria* species are subjected to may result in 'physiologically injured' microbial cells.

In addition to these, the natural flora present on the freshly harvested vegetables may have outgrown the *Listeria* spp., thus contributing to its poor isolation. It was believed that prolonged cold storage[3] will allow a small initial inoculum of *L. monocytogenes* to proliferate and cause a dying off of competitive micro-organisms. This kind of situation rarely occurs with Malaysian vegetables which are normally harvested and sold within 48 hours. Coincidentally, chilled fresh vegetables (0-4°C) purchased from the supermarket gave a slightly better recovery rate, with two Listeria positive out of the 70 samples analysed (2.9%). However, this is not statistically significant (P value = 0.5). It is assumed here that the refrigerated vegetables were kept for a longer period of time than that experienced in the wet markets, thus resulting in better recovery. A study[15] showed that prepacked salads sold in a supermarket demonstrated a two-fold increase of *L. monocytogenes* when left at 4°C for four days.

Natural flora in food products often complicate the detection of *Listeria* and there is no single known isolation medium which is satisfactory for all kinds of food. Both LSM[15] and LPM[16] are reputedly well established isolation media for *L. monocytogenes*.

Our results (Table II) showed LPM was a better isolation medium for *Listeria* spp. in vegetables than LSM. LPM has very good inhibitory properties as most natural flora found growing on B.A. are hardly detected on LPM. Its main disadvantage lies on the cumbersome use of a dissecting microscope (45° oblique transillumination) for detecting suspicious colonies. Enterococci and some gram positive organisms may give the same blue crushed glass appearance as that produced by the *Listeria* spp., and may lead to some erroneous initial identification. We were unable to recover the *Listeria* spp. colonies after 16-20 hours of incubation at 30°C as reported by Lee[17] and most of our recovery were obtained after 48 hours' incubation.

Contamination with other microbial flora was observed (Table II). The predominant contaminants seen were Staphylococci (large yellow colonies on LSM), enterococci and other gram positive organisms like *Bacillus* and diptheroids which were present on all three media. Gram negative bacteria were mostly found on Blood agar. Care must be exercised in the examination of suspicious listerial colonies on LSM, as enterococci, some aerobic sporeformers and diphtheroids may also give black colonies with blackish discoloration as *Listeria.* BA is not a good medium for isolating *Listeria* with microbial flora owing to its noninhibitory properties. Pretreatment with 0.5% KOH did help to reduce the amount of natural flora (Table II) in all the three media but it did not improve the recovery of *L. monocytogenes* as reported in another study with raw milk samples[18]. The ineffectiveness of 0.5% KOH in our study could be due to the presence of large amount of natural flora and the effect of suppressive substances in the vegetables.

It must be noted here that our first isolation of *L. monocytogenes* was obtained on LSM after 24 hours incubation and the colonies were easily recognisable while the same sample took 48 hours' incubation before the colonies were discernable on the LPM.

All five positive isolates were obtained after 48 hours' incubation or seven days' incubation in the EB and majority of the isolates were detected on LPM. Four out of five of the isolates required seven days' incubation in the EB, thus demonstrating a longer incubation period is needed. By extending the incubation period to one week at 30°C would allow sublethally injured listerial cells to recover[19] sufficiently for its isolation. Another study reported that to ensure full resuscitation of heat injured or sublethally injured listerial cells, it would be prudent to inoculate first into a nonselective medium 6-8 hours before transferring into a selective enrichment broth as EB[20].

In conclusion, this small study shows *L. monocytogenes* is present in our local market vegetables. The isolation medium for *Listeria* spp. in vegetables seem to be better with the use of LPM. Although the incidence of *Listeria* contamination is lower than that reported abroad, it is recommended that more studies be made on other food products, domestic and imported. With a more comprehensive list of foods and their contamination rates, a new awareness of the dangers towards this foodborne pathogen may be instilled among the local food suppliers and industries and consumers. Careful cleaning and washing of raw vegetables before consumption must be emphasised, especially so in our society where 'ulam' and 'rojak' are commonly served. Such good practices would go a long way in helping to prevent potential foodborne listeriosis in Malaysia.

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