International Food Research Journal 18: 459-463 (2011)

Biosafety assessment of *Listeria monocytogenes* in vegetarian burger patties in Malaysia

[1*]Wong, W. C., [1]Pui, C. F., [1]Chai, L. C., [1]Lee, H. Y., [1]Ghazali, F. M., [2]Tang, J. Y. H., [3]Ponniah, J., [3]Tuan Zainazor, T. C., [4]Cheah, Y. K., and [1]Son, R.

[1]*Center of Excellence for Food Safety Research, Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia*
[2]*Faculty of Food Technology, Universiti Sultan Zainal Abidin, Jalan Sultan Mahmud, 20400 Kuala Terengganu, Terengganu, Malaysia*
[3]*National Public Health Laboratory, Ministry of Health Malaysia, Lot 1835 Kampung Melayu, 47000 Sungai Buloh, Selangor Darul Ehsan, Malaysia*
[4]*Department of Biomedical Science, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia*

Abstract: The aim of this study was to examine vegetarian burger patties manufactured by two producers in Malaysia for the presence of *Listeria monocytogenes*. Brand A was produced by an established food manufacturer while Brand B was produced by a small-scaled food producer. A total of 108 samples of vegetarian burger patties produced by both manufacturers were sampled from retail market and were analyzed by combined MPN-PCR and MPN plating method. Of all the samples tested, ten (9.3%) were found to be contaminated with *L. monocytogenes*. The *L. monocytogenes* contamination level in vegetarian burger patties manufactured by producer A (20.9% of the samples were contaminated with 3-1100 MPN/g of *L. monocytogenes*) was significantly higher ($P<0.05$) than vegetarian burger patties manufactured by producer B (1.5% of the samples harbored 9.2 MPN/g of *L. monocytogenes*). Based on the detection and isolation rate obtained with MPN-PCR and MPN-plating, the recovery rate of the *L. monocytogenes* was estimated to be only 40.0% by MPN-plating approach.

Keywords: *L. monocytogenes*, prevalence, vegetarian burger patty, MPN-PCR, recovery rate

Introduction

Interest in vegetarianism appears to be increasing nowadays due to health reasons. In United Kingdom, the number of people who claimed to be vegetarian has increased dramatically from 0.2% in the 1940s (Spencer, 1994) to between 3 and 7% of the population in the year 2000 (Povey *et al*., 2001; Robinson, 2001). In the United States, a poll conducted by The Vegetarian Resource Group (Stahler, 2006) showed that 2.3% of the adult population consistently follows a vegetarian diet and 1.4% follows a strict vegan diet. In India, the population who follow a strict vegetarian diet is approximately 35% (International Vegetarian Union, 2006).

In Malaysia, there is no published data showing the percentage of vegetarians in the population. However, the expansion of vegetarian meals and frozen vegetarian food market indicates increasing demand for vegetarian food products, and hence indicate an increase in the number of vegetarians in the nation. Besides producing a vast variety of vegetarian products from soup to nuts, food manufacturer also bear the responsibility of ensuring the wholesomeness of the products. However, the widespread presence of *Listeria monocytogenes* in incoming ingredients as well as the ability of the bacterium to withstand wide range of pH, salt concentrations and storage temperatures results in its persistence in food processing environments (Liu *et al*., 2004).

In recent decades, *L. monocytogenes* has emerged as a significant foodborne pathogen. Most human foodborne infections are associated with high incidence rate but low morbidity and mortality rate. However, this is opposite for human listeriosis, which is a relatively rare but potentially severe infection, associated with a fatality rate of up to 30% (Lorber, 1997). Although the pathogens are normally killed by pasteurization, or other heating procedures, the possibilities for cross-contamination or undercooking are reasons for concern.

To date, many studies on biosafety of *L. monocytogenes* in various foods have been carried out (Ferguson and Shelef, 1990; Arumugaswamy *et al*., 1994; Beuchat, 1996; Aparecida de Oliveira *et al*., 2010; Ponniah *et al*., 2010). Several reports

*Corresponding author.
Email: *woanchwen85@hotmail.com*
Tel: +603-89468361; Fax: +603-89423552

on various food pathogens in Malaysia also have been reported (Tan *et al*., 2008; Tang *et al*., 2009; Jeyaletchumi *et al*., 2010; Suzita *et al*., 2010; Tunung *et al*., 2011) but there is no published data showing the prevalence of any of these pathogens vegetarians foods products. The objective of this study was to carry out a preliminary study on the occurrence of *L. monocytogenes* in vegetarian burger patties produced by two different manufacturers, by using the MPN-PCR method and MPN-plating method.

Materials and Methods

Sample

In this study, 'vegetarian burger patty' refers to a disc-shaped, compact serving of a meat substitute that is commercially available in frozen state in several retail shops in Malaysia. It is normally fried and served with burger buns as a 'vegetarian burger'. Generally it is made of soy protein, gluten, mushroom, and flavor enhancers.

Sample preparation

A total of 108 vegetarian burger patties were sampled from June to October 2009 and investigated for the presence of *L. monocytogenes*. Samples were transported to the laboratory in chilled condition and analyzed immediately. These samples consisted of 43 pieces of brand A and 65 pieces of brand B vegetarian burger patties. The two brands differed in terms of manufacturers. Brand A was manufactured by a large registered local manufacturer in Selangor, Malaysia; while Brand B was manufactured by a small-scale local food industry. Brand B did not fully conform to local food labeling regulations because some important information such as expiry date was not found. The samples were analyzed using the procedure adapted from FDA-BAM Standard for detection of *Listeria*, with modification (Ponniah *et al*., 2010).

Pre-enrichment

A sample was cut into small pieces and 10 g portion was homogenized with 90 ml of *Listeria* Enrichment Broth Base (Merck) for 120 s. At the fourth hour of incubation at 30°C, selective agents were added: acriflavin (final concentration: 10 mg/l); sodium nalidixate (final concentration: 40 mg/l); and cycloheximide (final concentration: 50 mg/l) (Sigma).

Detection and Enumeration of L. monocytogenes *by MPN-PCR*

Enriched broth (10^{-1}) was made up to 100-fold (10^{-2}) and 1000-fold (10^{-3}) dilution. For three-tube MPN method, 1 ml aliquot from each dilution was transferred into triplicate MPN tubes. All of the turbid MPN tubes after 48 h of incubation (30°C) were subjected to DNA extraction by boiled-cell method (Chai *et al*., 2007) with modification. The content in MPN tube was centrifuged at 10,000 g for 5 min to pellet the microorganisms. The pellet was resuspended in 500 µl of sterile distilled water and boiled for 10 min. After boiling, the content was cooled at -20˚C immediately for 10 min and then centrifuged at 10,000 g for 10 min. The supernatant that contained the template DNA was used in PCR assay. Two pairs of primers were used in this study (Border *et al*., 1990). Primer pairs of 5'-CCT-AAG-ACG-CCA-ATC-GAA-3' and 5'-AAG-CGC-TTG-CAA-CTG-CTC-3' was used to target *hlyA* gene at 702-bp region whereas primer pairs of 5'-CAG-CMG-CCG-CGG-TAA-TWC-3' and 5'-CTC-CAT-AAA-GGT-GAC-CCT-3' was designed to amplify 16S rRNA at 938-bp region. PCR amplification was performed in 25 µl of a reaction mixture: 5 µl of 5× PCR buffer, 1.5 mM $MgCl_2$, 0.08 mM dNTP mix, 1.5 U *Taq* Polymerase, 10 pM of each primers and 2 µl of DNA template. Amplification of DNA segment was performed with the Veriti 96-Well Thermal Cycler (Applied Biosystems) using the condition: initial denaturation (94˚C for 5 min); 30 cycles of denaturation at 94˚C (30 s), annealing at 53˚C (1 min) and elongation at 72˚C (2 min); followed by a final extension (72˚C for 7 min). *L. monocytogenes* ATCC 19155 was used as the positive control during PCR amplification. 5 µl of PCR products were loaded on 1.0% agarose gel (stained with ethidium bromide) and electrophoresized at 100 V for 30 min. The gel was viewed under UV light.

Isolation of L. monocytogenes *on culture media*

Prior to DNA extraction, culture from MPN tube was streaked onto Palcam agar and incubated at 30°C for 48 h. *L. monocytogenes* grows on Palcam agar as black-centered grayish-green sunken colony. At least five presumptive colonies were selected and purified onto Tryptose Soy Agar and subjected to PCR assay for confirmation.

Data analysis

The resulting data were subjected to Chi-Square test using SPSS software (version 16.0) to determine significant difference between the different brands of vegetarian burger patty. The sample that showed positive result upon plating was compared to MPN-PCR positive sample to determine the recovery rate of *L. monocytogenes* with the following equation:

$$\text{Recovery rate} = \frac{\text{Plating positive sample}}{\text{MPN - PCR positive sample}} \times 100\%$$

Results and Discussion

L. monocytogenes was detected in vegetarian burger patties in this study at a prevalence of 9.3%. The results of the detection and enumeration for *L. monocytogenes* in burger patties from different brands by using different methods are shown in Table 1. For brand A, *L. monocytogenes* was detected at a prevalence of 20.4% and 9.3%, using MPN-PCR and MPN-plating method, respectively. However for brand B, *L. monocytogenes* was only detected in 1 out of 65 (1.5%) using MPN-PCR method; and none was detected by MPN-plating method. The microbial load for *L. monocytogenes* in tested samples was found to range from 3 to more than 1100 MPN/g. Of ten MPN-PCR positive samples, almost 80% of positive samples harbored ≤19 MPN/g of *L. monocytogenes*.

Table 1. Prevalence (%) and microbial load (MPN/g) of *L. monocytogenes* in raw vegetarian burger patties, showed by both MPN-PCR and MPN-plating method

Brand	Number of sample tested	Number of MPN-PCR positive sample (Percentage, %)	Microbial load (MPN/g)			Number of MPN-plating positive sample (%)
			Min	Med	Max	
A	43	9 (20.4)	3	16	>1100	4 (9.3)
B	65	1 (1.5)	9.2	9.2	9.2	0 (0)

This finding is quite surprising especially to vegetarians group since their thought on vegetarian food products is generally healthier and more hygienic. But somehow, when look into their main ingredients, contamination is possible which might originated from the plant protein sources which include soy bean and mushroom. A study done by Welshimer and Donker-Voeat (1971) showed that *L. monocytogenes* was found in soil and decaying vegetation, especially dead and decayed soybean plants. On the other hand, *L. monocytogenes* has also been detected in mushrooms in a study carried out in Norway (Johannessen *et al*., 2002). In fact, Beuchat (1996) debated that *L. monocytogenes* is the most frequent pathogen associated with fresh produce among the foodborne pathogens. A number of studies have shown that *L. monocytogenes* is a common contaminant in fresh produce (Beuchat, 1996; Ferguson and Shelef, 1990; Aparecida de Oliveira *et al*., 2010). Several studies carried out in Malaysia also reported the incidence of *L. monocytogenes* in fresh produce (Arumugaswamy *et al*., 1994; Ponniah *et al*., 2010).

Montville and Matthews (2008) described the transmission route of *L. monocytogenes* from the surrounding environment into food handling areas through soil on workers' shoes and clothing, as well as naturally contaminated raw materials. This bacterium tends to attach onto surfaces such as stainless steel, glass, and rubber. At the same time, high humidity and nutrient levels inside food processing line support and promote listerial growth (Montville and Matthews, 2008). In the present study, *L. monocytogenes* was found to be more prevalent in brand A (20.9%) than brand B (1.54%) at the significant level of $P < 0.05$. This result suggested that the microbiological standard in well-established food processing plant (producer A) might be lower than the small-scaled food manufacturer (producer B). However, on-site investigation is necessary, before we can draw a conclusion on the hygienic status of these two manufacturing plants.

L. monocytogenes in food is an ongoing biosafety concern worldwide especially for the food manufacturing premise. As early as 1980s, extensive reviews had been done by numerous researchers on the persistence of *L. monocytogenes* in foods (Doyle *et al*., 1985; Grau and Vanderline, 1990; Ryser and Marth, 1988; Ryser *et al*., 1985). Beside the ability to grow over wide temperature and pH ranges, its biofilm formation ability also causes *L. monocytogenes* to persist in the food processing line. Development of biofilm on food handling or processing surfaces and food storage areas is a major concern as this can cause product contamination. Furthermore, according to Mah and O'Toole (2001) and Lewis (2001), elimination of biofilm from food processing environments is particularly difficult because they are more resistant to sanitizers and disinfectants compared to planktonic cells.

Out of ten MPN-PCR positive samples, only 40.0% of *L. monocytogenes* was assumed successfully recovered and isolated on culture media. This could be due to the bacterium's ability to enter VBNC (viable but nonculturable) state as a result of environmental stress which are lethal if the cells do not get into dormancy state, such as storage at temperature outside the range of growth, changes in osmotic concentration due to addition of seasoning or flavoring compounds (Oliver, 2000). VBNC cells are unable to grow and

form colony on routine bacteriological media on which they would normally grow but remain alive (Oliver, 1991). Thus far, it is uncertain if VBNC cells can cause human infection. However, it is possible to resuscitate VBNC to active cells and subsequently initiate infection in human host. This is of particular importance if the food safety inspection body still employs the conventional culture-dependent method in investigation of microbiological quality of food, as it tends to give false negative results. Moreover, Shearer *et al*. (2001) stated that PCR-based methods were better in detecting pathogens in contaminated foods as compared to culture-dependent methods due to their higher sensitivity. In recent year, MPN-PCR method has been widely applied in many studies to report the presence of foodborne pathogen in various foods quantitatively and qualitatively (Chai *et al*., 2007; Chai *et al*., 2009; Ponniah *et al*., 2010; Pui *et al*., 2010; Tunung *et al*., 2010).

L. monocytogenes was detected in vegetarian burger patties in this study. Although the incidence rate is comparatively lower than other foods, it could still pose a serious health risk to consumers who consumed undercooked contaminated vegetarian burger patties, especially pregnant women, the elderly and immune-compromised individuals. The present study showed that MPN-PCR is more useful and effective for detection of *L. monocytogenes* as compared to MPN plating method. Hence, the usage of PCR method in current practices is highly recommended in order to obtain more precise and reliable result.

Acknowledgement

This study was supported by Science Fund from the Ministry of Science, Technology and Innovation, Malaysia.

References

Aparecida de Oliveira, M., Guimarães Abeid Ribeiro, E., Maria Morato Bergamini, A. and Cristina Pereira De Martinis, E. 2010. Quantification of *Listeria monocytogenes* in minimally processed leafy vegetables using a combined method based on enrichment and 16S rRNA real-time PCR. Food Microbiology 27: 19-23.

Arumugaswamy, R. K., Ali, G. R. R. and Hamid, S. N. B. A. 1994. Prevalence of *Listeria monocytogenes* in foods in Malaysia. International Journal of Food Microbiology 23: 117-121.

Beuchat, L. R. 1996. Pathogenic microorganisms associated with fresh produce. Journal of Food Protection 59: 204-216.

Border, P. M., Howard, J. J., Plastow, G. S. and Siggens, K. W. 1990. Detection of *Listeria* species and *Listeria monocytogenes* using polymerase chain reaction. Letters in Applied Microbiology 11: 158-162.

Chai, L. C., Ghazali, F. M., Bakar, F. A., Lee, H. Y., Suhaimi, L.R. A., Talib, S. A., Nakaguchi, Y., Nishibuchi, M. and Radu, S. 2009. Occurrence of Thermophilic *Campylobacter* spp. contamination on vegetable farms in Malaysia. Journal of Microbiology and Biotechnology 19: 1415-1420.

Chai, L. C., Robin, T., Usha, M. R., Jurin, W. G., Fatimah, A. B., Mohammad Ghazali, F., Radu, S. and Pradeep, M. K. 2007. Thermophilic *Campylobacter* spp. in salad vegetables in Malaysia. International Journal of Food Microbiology 117: 106-111.

Doyle, M. O., Meske, L. M. and Marth, E. H. 1985. Survival of *Listeria monocytogenes* during the manufacture and storage of nonfat dry milk. Journal of Food Protection 48: 740-742.

Ferguson, R. D. and Shelef, L. A. 1990. Growth of *Listeria monocytogenes* in soy milk. Food Microbiology 7, 49-52.

Grau, F. H. and Vanderline, P. B. 1990. Growth of *Listeria monocytogenes* on vacuum-packaged beef. Journal of Food Protection 53: 739-741.

International Vegetarian Union. 2006. 37th IVU World Vegetarian Congress Goa, India, September 10-16, 2006. Healthy Lifestyle Vegetarian Way! Available at *http://www.ivu.org/congress/2006/whyindia.html.* Accessed on 9 June 2010.

Jeyaletchumi, P., Tunung, R., Margaret, S. P., Son, R., Ghazali, F. M., Cheah, Y. K., Nishibuchi, M., Nakaguchi, Y., and Malakar, P. K. 2010. Quantification of *Listeria monocytogenes* in salad vegetables by MPN-PCR. International Food Research Journal 17: 282-286.

Johannessen, G. S., Loncarevic, S. and Kruse, H. 2002. Bacteriological analysis of fresh produce in Norway. International Journal of Food Microbiology 77: 199-204.

Lewis, K. 2001. Riddle of biofilm resistance. Antimicrobial Agents and Chemotherapy 45(4): 999-1007.

Liu, D. Y., Lawrence, M. L., Ainsworth, A. J. and Austin, F.W. 2004. Comparative assessment of acid, alkali and salt tolerance in *Listeria monocytogenes* virulent and avirulent strains. FEMS Microbiology Letters 243: 373-378.

Lorber, B. 1997. Listeriosis. Clinical Infectious Diseases 24: 1-9.

Mah, T. F. and O'Toole, G. A. 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends in Microbiology 9(1): 34-39.

Montville, T. J. and Matthews, K. R. 2008. Food Microbiology, pp. 173-188. ASM Press, Wahington, DC.

Oliver, J. D. 2000. The viable but nonculturable state and cellular resuscitation. In Bell, C. R., Brylinsky, M., and Johnson-Green, P. (Eds.), Microbial Biosystems: New Frontiers, pp. 723-730. Atlantic Canada Society for Microbial Ecology, Halifax, Canada.

Oliver, J. D., Nilson, L. and Kjelleberg, S. 1991. The formation of nonculturable cells of *Vibrio vulnificus* and its relationship to the starvation state. Applied and Environmental Microbiology 57: 2640-2644.

Ponniah, J., Robin, T., Margaret, S. P., Radu, S., Mohammad Ghazali, F., Cheah, Y. K., Nishibuchi, M., Nakaguchi, Y. and Pradeep, K. M. 2010. *Listeria monocytogenes* in raw salad vegetables sold at retail level in Malaysia. Food Control 21(5): 774-778.

Povey, R., Wellens, B. and Connor, M. 2001. Attitudes towards eating meat, vegetarian and vegan diets: an examination of the role of ambivalence. Appetite 37(1): 15–26.

Pui, C. F., Wong, W. C., Chai, L. C., Nillian, E., Ghazali, F. M., Cheah, Y. K., Nakaguchi, Y., Nishibuchi, M. and Radu, S. 2010. Simultaneous detection of *Salmonella* spp., *Salmonella typhi*, and *Salmonella typhimurium* in sliced fruits using multiplex PCR. Food Control (In press, doi: 10.1016/j.foodcont.2010.05.021).

Robinson, F. 2001. The nutritional contribution of meat to the British diet: recent trends and analysis. Nutrition Bulletin 26(4): 283-295.

Ryser, E. T. and Marth, E. H. 1988. Survival of *Listeria monocytogenes* in cold-pack cheese food during refrigerated storage. Journal of Food Protection 51: 615-621.

Ryser, E. T., Marth, E. H. and Doyle, M. P. 1985. Survival of *Listeria monocytogenes* during manufacture and storage of cottage cheese. Journal of Food Protection 48: 746-750.

Shearer, A. E. H., Strapp, C. M. and Joerger, R. D. 2001. Evaluation of a polymerase chain reaction-based system for detection of *Salmonella enteritidis*, *Escherichia coli* O157:H7, *Listeria* spp. and *Listeria monocytogenes* on fresh fruits and vegetables. Journal of Food Protection 64: 788-795.

Spencer, C. 1994. The Heretic's Feast. A History of Vegetarianism. Fourth Estate, London.

Stahler, C. 2006. How many adults are vegetarians? The Vegetarian Resource Group, Vegetarian Journal. Available via *http://www.vrg.org/journal/vj2006issue4/vj2006issue4poll.htm*. Accessed on 9 June 2010.

Suzita, R., Abu Bakar, F., Son, R., and Abdulamir, A. S. 2010. Detection of *Vibrio cholerae* in raw cockles (*Anadara granosa*) by polymerase chain reaction. International Food Research Journal 17: 675-680.

Tan, Y. F., Haresh, K. K., Chai, L. C., Ghazali, F. M., and Son, R. 2008. Prevalence of *Campylobacter* spp. in retailed ready-to-eat sushi. International Food Research Journal 15: 331-336.

Tang, J. Y. H., Mohamad Ghazali, F., Saleha, A. A., Nishibuchi, M., and Son, R. 2009. Comparison of thermophilic *Campylobacter* spp. occurrence in two types of retail chicken samples. International Food Research Journal 16: 277-288.

Tunung, R., Margaret, S. P., Jeyaletchumi, P., Chai, L. C., Tuan Zainazor, T. C., Ghazali, F. M., Nakaguchi, Y., Nishibuchi, M. and Radu, S. 2010. Prevalence and quantification of *Vibrio parahaemolyticus* in raw salad vegetables at retail level. Journal of Microbiology and Biotechnology 20: 391-396.

Tunung, R., Ghazali, F. M., Noranizan, M. A., Haresh, K. K., Lesley, M. B., Nakaguchi, Y., Nishibuchi, M., and Son, R. 2011. Rapid detection and enumeration of pathogenic *Vibrio parahaemolyticus* in raw vegetables from retail outlets. International Food Research Journal 18: 67-78.

Welshimer, H. J. 1968. Isolation of *Listeria monocytogenes* from vegetation. The Journal of Bacteriology 95: 300-303.