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Quantification of *Listeria monocytogenes* in salad vegetables by MPN-PCR

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Abstract: The aim of this study was to assess the most probable number-polymerase chain reaction (MPN-PCR) technique for detection of *Listeria monocytogenes* in salad vegetables in comparison with reference EN ISO 11290-2 and Food Drug Administration Bacteriological Analytical Manual method using artificial and naturally contaminated samples. Based on recovery of *L. monocytogenes* from artificially contaminated samples, MPN-PCR showed a moderate correlation (R=0.67) between spiking concentration and microbial levels which was better than the FDA-BAM method (R=0.642) and ISO 11290-2:1998 method (R=0.655). With naturally contaminated samples, it was found that *L. monocytogenes* was detected in 25% of the vegetable samples using MPN-PCR; 15% of the samples by the FDA-BAM method and 8% of samples using ISO 11290-2:1998 method. Overall, MPN-PCR was found to be a rapid and reliable method that could facilitate the enumeration of *L. monocytogenes* in vegetables.

Keywords: ***Listeria monocytogenes*, enumeration, salad vegetables, MPN-PCR**

Introduction

Listeria monocytogenes is an important opportunistic food-borne pathogen that can cause especially severe problems in pregnant women, neonates, the elderly and immunocompromised individuals (Liu, 2006). With mortality rates on average approaching 30%, *L. monocytogenes* far exceeds other common foodborne pathogens in terms of disease severity (Altekruse *et al.,* 1997). The high prevalence of *L. monocytogenes* in foods and the high fatality rates of listeriosis suggest that this pathogen represents an important hazard to human health.

Recently, there have been several foodborne outbreaks that are linked to the consumption of fresh vegetables (Doris and Seah, 1995). Little *et al.* (2007) reported that *L. monocytogenes* had been detected in ready-to-eat mixed salads in the United Kingdom. There is some data on the prevalence of *L. monocytogenes* in minimally processed vegetables in Malaysia and surrounding regions (Arumugaswamy *et al.*, 1994; Doris and Seah, 1995) although they are limited and somewhat outdated. The presence of *L. monocytogenes* in vegetables would be of public concern since vegetables are often consumed in the minimally processed form as a local salad dish, commonly known as 'ulam' in Malaysia, Singapore and Indonesia. Consumption of fresh fruits and vegetables is also actively promoted by health agencies as part of a healthy lifestyle therefore it is important to ensure their safety.

Analysis of *L. monocytogenes* based on conventional culture and biochemical methods is a laborious and time consuming process which can take up to a week when carried out according to long-established methods of cultivation-based microbial identification. In contrast, PCR-based techniques allow for the identification of microorganisms regardless of their culturability. Among techniques that have been applied for *L. monocytogenes* quantification include Listeria-SELeCT (Carrol *et al.*, 2000) ISO-GRID filter method (Entis and Lerner, 2000) and real-time PCR (Hein *et al.*, 2001). However, these methods are generally too expensive and unsuitable for routine analysis in many laboratories.

PCR has been successfully used in conjunction

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with Most Probable Number (MPN) for quantitative determination of pathogens in foods (Miwa *et al.,* 2003; Chai *et al.*, 2007; Su and Liu, 2007; Hai-Yen *et al*., 2009; Tang *et al*., 2009; Usha *et al*., 2010). Martin *et al.* (2004) reported that the MPN-PCR method facilitated the enumeration of *L. monocytogenes* in fermented sausages by combining the high sensitivity of the MPN technique with the specificity of PCR. For this study, we developed a most probable number–polymerase chain reaction (MPN-PCR) enumeration protocol for *L. monocytogenes*, which is fundamentally similar to the FDA-BAM method but which identifies the pathogen from MPN tubes using a species-specific PCR technique. The rapid PCR identification step combined with the most probable number (MPN) method is supplemented by the plate count method.

The aim of this study is to assess the MPN-PCR method for the detection and quantification of *L. monocytogenes* in vegetables that are consumed as salads in comparison with the ISO 11290-2:1998 and FDA-BAM enumeration procedure. Evaluation will also be carried out on artificially contaminated samples. This would enable a more accurate evaluation of the performance of the MPN-PCR method since it would enable assessment of naturally contaminated as well as artificially contaminated samples.

Materials and Methods

Bacterial strains and preparation of inocula

L. monocytogenes strain ATCC 19115 was used in this study to inoculate the vegetable samples and as a positive control in PCR assays. Pure cultures of *L. monocytogenes* were grown at 37°C for 24 hours 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 artificial spiking. All decimal dilutions were prepared in Tryptone Salt (Oxoid, United Kingdom) and ranged from 3 to 3 x 10^6 cfu/ml.

Sample collection

A total of 81 samples from five types of vegetables that are commonly consumed as 'ulam', a local salad dish, were randomly purchased from two retail outlets. All samples were transferred to sterile plastic bags for transportation and analyzed immediately on arrival to the laboratory. The types of raw vegetable samples that were subsequently classified into six types are as shown in Table 1.

Table 1. Vegetable samples examined in the study

English name	Local name	Scientific name	Number of samples examined
Indian pennywort	Pegaga	*Centenella asiatica*	16
Japanese parsley	Selom	*Oenanther stolonifera*	16
Wild parsley	Ulam raja	*Cosmos caudatus*	17
Winged bean	Kacang botol	*Psophocarpus tetragonolobus*	16
Yardlong bean	Kacang panjang	*Vigna unguiculata*	16
Total			81

Artificial contamination of vegetables

For experiments using artificial contamination, absence of *L. monocytogenes* was first checked using the ISO 11290-1 reference method (Anon, 1996). Only vegetables samples that were not found to contain *Listeria* spp. were used. Artificial contamination was achieved by spiking 25 g samples with a low volume (1 ml) of appropriate TS dilution of *L. monocytogenes* culture. Analysis was carried out in triplicates. Rates of contamination ranged from 3 to 3 x 10^6 cfu/g.

Detection and enumeration of L. monocytogenes

The analytical method performed in this study was generally similar to the Food Drug Administration Bacteriological Analytical Manual standard method for detection of *L. monocytogenes* in foods with slight modifications. 25 g of sample was placed in a stomacher bag and 225 ml of Buffered Listeria Enrichment Broth (BLEB, Merck, Darmstadt, Germany) was added. The sample was homogenised in a stomacher (Interscience, France) for 60s, followed by pre-enrichment at 30°C for 4 hours. Then acriflavin (final concentration: 10 mg/l); sodium nalidixate, final concentration: 40 mg/l) and cycloheximide (final concentration: 50 mg/l (Sigma, St. Louis, USA) were added as selective agents. Tenfold and hundredfold dilutions of the stomacher fluid were prepared with Buffered Listeria Enrichment Broth (BLEB). 100 µl of each dilution was directly plated onto PALCAM agar (Oxoid, United Kingdom) and incubated for 48 h at 30°C. At least 5 presumptive colonies (black with grey zone) were picked after 24 hours and 48 hours and subcultured onto Tryptone Soy Yeast Extract Agar at 35°C before being subjected to confirmatory biochemical tests.

For MPN enumeration, 1ml of three successive 10-fold dilutions was transferred into a three test tube set containing 9 ml of half Fraser (Oxoid) broth. The

tubes were incubated at 30°C for 48 hours. Turbid MPN tubes are considered positive. The numbers of turbid tubes were counted. All tubes were also subjected to DNA extraction to enable PCR detection of LLO gene specific for *L. monocytogenes*. Turbid tubes and tubes identified as *L. monocytogenes* positive by PCR was streaked on PALCAM agar and the plates were incubated for another 48 hours. Analysis was also carried out in parallel according to the ISO 11290-2:1998 and FDA-BAM enumeration procedure.

DNA extraction and confirmation of L. monocytogenes

DNA extraction was carried out using a boiled-cell method. A 500 µl portion of each broth was subjected to centrifugation 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 sample was cooled at − 20°C for 5 min before it was centrifuged at 10,000 ×*g* for 10 min. The supernatant was obtained for use as the template DNA solution in the PCR, for detection of *L. monocytogenes*. 5 µl of the supernatant was used as a template for PCR amplification. For confirmation of *L. monocytogenes* from PALCAM plates, suspected colonies were resuspended in 50 µl of sterile distilled water. 5 µl of the suspension was used as the template for PCR amplification. The nucleotide sequences of the primer used in the PCR which specifically amplifies the 701 bp region in the listeriolysin O (LLO) gene were *hly* gene LM1:5'-CCT AAG ACG CCA ATC GAA- 3' and LM2:5'-AAG CGC TTG CAA CTG CTC-3' obtained from Invitrogen.

PCR amplification

PCR amplification was performed in 25 µl of a reaction mixture containing 1.5 µl of 10 x PCR buffer, 0.2 µl of 10 mM of deoxynucleoside triphospate mix, 0.5 µl of each primer, 15 µl of sterile distilled water, 0.2 µl *Taq* DNA polymerase, 2.5 mM $MgCl_2$ and 2 µl of the DNA template solution. 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, followed by 1 min denaturation at 95°C, 1 min amplification at 52°C and 2 min elongation. The reaction was terminated after a 7 min extension period at 72°C. All thermal cycling reactions were performed with 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 ethinidium bromide and viewed under ultra-violet light. A DNA-molecular ladder (100 bp ladder) (Vivantis Technologies) was included in each gel.

PCR sensitivity, specificity and reproducibility

Sensitivity of PCR detection from vegetable samples was determined using various concentrations of artificially contaminated samples to determine lowest limit of detection. Reproducibility of PCR detection was carried out by analysing vegetable samples in triplicates with the lowest limit of detection as determined by the sensitivity assay. A negative PCR (no amplification) was considered as negative for *L. monocytogenes*; considerations for positive results were the amplification fragments of 701bp. Specificity of the PCR detection was determined with 5ml of tryptone soy broth artificially contaminated with 10^8 cfu / ml of two different microorganisms i.e. *L. innocua* ATCC 33090 and *S. aureus* ATCC 29213.

Comparison of MPN-PCR method with the ISO 11290-2:1998 and FDA-BAM enumeration procedure

Four sets of the same 10-fold dilution of vegetable artificially contaminated at a concentration of 300 cfu/ml was analysed five times in parallel with the three methods. This concentration was used to ensure it would be detected by all the three methods thus enabling a better comparison.

Statistical analysis

SPSS software (version 16.0) was utilised to analyze the data. Several tests were carried out i.e. stability of precision was tested with a Fischer test; relative accuracy of methods was evaluated by a comparison of means by one-way ANOVA, relationship between the known inoculum level and microbial counts was compared by simple linear regression.

Results

Based on recovery of *L. monocytogenes* from artificially contaminated samples, there was a moderate and better correlation between the known inoculum spiking levels and microbial levels as enumerated for the MPN-PCR method (R=0.670) as compared to the FDA BAM method (R=0.642) and ISO 11290-2:1998 method (R=0.655). Regression analysis showed that there was a good linear relationship between results obtained using MPN-PCR and inoculum concentration up to 300 cfu/g (equation of linear relationship: MPN/g (MPN-PCR)

= 0.609 + 0.702CFU/g inoculum concentration; R=0.902). Figure 1 shows that MPN-PCR yielded more accurate results (nearer to true value) at this concentration than the FDA-BAM method and ISO 11290-2:1998 method.

During analysis of *L. monocytogenes* from 81 vegetable samples with the three methods, *L. monocytogenes* was detected in 25% of the vegetable samples using MPN-PCR; 15% of the samples by the FDA-BAM method and 8% of samples using ISO 11290-2:1998 method. The microbial load ranged from 0 to 240 $MPNg^{-1}$. The microbial load as detected by MPN-PCR is shown in Table 2. The low detection rate using the ISO 11290-2:1998 method is possibly due to the presence of the pathogens in low numbers in vegetables that, as we can see from the artificially contaminated samples, the ISO 11290-2:1998 method is not very adept at detecting. The quantification of *L. monocytogenes* in vegetable samples using the ISO 11290-2:1998 method was also found to be difficult due to the high populations of other competitive microflora and the low levels of *L. monocytogenes.* The ALOA (Oxford, United Kingdom) agar was not selective enough and enabled the growth of other background micro-organisms which made the counting of *Listeria* colonies difficult. The FDA-BAM method is only presumptive for *L. monocytogenes* and involves further testing of isolated *Listeria* colonies to determine the proportion of *L. monocytogenes* among the *Listeria* colonies, thereby possibly increasing the probability of error. The MPN-PCR method, on the other hand, enables the direct enumeration of *L. monocytogenes* in vegetables without interference of background micro-organisms.

A comparison of the statistical evaluation of enumeration results obtained by the three methods artificially contaminated at a concentration of 300 cfu/ml per sample are shown in Table 3. According to statistical analysis of the data, the precision was found to be stable for all samples tested for the three methods (sig. F=0.091) at this concentration. The accuracy of the MPN-PCR method was also not significantly different from the other two methods.

The detection limit of the PCR using various concentrations of artificially contaminated samples was found to be 30 cfu/g. The coefficient of variation (C.V.) which gives a measure of the reproducibility of the method had values between 3-10% which indicate acceptable variation for the method. Figure 2 shows that PCR was able to achieve specificity through positive detection of the band at 701 bp for isolates of *L. monocytogenes* and negative detection for the negative control and two other bacteria tested.

Table 2. *L. monocytogenes* contamination in raw salad vegetables as quantified by MPN-PCR

Microbial load (MPN/g)	Number	Percentage (%)
Not detected (<3)	61	75.3
3-100	16	19.8
100-1000	4	4.9

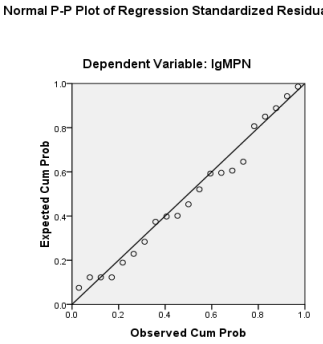
Table 3. Comparison of enumeration results obtained by three methods: result of five counts obtained by each method on four sets of samples of the same type.

Number of samples (N)	MPN-PCR	FDA-BAM	ISO11290:2
	20	20	20
Geometric mean	150.5861	142.5374	132.8047
Standard Error	3.71873	5.95762	6.61338
Standard Deviation	16.63066	29.576	26.62093

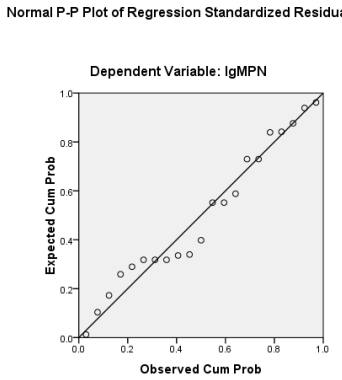
Discussion

The growing importance of *L. monocytogenes* on a global scale has prompted improved analytical procedures for the detection and enumeration of this pathogen in foods. Conventional methods based on culture plates have limitations in terms of overgrowth of other natural flora present in vegetables. Molecular methods have been shown to be less time-consuming and more sensitive for *L. monocytogenes* detection in foods. The present study utilized a highly sensitive PCR-based method to detect and quantify *L. monocytogenes* in vegetables that are consumed in the minimally processed state as salads. The MPN-PCR method that was used is faster than the standard method since it can be completed in 2 days. It is easy to implement, cheap and able to achieve a higher sensitivity wherein it is able to detect *L. monocytogenes* at the low levels that is normally present in vegetables. It also overcomes the problem of interfering microflora with similar morphology that is found in abundance when certain vegetables are plated on PALCAM or ALOA agar. This problem of interfering background microflora has also been reported in other foods like sausages, fresh poultry and milk (Slade *et al.,* 1988; Capita and Alonso, 2003; Martin *et al.*, 2004)

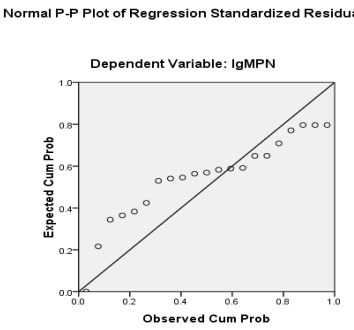
The PCR amplification enables detection of *L. monocytogenes* in BLEB enrichment broth and confirmation of suspect colonies on PALCAM plates. The simple pretreatment of the samples through centrifugation, washing and resuspension in distilled



A: Regression plot obtained for microbial load and inoculum size using MPN-PCR method



B: Regression plot obtained for microbial load and inoculum size using FDA-BAM method



C: Regression plot obtained for microbial load and inoculum size using ISO 11290-2 method

Figure 1. Linearity study using artificially contaminated vegetables: comparison of enumeration results obtained using ISO 11290-2 method , FDA-BAM method and MPN-PCR method.

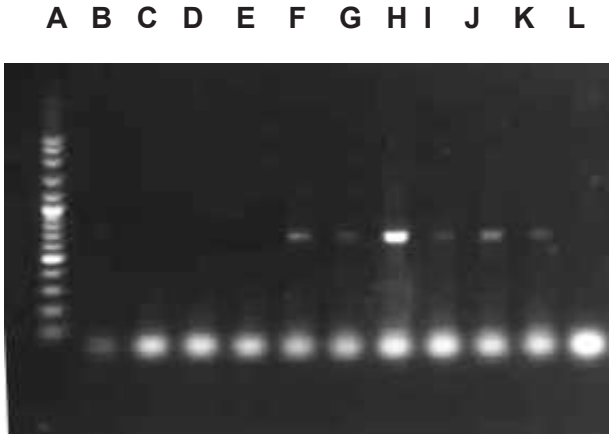


Figure 2. Representative amplification of the listeriolysin O (hlyA) gene for identification of *L. monocytogenes*. Lane A shows the ladder. Lane B and C shows DNA from *L. innocua* isolate ATCC 33090. Lane D and E shows DNA from *S. aureus* ATCC 29213. Lane E shows DNA from MPN broth that is negative. Lane F and G show DNA from MPN broth that is positive. Lane H shows *L. monocytogenes* reference strain ATCC 19115. Lane I to K shows DNA from MPN broth that is positive. Lane L shows the negative control.

water which is necessary to prevent inhibition of the reaction, was sufficient to obtain successful amplification from vegetable samples.

Conclusion

The MPN-PCR method is an efficient analytical technique for the detection of *L. monocytogenes* in vegetables since it can significantly reduce time and labour with analysis completed within 2 days, as opposed to the traditional confirmation method that can take up to 5 days for unequivocal identification of species.

Acknowledgements

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References

Altekruse, S. F., Cohen, M. L. and Swerdlow, D. L. 1997. Emerging foodborne diseases. Emerging Infectious Diseases 3: 285-293.

Anonymous. 1996. Microbiology of Food and Animal Feeding Stuff- Horizontal Method for the detection and enumeration of *Listeria monocytogenes*: Part 1.Detection Method. International Standard ISO 11290-1 International Organization for Standardization. Geneva, Switzerland.

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

Capita, R. and Alonso, C. 2003. Comparison of different most-probable-number methods for enumeration of *Listeria* in poultry. Journal of Food Protection 66: 65-71.

Carrol, S. A., Carr, I. E., Mallinson, E. T., Lamichanne, C., Rice, B. E., Rollins, D. M. and Jospeh, S. W. 2000. Development and evaluation of a 24-hour method for the detection and quantification of *Listeria monocytogenes* in meat products. Journal of Food Protection 63: 347-353.

Chai, L. C., Tunung, R., Usha, M. R., Jurin, W. G., Fatimah, A. B., Farinazleen, G., Son, R. and Malakar, P. K. 2007. Thermophilic *Campylobacter* spp. in salad vegetables in Malaysia. International Journal of Food Microbiology 117: 106-111.

Doris, L. K. N. and Seah, H. I. 1995. Isolation and identification of *Listeria monocytogenes* from a range of foods in Singapore. Food Control 6(3): 171-173.

Entis, P and Lerner, I. 2000. Twenty-four-hour direct presumptive enumeration of *Listeria monocytogenes* in food and environmental samples using the ISO-GRID method with LM-137 agar. Journal of Food Protection 63: 354-363.

Gorny, J. 2006. Microbiology of fruits and vegetables: Chapter 1, Microbial contamination of fresh fruits and vegetables. pp. 3-32.7th Ed. Taylor and Francis, USA.

Hai-Yen, L., Lay-Ching, C., Sui-Yan, T., Jinap, S., Ghazali, F. M., Nakaguchi, Y., Nishibuchi, M. and Son, R. 2009. Application of MPN-PCR in biosafety of *Bacillus cereus* s.l. for ready-to-eat cereals. Food Control 20: 1068-1071.

Hein, I., Klein, D., Lehner, A., Bubert, A., Brandl., E. and Wagner, M. 2001. Detection and quantification of the *iap* gene of *Listeria monocytogenes* and *Listeria innocua* by a new real-time quantitative PCR assay. Research in Microbiology 152: 37-46.

Little, C. L., Taylor, F. C., Sagoo, S. K., Gillespie, I. A., Grant, K., McLauchin. 2007 Prevalence and level of *Listeria monocytogenes* and other *Listeria* species in retail pre-packaged mixed vegetable salads in the UK. Food Microbiology 24: 711-717.

Liu, D. 2006. Identification,subtyping and virulence determination of *Listeria monocytogenes,* an important foodborne pathogen. Journal of Medical Microbiology 55: 645-659.

Martin, B. Jofre, A., Garriga, M., Huges, M and Aymerich, T. 2004. Quantification of *Listeria monocytogenes* in fermented sausages by MPN-PCR method. Letters in Applied Microbiology 39: 290-295.

Meldrum, R. J., Little, C. L., Sagoo, S., Mithani, V., McLauchlin, J. and de Pinna, E. 2009. Assessment of microbiological safety of salad vegetables and sauces from kebab take-away restaurants in the United Kingdom. Food Microbiology 12: 1-5.

Miwa, N., Nishio, T., Arita, Y., Kawamori, F., Masuda, T. and Akiyama, M. 2003. Evaluation of MPN method combined with PCR procedure for detection and enumeration of *Vibrio parahaemolyticus* in seafood. Journal of Food Hygiene Society of Japan 44: 289–293.

Slade, P. L. and Collins-Thompson, D. L. 1988. Enumeration of *Listeria monocytogenes* in raw milk. Letters in Applied Microbiology 6: 121-123.

Su, Y. C. and Liu, C. 2007. *Vibrio parahaemolyticus*: A concern of seafood safety. Food Microbiology 24: 549-598.

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: 277-289.

Usha, M. R., Tunung, R., Chai, L. C., Ghazali, F. M., Cheah, Y. K., Nishibuchi, M. and Son, R. 2010. A study on *Campylobacter jejuni* cross-contamination during chilled broiler preparation. International Food Research Journal 17: 107-115.