Application of molecular technique for risk assessment of *Listeria monocytogenes* in a frozen cooked chicken processing plant

Chirapiphat Phraephaisarn[1] and Suwimon Keeratipibul[2*]

ABSTRACT: *L. monocytogenes*, the causative agent of listeriosis in humans and animals, is one of the most concerned pathogen affecting frozen ready-to-eat food industry worldwide. It could remain in the processing environment for years and cause cross-contamination into the products. The objectives of this study were to evaluate occurrence of *Listeria* spp., and to investigate contamination sources of *L. monocytogenes*. In this study, a total of 100 environmental swabs, and 100 finished product samples were analyzed by a conventional method and subjected to multilocus variable number of tandem repeat analysis (MLVA) for subtyping *L. monocytogenes*. From a total of 100 environmental swab samples taken from before (50 samples) and after (50 samples) cleaning, 28 (56%) and 16 (32%) samples were positive for *Listeria* spp., respectively. The occurrence of *L. monocytogenes* was 20%. Meanwhile, 20 samples the finished products were positive for *Listeria* spp., accounting for 20% occurrence. The occurrence of *L. monocytogenes* was 6%. Then, the MLVA with selected 9 VNTR loci was applied to evaluate sources of contamination of 25 *L. monocytogenes* isolates found in the environmental swabs and product samples. *L. monocytogenes* isolates were classified into 3 strains which were LM1, LM2, and LM3. In the environmental swabs, the LM1 was the dominant strain represented by 74% of all *L. monocytogenes* positive samples. Other *L. monocytogenes* strains were LM2 (5%) and LM3 (21%). However, only LM1 strain was found in both finished product and environmental swab samples. There was correlation between *L. monocytogenes* strain LM1 found in processing environment and in finished products. The cross contamination from processing environment to finished products may occur due to improper cleaning procedure and staff straining. In this study, it clearly showed that the MLVA can be used as a useful tool for investigation of *L. monocytogenes* contamination in food products.
Keywords: *Listeria monocytogenes*, molecular technique, risk assessment

Introduction

Thailand is one of the world's largest cooked chicken meat exporting country. In 2016, the value of export for cooked chicken meat in Thailand was 2.14 billion U.S. dollars (FAS/USDA, 2017). Hence, a pathogen contamination in the products can cause serious economic losses due to product rejection and/or elimination. *Listeria monocytogenes* contamination is one of the most food safety concerns in frozen-cooked chicken meat industry. *L. monocytogenes* associates with listerosis which often affects severe underlying condition such as encephalitis, abortion or blood poisoning in human. The overall mortality rate associated with the disease is 30-40% in susceptible groups of people (Siegman-Igra et al., 2002). Due to high disease

[1] Program in Biotechnology, Faculty of Science, Chulalongkorn University, Bangkok, 10330, Thailand
[2] Department of Food Technology, Faculty of Science, Chulalongkorn University, Bangkok, 10330, Thailand
* Corresponding author: suwimon.k@chula.ac.th

severity of the pathogen, the U.S. Food and Drug Administration standards require zero tolerance for *L. monocytogenes* contamination in processed foods (Swaminathan et al., 2001). Likewise, the contamination level of *L. monocytogenes* in processed foods is also strictly regulated in the EU at < 100 cfu/g (Jadhav et al., 2012). Therefore, many attempts worldwide have been tried to control and eliminate the contamination of *L. monocytogenes* in their processing factories and food products. Many researchers have presented that the sources of contamination from *L monocytognes* are usually contaminated environmental surfaces in the processing line (Keeratipibul and Techaruvichit, 2011, Lekroengsin et al., 2007; Tompkin et al., 1999). *L. monocytogenes* have been reported to be found in dicers, chillers (Van den Elzen and Snijders, 1993), workers' hands (Kerr et al., 1995), conveyor belt rollers (Tompkin, 2002), and other processing equipment (Lawrence and Gilmour, 1995). The microorganism can persist some industrial sanitizers, colonize the whole environment, and form biofilms that make them even more resistant. They may remain in the processing environment for months to years and cause cross-contamination to the product (Carpentier and Cerf, 2011). Hence, potential sources of *L. monocytogenes* contamination need to be identified and eliminated to effectively control the pathogen contamination. However, microbiological testing methods used in food factory such as BAM (Hitchins, 2001), ISO11290 method (Anon, 1996), and AOAC/IDF method 993.09 (AOAC, 2000) identify only the species level which restrict ability to identify the real source of product contamination.

As a consequence, higher degree of discrimination requires a molecular typing method to provide additional strain level. Recent studies have been facilitated by the use of multiple-locus variable number tandem repeat analysis (MLVA) as an efficient tool for a rapid assessment of bacterial source tracking (Otero et al., 2013; Prendergast et al., 2011; Chenal-Francisque et al., 2013; Kimura et al., 2008; Lindstedt et al., 2004; Sabat et al., 2003).MLVA is based on the variation in the number of tandem repeated sequences found in many different loci in the genome of bacteria. Variable number tandem repeats (VNTRs) are short segments of DNA that have variable copy numbers. The difference in copy numbers at specific loci is used to measure relatedness of strains in this subtyping scheme. The number of tandem repeats is assessed based on the size of the PCR products. The MLVA profile is defined by the number of tandem repeats of the VNTR loci. Each unique MLVA profile coded by multi-digit is assigned a MLVA pattern.

To enhance a better monitoring and control of *L. monocytogenes* in food production chain, this study was aimed to apply MLVA as a subtyping method to evaluate occurrences of *Listeria* spp. and to investigate potential contamination sources of *L. monocytogenes* in a Thai frozen cooked chicken meat factory.

Materials and Methods

Sampling

The sampling in this study was performed in a cooked chicken meat processing plant in Thailand. One hundred environmental areas from 50 surface areas in the processing line (**Table 1**) were sampled before (50 samples) and after (50 samples) a big cleaning day. The cleaning followed the factory standard of operating procedure including disassembly of equipment and deep cleaning specialized parts of machines. Moreover, 100 finished product samples were also collected. All samples were swabbed by

sterile cotton swabs moistened with 0.85% NaCl (w/v). After sampling, the swabs were soaked in 10 ml of Dey/Engley (D/E) Neutralizing Broth and kept in a cooler during transport to the laboratory.

Table 1 Sampling location of the environmental swabs

Zone*	Area	Location	Zone*	Area	Location
1	cooking	Spiral Superlene belt in oven	3	cooking	Bar stool
1	cooking	Net conveyor no. 1	3	cooking	Water hose
1	cooking	Net conveyor no. 2	3	cooking	Wall
1	cooking	Net conveyor no. 3	3	cooking	Wall cornice
1	cooking	Net conveyor no. 4	3	cooking	Washing basin (inside)
1	packing	Net conveyor no. 18	3	cooking	Washing basin (outside)
1	packing	Net conveyor no. 24	3	cooking	Floor drain nearby dicer
1	packing	Conveyor belt no. 24	3	cooking	Floor nearby dicer
1	packing	Declining belt no.1	3	packing	Declining wheel
1	packing	Declining belt no.2	3	packing	Male power plug
1	packing	Funnel cone	3	packing	Female power plug
2	cooking	Table	3	packing	Wire
2	cooking	Weight balance	3	packing	Floor
2	cooking	Shelf for cleaned equipment	3	packing	Floor drain
2	cooking	Container for cleaned equipment	3	packing	Bar stool
2	cooking	Equipment cabinet	3	packing	Apron rack
2	cooking	Cooking and packing cart	3	packing	Staff shoes no. 1
2	cooking	Conveyor controller cabinet 1	3	packing	Staff shoes no. 2
2	cooking	Conveyor controller cabinet 4	3	packing	Cart wheel QC
2	cooking	Operating controller cabinet	3	packing	Cart wheel QC line
2	cooking	Male power plug of dicer	3	packing	Fire extinguisher (inside)
2	cooking	Female power plug of dicer	3	packing	Shaft set (declining motor)
2	cooking	Wire	3	packing	Cornice (Freezer)
3	cooking	Conveyor belt supporter no.1	3	packing	Washing basin
3	cooking	Air sock	3	packing	Washing basin (outside)

*Zone concept: Zone 1 is the product-contact surfaces; Zone 2 is the non-product contact surfaces in close proximity to the product; and Zone 3 is the non-product contact surfaces that are further away from the product.

***Listeria* spp. identification and DNA extraction**

Upon arrival at the laboratory, swab samples were handled according to VIDAS® Method. The swab sample was each homogenized for 1 min in 225 ml Half-Fraser (HF) broth (bioMérieux) in a stomacher, and incubated at 30 °C for 20 to 26 h as a pre-enrichment step. One ml of the suspension was transferred to tubes containing 10 ml of Fraser Broth and incubated at 30 °C for 20 to 26 h. One loop of all positive samples were streaked on *Listeria* selective agar (Oxford; OXOID) and Ottaviani Agosti agar (OAA) plates (bioMérieux), incubated at 37 °C for 48 ± 2 h and then observed for the presence of typical *Listeria* colonies according to ISO 11290-1. From each plate, three colonies with morphological characteristics of *Listeria* were picked off, streaked onto TSAYE (Tryptone Soy Agar; OXOID) with 0.6% (w/ v) Yeast Extract (Merck) plates and incubated at 37 °C for 18 to 24 h. Colonies presumptive for *Listeria* spp. on TSAYE were selected and subjected to Gram staining, c3atalase test and motility at 25 °C for 48 h. The API *Listeria* System incubated at 35 °C for 18 to 24 h was used to confirm the identified species (bioMérieux S.A.). All *L. monocytogenes* strains

were then individually grown in Trypticase Soy Broth (TSB) (Becton Dickinson, U.S.A.) overnight at 30 °C. Bacterial cells were harvested from 1 mL TSB medium by centrifugation at 8,000×*g* for 3 min and the supernatant was removed. Total genomic DNA was extracted using NucleoSpin Tissue (Macherey-Nagel, Germany) according to the manufacturer's protocol.

Molecular subtyping of *L. monocytogenes* using MLVA

The MLVA protocol used in this study was modified from the protocol in the study of Chenal-Francisque et al. (2013). Nine primer sets which provided the high discriminatory power were selected (**Table 2**). The PCR amplification was performed in a final volume of 50 µL, containing 10 mMTris-HCl (pH 8.3), 50 mMKCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 100 nM forward primer, 100 nM reverse primer, 25 ng template DNA, and 0.5 U Takara Taq DNA polymerase (Takara Bio, Japan). Amplification was performed using the GeneAmp PCR System 9700 thermalcycler (Life Technologies, U.S.A.). The following parameters were used for amplification: 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 60 °C for 40 s, 72 °C for 1 min, and 72 °C for 4 min. PCR products were visualized and confirmed by electrophoresis in a 2% agarose gel. To analyze the variants further, the observed amplicons were subjected to capillary electrophoresis (CE; QIAxcel Advanced; Qiagen, Tokyo, Japan) for fragment analysis.

Table 2 Primer sequences of the selected VNTR loci

		Primer sequence (5'to 3')	Simpson index	Reference
JLR1	F:	AGTTCCTCCATTGGTAGAAGGATT	0.87	Larsson, 2008
	R:	TTTGAAAGCTGGAGATGTTATTCA		
JLR2	F:	CCTTCCAGAGAAAGACAAAACAG	0.72	Larsson, 2008
	R:	RCTAATCCACCAGCAAATAGC		
Lis-TR1317	F:	TGATTTGCAAAAAGCTGCACC	0.47	Chenal-Francisque et al., 2013
	R:	ACTTGGMACTTCTGGTTTA		
Lis-TR881	F:	TGTAAATAAAGCTGGTACGTAC	0.65	Chenal-Francisque et al., 2013
	R:	GTATGTTGCTTGTTATCAACTAC		
JLR4	F:	AGAAATTCCAGTCCGCCAG	0.59	Larsson, 2008
	R:	GGARCAACAGAAGCTGATCCA		
LM-TR4	F:	AAAAGACGAAGAACCAGTAGGTAAA	0.42	Murphy et al., 2007
	R:	CAGCCTCTTTGATTTTATTCGTCT		
LMV1	F:	CGTTACCACCCCATGAATAAG	0.63	Lindstedt et al., 2008
	R:	CAACAAACAGCACCTAAARCAC		
LMV6	F:	ATATGAACTYGATACGACSCCAGT	0.63	Lindstedt et al., 2008
	R:	YTCGCTGTTTTCTGWTTTCTKAGG		
LMV9	F:	GCACAGAGGCACTAAACGTAACCG	0.67	Lindstedt et al., 2008
	R:	ATTAAKCCRCTCGCTGAAAT		

Results and discussion

MLVA profiling and occurrence of *Listeria* species and strains

From a total of 100 environmental swab samples taken from surface of processing line before (50 samples) and after (50 samples) cleaning, 28 (56%) and 16 (32%) samples were positive for *Listeria* spp., respectively. The species compositions were *L. monocytogenes* (20%), *L. innocua* (18%), *L. welshimeri* (6%), and *L. seeligeri* (1%). Meanwhile, 20 samples of finished product were positive for *Listeria* spp., accounting for 20% occurrence. The species compositions were *L. monocytogenes* (6%), *L. innocua* (8%), and *L. welshimeri* (6%). The occurrence of *Listeria* spp. isolated from environmental surfaces and finished products of chicken meat processing plant, is shown in **Table 3**.

Table 3 The number of *Listeria* spp. samples isolated from environmental surfaces and finished products of the chicken meat processing plant

Species	Environmental samples		Finished product	Total
	Before cleaning	After cleaning		
L. monocytogenes	13	6	6	25
L. innocua	11	7	8	26
L. welshimeri	4	2	6	12
L. seeligeri	0	1	0	1
Total	28	16	20	64

In this study, 9 developed VNTR loci of 25 *L. monocytogenes* isolated from the environmental swab and finished product samples were amplified. The numbers of repeat units in each of the VNTR in coding regions of *L. monocytogenes* were detected by MLVA-CE approach. As a result, 25 *L. monocytogenes* isolates were classified into 3 strains which were LM1, LM2, and LM3. MLVA patterns of all 3 strains are shown in **Table 4**. The *L. monocytogenes* strain found in the finished products was strain LM1. In environmental swabs, *L. monocytogenes* LM1 was the dominant strain with 74% occurrence, followed by LM3 (21%) and LM2 (5%) (**Table 5**). The *L. monocytogenes* strains LM1 and LM3 were detected along the processing line from cooking to packing areas, while strain LM2 was found only at washing basin in cooking area (**Table 6**). Even after cleaning, strain LM1 was still detected in both cooking and packing area and strain LM3 was also detected on conveyor belt supporter in cooking area. Whilst, strain LM2 was not found on the surface after cleaning. Since identical *L. monocytogenes* MLVA strains was obtained from both before and after cleaning, it appeared that *L. monocytogenes* strain LM1 and LM3 were able to persist in the processing environment. In addition, these areas which were found to be contaminated with LM1 and LM3 after cleaning were in zone 3.According to zone concept, zone 3 is the non-product contact surfaces that are further away from the product. These areas are not often in people attention to clean seriously resulting in reintroducing of the bacteria (Grocery Manufacturers Association, 2016).

Table 4 The MLVA patterns of each *L. monocytogenes* strain considered by the observed PCR product sizes (bp)

Primer	LM1	LM2	LM3
JLR11	350	356	386
JLR21	496	556	520
JLR41	246	300	309
TR13171	nd*	nd	nd
TR8811	368	290	296
LMV11	386	356	356
LMV61	312	312	294
LMV91	286	312	312
TR41	246	234	246
LM111	222	240	240

*nd = not detected

Table 5 The number of *L. monocytogenes* isolates found in this study

Strain	Environment before cleaning	Environment after cleaning	Finished product	Total
LM1	9	5	6	20
LM2	1	0	0	1
LM3	3	1	0	4
Total	13	6	6	25

Table 6 Detection of *L. monocytogenes* strains on different environmental areas

Swab area	Zone*	Area	Before cleaning	After cleaning
Conveyor belt no.24	1	Packing	LM1	
Net conveyor no.18	1	Packing		LM1
Cooking and packing cart	2	Cooking		LM1
Conveyor belt supporter no.1	3	Cooking	LM3	LM3
Washingbasin (outside)	3	Cooking	LM2	
Floor drain nearby dicer	3	Cooking	LM1	
Bar stool	3	Cooking	LM1	
Water hose	3	Cooking	LM3	
Declining wheel	3	Packing	LM1	LM1
Wire	3	Packing	LM1	
Floor	3	Packing	LM1	
Floor drain	3	Packing	LM1	LM1
Washing basin	3	Packing	LM3	LM1
Bar stool	3	Packing	LM3	
Cart wheel QC	3	Packing	LM1	
Fire extinguisher (inside)	3	Packing	LM1	

*Zone 1 is the product-contact surfaces. Zone 2 is the non-product contact surfaces in close proximity to the product. Zone 3 is the non-product contact surfaces that are further away from the product.

Correlation of *Listeria* strains in cooked chicken meat and in the processing environment

The correlation of *L. monocytogenes* strain between finished products and processing environment was found with contamination of *L. monocytogenes* strain LM1. In cooking area, *L. monocytogenes* LM1 was found on cooking and packing cart, floor drain nearby dicer, and bar stool. In packing area, *L. monocytogenes* LM1 was found on conveyor belt, net conveyor, declining wheel, wire, floor of the cooking area, floor drain, fire extinguisher, and cart wheel. Most of these areas, except conveyor belt No. 24, were not product contact surfaces and being far away from the products (**Table 6**). The cross contamination between the surfaces and finished products may occur due to improper cleaning sequence. The staff might clean food contact surfaces before cleaning non-food contact surfaces such as floor and drain. So recontamination of the dirty splashed water from floor and drain could cause the cross contamination as reported by Keeratipibul and Techaruvichit (2011).

Suggestion on risk assessment and management of *L. monocytogenes*

Previous studies have emphasized that the processing environment represents significant sources of *L. monocytogenes* contaminated in finished products (Lekroengsin et al., 2007; Tompkin, 1999). Therefore, source tracking of *L. monocytogenes* contamination is necessary for assessing the risk of product contamination. The accurate and reliable MLVA has been successfully used for many years to track sources of microbial contamination (Bertrand et al., 2015; Chiou et al., 2010; Takahashi et al., 2014). In this study, the MLVA results revealed that the clonal *L. monocytogenes* strain LM1was cross contaminated from zone 3 (which was remoted from the finished products). As mention earlier, this may due to the improper sequence of cleaning and staff training. Therefore, to manage the risk of product contamination, cleaning procedure and staff training should be reconsidered. Sequence of cleaning should be rearranged to avoid cross contamination of dirty water on the floor splashed onto food contact surfaces. In addition, high pressure cleaning should be avoided to prevent aerosols which may trap dirts and microorganisms and eventually dropped and contaminated on the products. Staffs should be trained to highly aware on consequence of *L. monocytogenes* contamination such as rejection of the products and may cause listeriosis to the consumers. Training can be both OJT (on the job training) and in-house training. Awareness of the staffs and consistent implementation of comprehensive good practice systems are key elements to prevent *L. monocytogenes* contamination in the products.

Conclusions

The MLVA with 9 selected VNTR loci was successfully subtyped and used for tracking *L. monocytogenes* in environment which were the sources of product contamination in the frozen cooked chicken meat processing plant. The MLVA can overcome the obstacle of conventional bacterial culture method which has low discriminatory power because of a very similar phenotype of bacterial colonies. This study demonstrated that the MLVA can be used as an effective monitoring tool to identify cross contamination of *L. monocytogenes* in processing environment and products. This subtyping method provided useful information for risk assessment and management to address the problem of product contamination, ensure the safety of food products, and eventually reduce economic losses.

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