Evaluation of Loop Mediated Isothermal DNA Amplification (LAMP) on Detection of *Salmonella* spp. in Foods and *Listeria monocytogenes* on Environmental Surfaces

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A THESIS SUBMITTED

FOR THE DEGREE OF MASTER OF SCIENCE (RESEARCH)

FOOD SCIENCE & TECHNOLOGY PROGRAMME

DEPARTMENT OF CHEMISTRY

NATIONAL UNIVERSITY OF SINGAPORE

2014

THESIS DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety, under the supervision of Assistant Professor Yuk Hyun-Gyun (in the laboratory S14-05-04a), Food Science and Technology Programme, c/o Department of Chemistry, National University of Singapore between January 2013 and August 2014.

I have duly acknowledged all the sources of information which have been used in this thesis.

This thesis has also not been submitted for any degree in any university previously.

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Name	Signature	Date

ACKNOWLEDGEMENTS

First and foremost, I would like to extend my sincere gratitude to my supervisor, Dr Yuk Hyun-Gyun for his acceptance of me to be part of his research team, hence granting my childhood dream to study in NUS. I would like to thank him for his supervision, guidance and patience during my research along with his many words of encouragement. His openness to give constructive, critical comments aids me to think deeper and more extensively on how to improve and enhance my research work. I am also highly grateful to Dr Miks-Krajnik, Marta Hanna for her sound advice and suggestion in all areas of my research work, most importantly is her faith in me that I can complete the research project.

I would also like to thank the faculty and staff of Food Science and Technology Programme including Mdm Lee Chooi Lan, Ms Lee Huey Lee, Ms Jiang Xiao Hui and Mr Abdul Rahaman Bin Mohd Noor for their kind assistance and support.

Also, my great thanks to all my fellow lab mates, QianWang, Yishan, MinJeong, Vinayak and Amit, for their kind words and supports. I would also like to thank internship students from Korea So –Yeon and FST 1st year student, Zhi Hwa for their great assistance and contribution in this research work. It was such an honor to work with such fine students who are smart yet humble. Next, I would also like to extend my thanks to the Matt Turner and Norman Kok from 3M Food Safety for their strong support in this research work.

Last but not the least; I would like to give my whole hearted thanks to my family who has been extremely tolerant and patient during this period of my frequent absenteeism at home. Lucas, Skyler and Ethan are my greatest motivation to complete this research work , thereby I would like to dedicate this thesis to my three lovable boys who missed their mother during this time due to the commitment to finishing this research work the soonest to be with them.

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SUMMARY

Salmonella spp. and *Listeria monocytogenes* are listed in top five pathogens contributing to domestically acquired foodborne illnesses resulting in death according to Centre for Disease Control (CDC). Food is an excellent vehicle of transmission for pathogens to grow and make consumers sick. Hence, monitoring of contamination in the food is crucial. Advances in detection methods of pathogens in foods and environmental samples using molecular detection improve response time to prevent food contaminated with pathogens reaching consumers.

A simple and cost effective novel detection method combining loop mediated isothermal DNA amplification (LAMP) method with bioluminescence named as 3M™ molecular detection system (MDS) has recently been developed. 3M™ molecular detection assay (MDA) is used with the 3M™ MDS for qualitative analysis of pathogens in foods and environmental samples the next day after enrichment. Hence, testing time is much reduced in comparison to ISO methods that typically require 5 – 7 days.

In this study, the comparison of 3M™ MDA to standard ISO methods on *Salmonella* spp. and *Listeria monocytogenes* were performed to determine the sensitivity and specificity at various inoculum levels. For the first study, a healthy *Salmonella* cocktail was inoculated on raw duck wings, raw bean sprouts and processed fish balls to achieve two inoculation levels: 10^0 and 10^1 CFU/25g. To simulate real food processing scenario, a *Salmonella* cocktail culture was subjected to heat and sanitizer processes to achieve 80% - 85% sub-lethal heat and sanitizer injury, respectively, followed by inoculation on

food matrices. Validation on the naturally contaminated food matrices was conducted as well.

The second study was the detection of *L. monocytogenes* on environmental surfaces at 3 inoculum levels: 10^0, 10^1 and 10^2 CFU/100 cm^2. Often, food preparation surfaces are contaminated with food likely due to poor hygiene hence it is of interest to determine whether the presence of organic load affects the viability of *Listeria monocytogenes* on stainless steel (SS) and polyethylene (PE) surfaces.

It is evident from the first study; time to result for rapid pathogen detection methods is generally shorter due to more sophisticated technology and also shorter enrichment time. This shorter enrichment time may result in level of target pathogens not reaching the limit of detection level due to the low numbers of target pathogens present, the presence of background microflora competing for nutrients or insufficient time for injured target pathogens to grow to detectable level. Hence, it is important to have optimized enrichment protocol for food sample of high background microflora. Other optimization methods to be considered could be increasing the sample volume or increase the sample concentration via centrifugation.

Other than testing for pathogens in food matrices, it is also important for food manufacturers to choose materials of construction that do not support cell viability in food processing plants and to maintain plant hygiene at all times to minimize cross contamination due to contact or handling.

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CHAPTER 1

INTRODUCTION

Food safety is the global goal of food producers and food industry at large as food is consumed daily, from the young to elderly, whereby these two groups are the most vulnerable to foods contaminated with pathogens due to weak immunity (Kärkkäinen et al., 2011; Kothary and Babu 2001). As such, many countries have adopted a zero tolerance policy regarding the presence of foodborne pathogens such as *Salmonella* spp. and *Listeria monocytogenes* in foods.

To ensure microbiological food safety, a wide range of pathogen intervention strategies along with control measures such as Good Agricultural Practices (GAP), Good Manufacturing Practices (GMP) and Hazard Analysis and Critical Control Point (HACCP) are in place to minimize opportunities for the introduction, persistence, and transmission of pathogenic microorganisms during farm to fork process (Velusamy et al., 2010; Doyle and Erickson, 2012).

Despite such effort, foodborne illnesses by consumption of foods contaminated with pathogens are still relatively common even in developed countries like the United States (US) and Singapore. In 2012, Communicable Disease Surveillance in Singapore reported 1,499 laboratory confirmed salmonellosis cases (MOH, 2012). In 2011, Center for Disease Control and Prevention (CDC) in US reported that known pathogens caused an estimated

9.4 million cases of foodborne illness, 55,961 hospitalizations and 1351 deaths in United States (CDC, 2011d). Likely factors are trading of contaminated foods between countries/states which increases the likelihood of outbreak and illness coupled with changes in lifestyle and consumer demands such as increasing consumption of fresh vegetables and frequent outdoor dining (Rocourt, 2003). Among foodborne pathogens, nontyphoidal *Salmonella* spp. and *L. monocytogenes* were responsible for 47% of the reported deaths in 2011 (CDC, 2011). These alarming reported data support the fact that failure to detect foodborne pathogens would lead to a dreadful effect.

Despite national monitoring and surveillance programs, reasons for failure to detect pathogens in foods could be due to the presence of low numbers of pathogens, food composites such as fats and phenolic compound that could inhibit detection methods, and injured cells that were not given enough time to resuscitate to be detected (Dwivedi et al., 2014). This is a valid concern since selective media contain agents such as antibiotics that were designed to select for healthy target microorganisms and the presence of these agents could lead to extended lag phases in injured target microorganisms. During the food process, treatments such as heating, freezing and sanitizing to microbial population cause dead, uninjured (healthy cells) or injured cells (Wu and Fung, 2001). Injured cells are as important as the healthy cells as they can resuscitate and become healthy again in favorable conditions, resulting in foodborne outbreak (Wu, 2008).

Conventional culture methods for the detection and identification of foodborne pathogens are laborious, time consuming and slow to obtain results.

These methods depend on several steps including enrichment, selective plates and biochemical confirmation that require long time for microbial pathogens to grow to react (Lee et al., 2015). Nevertheless, such methods are inexpensive and sensitive which explains why many food laboratories are still following such methods.

To overcome these drawbacks of conventional culture methods, rapid immunological or molecular-based assays such as enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR) (Dwivedi and Jaykus, 2011) and loop mediated isothermal DNA amplification method (LAMP) have been developed. ELISA relies on the specific binding of an antibody to an antigen and is designed as sandwiched assay with detection limits from 10^3 to 10^5 CFU/ml (Mandal et al., 2011), while PCR is an *in vitro* method that amplified specific DNA fragments with the cyclic 3-step process namely denaturation, annealing and extension (Cornett et al., 2001). With advances in PCR, real-time quantitative PCR (qPCR) has been developed to monitor the progress of reactions as it occurs in real time. LAMP, a newly developed method, uses multiple primers to recognize distinct regions of the genome (*invA*) with amplification taking place by auto-cycling strand displacement DNA synthesis in the presence of *Bst* DNA polymerase under isothermal conditions at 60°C (Wang et al., 2008).

Recently, a user-friendly rapid detection system using LAMP coupled with bioluminescence named as 3M™ molecular detection system (3M™ MDS) has been commercialized. LAMP is known for its specificity and ability to handle more complex samples while bioluminescence is predominantly

used in hygiene monitoring (Murphy et al., 1998). 3M™ Molecular Detection Assay (MDA) is used with 3M™ MDS for qualitative analysis of pathogens in samples the next day after enrichment (Bird et al., 2013). This is the first commercially available assay of its kind that combines these two technologies. Studies have been performed on this system with various foods in USA and Europe. However, limited testing has been studied with foods in Southeast Asia. Hence, it would be of interest to conduct a comprehensive study of artificially inoculated foodborne pathogens at different inoculum levels on local food matrices in Southeast Asia along with surfaces commonly used at food processing facilities.

Therefore, the objective of this study was to evaluate the performance of 3M™ MDA to ISO standard methods for the detection of *Salmonella* spp. on raw duck wings, raw mung bean sprouts and processed fishballs at low inoculum levels of 10^0 and 10^1 CFU/25g, respectively. In addition, the performance of 3M™ MDA on the detection of thermally- or sanitizer-injured *Salmonella* spp. in each food matrix was also conducted. For the application on environmental samples, 3M™ MDA was evaluated for the detection of *L. monocyotogenes* artificially inoculated at 3 inoculum levels of 10^0 ,10^1 and 10^2 CFU/100 cm^2 on two food contact surfaces: stainless steel and polyethylene with or without organic load.

CHAPTER 2

LITERATURE REVIEW

2.1 *Salmonella* spp.

2.1.1 Bacteriology

Salmonella spp. is Gram-negative, motile, rod-shaped bacteria that can grow both aerobically and anaerobically belonging to the family Enterobacteriaceae (Baird-Parker, 1990). They are catalase positive, oxidase negative, and generally produces hydrogen sulfide. *Salmonella* spp. can utilize citrate as a sole carbon source and can decarboxylate lysine.

2.1.2 Sources and transmission

Salmonella spp. is able to colonize a wide range of hosts and all the major livestock species (poultry, cattle, and pigs) and are often asymptomatically (Newell et al., 2010). During the transportation to slaughter houses, *Salmonella* cells are readily transferred to carcasses through fecal contamination. Further spread of cells may occur during processing if carcasses become cross-contaminated (Carrasco et al., 2012).

Fresh produce grown in developing countries where manures from these infected animals are frequently used as natural fertilizers introduce pathogens directly to the field, and run-off can contaminate irrigation water (Heaton and Jones, 2008). For irrigation use, wastewater should receive

treatment, but in lower-income countries, raw sewage is often used directly (WWDR, 2003).

Handling processes from storage and rinsing to cutting are also possible sources of contamination. Experimental work has demonstrated clearly that passing a knife through a contaminated surface inoculates the newly exposed surfaces and allows pathogen to grow (Lin and Wei, 1997). Insects are another possible source as contaminated files have been shown to be a potential vector of *Salmonella* spp. to fruits (Sela et al., 2005).

2.1.3 Outbreaks associated with *Salmonella* spp.

Fresh vegetables are part of a healthy balanced diet and are generally consumed as raw like salad or side dishes. However, fresh produce could be contaminated during pre- or post-harvesting and becomes vehicles for transmission of *Salmonella* spp. (Guo et al., 2002).

Many studies have shown that outbreaks associated with raw sprouts are originated from contaminated seeds (Mahon et al., 1997). Sprout seeds could be contaminated via contact with pests such as rodents during storage or shipping or on the farms via animal feces (Taormina et al., 1999). *Salmonella* spp. can survive for months on seeds, along with a lack of microbiological control steps. Moreover, sprouting processes under high humidity and moderate temperature conditions may allow cells of low concentrations on seeds to proliferate to high levels (Erdozain et al., 2013). In response to this lack of control , US Food and Drug Administration (FDA) issued a guideline named “Reducing Microbial Food Safety Hazards for Sprouted Seeds” for industry in 1999 to minimize pathogen contamination in sprouts (FDA, 1999). Despite the guidelines entailing the need for pathogen testing, the FDA

inspection report stated that farm microbiological testing was not adequate to monitor pathogen contamination (FDA, 2010).

Poultry are a known reservoir of *Salmonella* spp. and the risk of salmonellosis after contact with live poultry, especially chicks and ducklings, has been verified by stated outbreaks in **Table 1**. Since most outbreaks are associated with purchase and/or contact with live poultry in agricultural feed stores, agricultural feed stores should provide handwashing facilities and information on salmonellosis to persons considering a live poultry purchase.

In Singapore, the documented foodborne outbreaks related to *Salmonella* spp. were due to the presence of eggs as the main ingredient for two reported cases and the other two cases were pre-prepared foods. Consumption of eggs are a frequent cause since eggs is a major source of *S.* Enteritidis. One study reported that when identifying the vehicle of transmission in *Salmonella* outbreak, 77% were caused by food that contained eggs or by eggs alone and were likely to be inadequately cooked (Louis et al., 1988). For the reported prepared meals, the kitchen was found to be dirty and poor hygiene of food handlers was observed. Food handlers were tested positive for *S.* Enteritidis and a high standard plate count was reported from food trays. In addition, it was observed that the same food trays and food scoops were used across raw and cooked foods resulting in cross contamination. As for foods prepared by caterers, the absence of soap on the premises and handling of food by the handlers without hand gloves were reported.

With effect from 15 Feb 2012, National Environment Agency (NEA) in Singapore had a mandatory requirement to have a time stamp on packed

foods and catering foods. The time stamp includes the time and date the food is cooked and when it should be "CONSUMED BY" time not more than 4 hours from the food is cooked (NEA, 2013). Foodborne outbreaks in Singapore associated with *Salmonella* spp. are summarized in **Table 2.**

Table 1. Summary of food borne outbreaks associated with *Salmonella* spp. in US from 2006 to 2013

Food Type	Food borne Pathogens	Year	No of cases	Hospitalization	Reference
Tomatoes	*Salmonella* Typhimurium	Nov 2006	183	22	CDC, 2006
Sprouts, Tiny Greens Organic Farm	*Salmonella serotype* I 4,[5],12:i	Nov 2010	140	34	CDC, 2010
Chicks and Duckling	*Salmonella* Altona	Oct 2011	68	19	CDC, 2011b
Raw Scraped Ground Tuna Product	*Salmonella* Bareilly, *Salmonella* Nchanga	Jan 2012	425	55	CDC, 2012a
Foster Farms Brand Chicken	*Salmonella* Heidelberg	Mar 2013	574	212	CDC, 2013c
Live Poultry in Backyard Flocks	*Salmonella* Typhimurium	Nov 2013	356	62	CDC, 2013a

Table 2 .Summary of food borne outbreaks associated with *Salmonella* spp. in Singapore from 2002 to 2011

Food Type	Foodborne pathogens	Year	No of cases	Hospitalization	Reference
Fried Egg Omelette	Clinical and epidemiological data suggestive of *Salmonella* spp.	Nov 2002	32	0	MOH, 2002
Prepared Meals	Handler tested positive for *S.* Enteritidis and *Salmonella* spp.	May 2006	77	0	MOH, 2006
Food Caterer	Likely to be of bacterial nature such as *Salmonella* spp.	Mar 2007	68	49	MOH, 2007
Omelette Floss Bread	*S.* Enteritidis	Sep 2011	14	4	MOH, 2011

2.2 *Listeria monocytogenes*

2.2.1 Bacteriology

Listeria monocytogenes are non-sporeforming, Gram-positive rods and facultative anaerobe. They are catalase positive, oxidase negative and secrete beta haemolysin that produces clear zones on a sheep plate with *Staphylococcus aureus* termed as Christie, Atkins, and Munch-Peterson (CAMP) test (Farber and Peterkin, 1991). *L. monocytogenes* can utilize glucose, lactose and rhamnose, and cannot utilize xylose under the aerobic condition, hence rhamnose and xylose serves as a key test to differentiate *L. monocytogenes* from other *Listeria* spp. (Gasanov et al., 2005).

2.2.2 Adaptability

L. monocytogenes are slow growing and can be rapidly out-grown by competitors; however, they are also adaptable to harsh conditions compared to their competitors, which explains the difficulty in controlling their presence in foods and environments (Duché et al., 2002). This pathogen is a psychrotophic bacterium that is able to grow at 4°C. When present in low numbers, they can grow to considerable numbers to make consumers sick upon eating refrigerated "ready-to-eat-food" such as packed smoked salmon (Rørvik et al., 1991). Studies have shown an increased expression of bacterial cold shock proteins (Csps) in response to reduced temperatures (Wouters et al., 2000). This pathogen also has the ability to grow in high salt concentrations, which is a common food preservative in smoked salmon.

2.2.3 Sources and transmission

Some food types are closely linked to *L. monocytogenes* contamination, namely cheese, poultry and fish. Studies have demonstrated that *L. monocytogenes* were typically isolated externally such as the surface of the cheese rind and meat (Farber and Peterkin 1991; Eklund et al., 1995). This affirms that in food processing plants, bacterial attachments to food contact surfaces are significant as the vehicle of transmission (Herald and Zottola 1988). One study concluded that excretion of *L. monocytogenes* by farm animals was linked to their diet especially if their diet source was contaminated with *L. monocytogenes* such as silage (Skovgaard and Morgen, 1988). Similar to *Salmonella* spp., when the animal is stressed by situations such as long hours of travelling, increased excretion of *L. monocytogenes* would occur. It is highly likely that contamination takes place on the farm and this potentially carries the bacterial to the food processing environment to be become adaptable and eventually established. This act leads to unwanted contamination of food especially if proper hygiene and testing is not in place and followed dutifully (Fenlon et. al., 1996).

2.2.4 Outbreaks associated with *Listeria monocytogenes*

Out of the four food borne outbreaks associated with *L. monocytogenes,* three were related to cheese products and one was fresh produce as summarized in Table 3. Investigation report stated that pasteurized milk was used in cheese. Although it had been perceived that pasteurized milk would be safer than raw milk, however, there had been studies with findings that stated more *L. monocytogenes* positive cheeses were made from pasteurized milk (8%) compared to the raw milk cheeses (4.8%) (Rudolf and

Scherer, 2001). Investigation report also states that other cheeses associated with products manufactured in the affected food company together with environmental samples harbored *L. monocytogenes*.

Cross contamination from food contact surfaces is likely to cause listeriosis as well. One study concluded that normal pasteurization of milk would prevent contamination of cheeses with *L. monocytogenes*, provided that recontamination during production is prevented (Beckers et al., 1987). Detached *L. monocytogenes* cells from soiled surfaces may contaminate foods and proliferate under refrigeration (Poimenidou et al., 2009).

The external epidermal layer of fruits protects it against microorganisms; once this barrier is broken by actions such as slicing; the internal of the fruit can be contaminated, which allows bacterial growth (Penteado and Leitão, 2004). As such, it is an industrial practice for the produce to be subjected to sanitizer treatments in order to reduce the microbial load. A reported study on inoculation of high levels of *L. monocytogenes* on the external of fresh produce had at least five log reductions after sanitizer treatments. Subsequently, fresh produce was stored in the cold for nine days and *L. monocytogenes* was successfully isolated from treated samples. This finding indicated that sanitizer treatments could only diminish the level of contamination but did not totally eliminate its presence during storage. In addition, this study also showed that *L. monocytogenes* was able to multiply and grow if there were survivors after sanitizer treatments (Rodgers et al., 2004). Hence, it is highly likely that the interior of fruits could be contaminated through improper handling via cutting on cutting board and knife as it had been in contact with the external surface of the fruit.

Recent foodborne outbreaks in US associated with *L. monocytogenes* are summarized in **Table 3**. It should be noted that *L. monocytogenes* cause high mortality rates of 27.6% compared to other pathogens, making elderly, children, immunocompromised people and pregnant woman particularly vulnerable (Mead et al., 1999).

Table 3.Summary of food borne outbreaks associated with *Listeria monocytogenes* in US from 200 to 2013

Food Type	Year	No of cases	Hospitalization	Death	Reference
Cheese (Roos Foods Dairy)	April 2014	8	7	1	CDC, 2014
Crave Brothers Farmstead Cheese	Sep 2013	6	6	1	CDC, 2013b
Frescolina Marte Brand Ricotta Salata Cheese	Nov 2012	22	20	4	CDC, 2012b
Cantaloupe from Jensen Farms	Sep 2011	145	143	33	CDC, 2011c

2.3 Nucleic acid based sequence amplification (NASBA) for the detection of foodborne pathogens

2.3.1 Real-time polymerase chain reaction

The real time PCR (qPCR) consists of three steps namely i) denaturation of the double standed DNA whereby two stands of DNA template are separated from each other at 94°C; ii) temperature is lowered to 55°C to allow annealing of short DNA fragments (primers) to single DNA stands; (iii) lastly, temperature increases to 72°C that is the optimal temperature for extension of the primers with a thermostable DNA polymerase till a double stranded DNA is formed (Scheu et al., 1998; Cornett et al., 2001). Real-time monitoring on amplification is possible with the presence of sequence specific fluorescent probes bound to the amplicon (the fragment of DNA replicated by PCR) and visualized as the amplicons accumulate. A PCR instrument detects the intensity of the fluorescent signal during each replication cycle of the PCR (Hanna et al., 2005). The amplification cycle at which the fluorescence exceeds a defined threshold level that is known as the threshold cycle (Ct) is a measure of the dye fluorescence generated by the cleavage of a probe against a fixed baseline threshold (Corless et al., 2000).

2.3.2 Loop-mediated isothermal amplification

Loop-mediated isothermal amplification (LAMP) was developed by a group of scientists from Tokyo, Japan (Notomi et al., 2000). The motivation came about to develop this novel method was due to the high cost of precision thermal cyclers and the complex method for the detection of amplified products which acts as a hurdle for nucleic acid-based amplification to be

widely used and adopted. The LAMP method does not require costly thermal cyclers and relies on auto cycling strand displacement DNA synthesis that is performed by a DNA polymerase derived from *Bacillus stearothermophilus* (Bst) with high stand displacement activity at optimal temperatures of 60-65°C along with a set of two specially designed inner and outer primers (Notomi et al., 2000). During the initial reaction, all four primers are used, but later on during the amplification, only the inner primers are used for strand displacement DNA synthesis (Notomi et al., 2000; Wang et al., 2008). End amplification products have variously sized structures consisting of alternate inverted repeats of the target sequence on the same strand in large amounts (Hara-Kudo et al, 2005). A schematic diagram of LAMP reactions is shown in **Figure 1**.

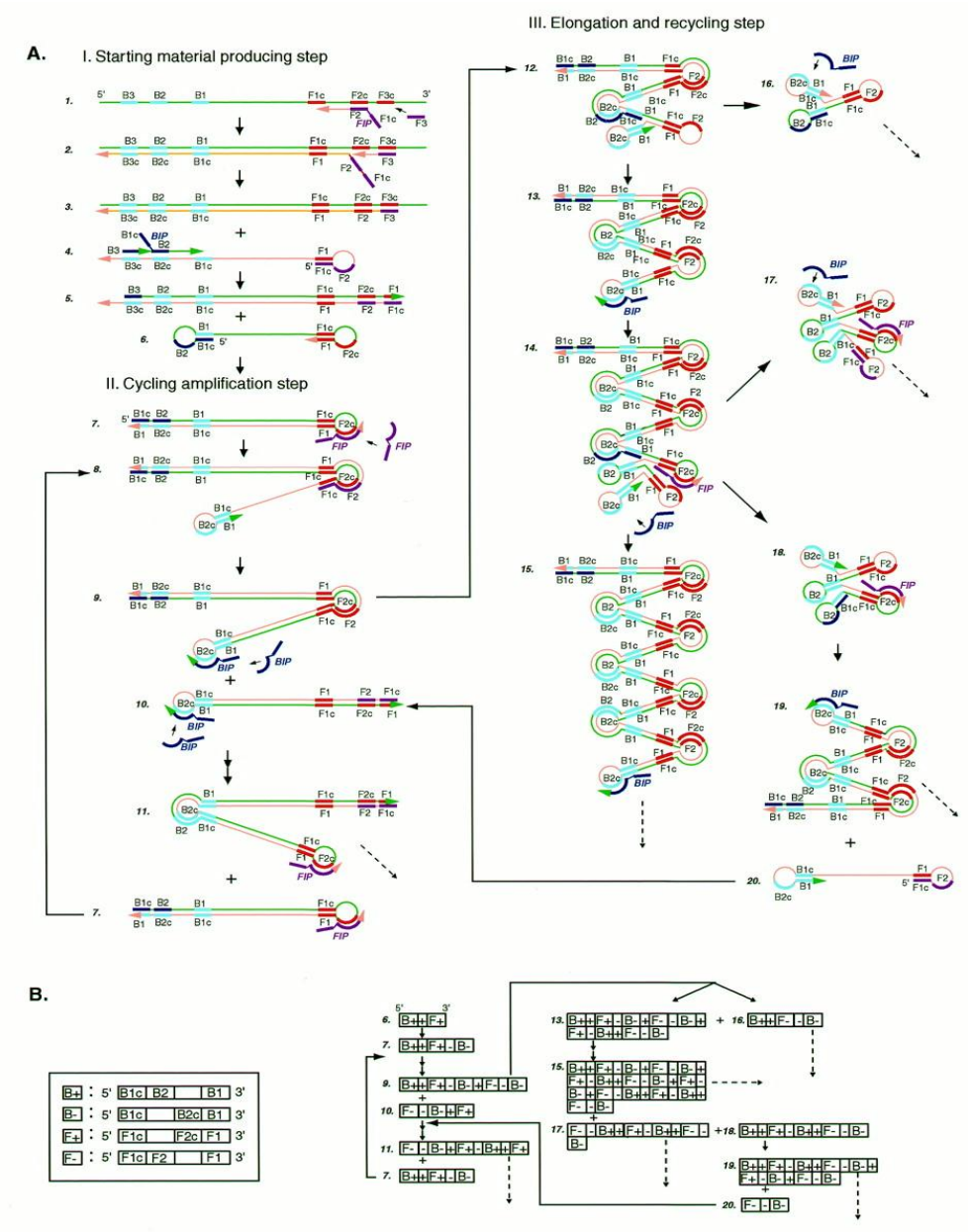

Figure 1. Schematic diagram of loop-mediated isothermal amplification (LAMP) reactions (Adapted from Notomi et al., 2000).

2.3.3 Loop-mediated isothermal amplification with bioluminescence

After DNA amplification, an inorganic pyrophosphate (PPi), which is a by-product of DNA amplification, is converted enzymatically to adenosine bioluminescence by ATP sulfurylase (**Figure 2**). This chemistry simplifies data interpretation and hardware requirements as it allows changes in pyrophosphate levels which result in the level of bioluminescence to increase to a peak .Unlike fluorescence techniques, bioluminescence is not dependent on absolute light intensity produced which makes it possible to have different colored assay tubes for each pathogen assay (Kiddle et al., 2012). This aids in minimizing lab errors as fluorescence techniques only allow clear tubes that could lead to pathogen assay mix-up.

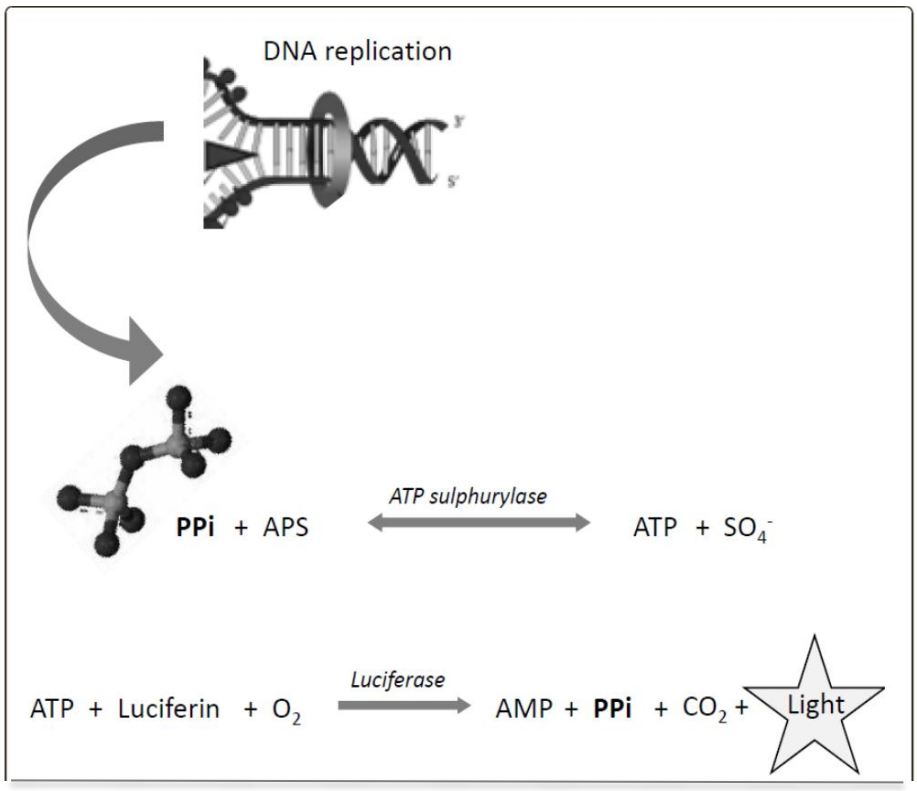

Figure 2. Chemistry of loop-mediated isothermal amplification (LAMP) with bioluminescence pathway (Adapted from Kiddle et al., 2012).

2.3.4 Limitation of PCR based detection methods in food safety

One major concern is obtaining false positive results from nucleic acid based microbiological methods since these methods also can detect intact DNA from non-viable cells. One alternative method to detect only viable cells is to use messenger RNA (mRNA) instead of DNA, however this is not favorable by the industry due to the fact that mRNA have a short half-life, which is less stable and difficult to extract (Bustin and Nolan, 2004). To overcome this shortcoming of the PCR bassed methods, a novel method using dye such as propidium monoazide (PMA) has been developed to distinguish viable and non-viable cells (Rawsthorne et al., 2009; Josefsen et al., 2010). However, this method is still very much at a development state and has not been commercialized for industry use yet. Another possible cause for false

positive results is the large quantities of PCR products formed during the PCR and this poses a potential risk of contamination via transfer by pipette tips, gloves and bench surfaces. Hence, it is important to dispose PCR by products properly, sanitize the working bench thoroughly or ideally to have a dedicate work space for PCR work if space permits. Nevertheless, most PCR reagents nowadays come in a closed tube format, as a result, the risk of contamination is minimized (Lantz et al., 1994).

False negative results would be another greater concern if it is undetected since there is a risk of releasing food contaminated with foodborne pathogens to the market that could lead to unwanted foodborne outbreaks. Most nucleic acid-based microbiological methods rely on an enrichment process to enable target pathogens to reach detection limits of at least 10^4 CFU (Knutsson et al., 2002). Some studies reported that the use of different enrichment broths would lead to different recovery rates of target pathogens during the enrichment process (Zheng et al., 2013). For instance, acid-injured *Salmonella* cells recovered at a much faster rate in universal pre-enrichment broth (UPB) than in lactose broth (LB) or buffered peptone water (BPW) (Liao and Fett, 2005). In fact, it was observed to have a more selective enrichment along with an extended incubation time, would resulted in a better chance to improve sensitivity as well as to achieve detection limits within a given time. Since most PCR methods include a enrichment step before qualitative analysis to ensure the detection of the presence of viable cells only, it would be worthwhile to consider increasing sensitivity by centrifugation, filtration or immunomagnetic separation techniques (Zheng et al., 2014).

Another contributor to false negative results is the presence of inhibitors in foods especially in complex foods such as cheeseburger and milkshake. For instance, soft cheese completely inhibited PCR at all concentrations, along with milk powder as the presence of calcium ions could be a potential inhibitor (Scheu et al.,1998; Bickley et al.,1996). Hence, it is common for industry to dilute the inhibitors. However, there could also be a possibility that target pathogens be diluted (Lantz et al.,1994). In response to this concern, scientists have successfully removed inhibitors via mixed-bed resins and chelating resins without comprising on the target pathogens (Abbaszadegan et al., 1993).

2.3.5 Performance characteristics in selections of rapid methods

NASBA has been very successful for research work among the highly skilled research worker. However, it posed a challenge to be the preferred method for routine lab largely due to high cost of machine and lack of skilled workers to perform the test. In this section, performance characteristics (PC) in selections of rapid methods is reviewed (Jasson et al., 2010).

Determination of PC is often in comparison to conventional method. PC encompasses of sensitivity, specificity and validation. Sensitivity is important as often low numbers of pathogens are present in biological environment along with high background microflora. Hence, if rapid method is not sensitive, it could result in a false negative result and risk in sending pathogen contaminated food to the market. Often, this is minimized by having primary and secondary enrichment that give an environment that favors the target pathogen to grow and suppressed the background microflora. Sensitivity

can also be determined via the limit of detection (LOD) which is the lowest level of pathogens present to be detected by the method.

Specificity refers to that only the target pathogen is detected and non-target pathogens should not been detected. In the event that rapid method is not specific, it could lead to false positive result that could lead to delay in releasing shipment to the market due to further investigation work. It is an industry practice for positive results obtained from rapid methods to be subjected to conventional methods for confirmation.

Validation is the process whereby the performance characteristics of an analytical method meet the requirements for the intended applications (Biringanine et al., 2006). In chapter 3, validation entailed the analysis of artifically inoculated food pathogens to food samples and naturally contaminated samples with both results compared to those obtained when using conventional methods. "Naturally contaminated" is defined as presence of pathogens are not artificially inoculated and are present as a result of production or environmental contamination (FAO, 1995).

CHAPTER 3

COMPARISON OF 3M™ MOLECULAR DETECTION ASSAY (MDA) *SALMONELLA* WITH STANDARD ISO METHOD FOR RAPID DETECTION OF *SALMONELLA* SPP ON RAW DUCK WINGS, RAW MUNG BEAN SPROUTS AND PROCESSED FISHBALLS

3.1 Introduction

In 2011, Center for Disease Control and Prevention in US reported nontyphoidal *Salmonella* spp. caused an estimated 1 million cases of foodborne illness, approximately 19,336 cases require hospitalization and 378 cases result in deaths (CDC, 2011d). The incidence rate is 16.8 per 100,000 population and exceeded the target of 11.4 incidences per 100,000 population that US set to achieve (CDC, 2011a). In Singapore, the mandatory notification for nontyphoidal salmonellosis started in 2008 with 14.9 incidence rate per 100,000 population with the highest incidence rate of 29.2 per 100,000 population reported in 2010 (Kondakci and Yuk, 2012). This alarming increase in incidence rates clearly demonstrates that salmonellosis is a severe problem and a public threat. The major vehicles for the transmission of *Salmonella* spp. to humans are fresh produce, poultry and seafood products in particular (Fratamico, 2003; Velusamy et al., 2010). Hence, rapid and reliable laboratory testing is a critical component in food safety monitoring to prevent salmonellosis and to find a causative agent in the event of outbreaks through the food supply (Yang et al., 2014).

Although, typically a bacterial dose of more than 10^5 *Salmonella* cells can cause an infection in humans, there are evidences that an ingestion of as little as 10^0 to 10^1 cells is capable of causing illnesses in susceptible hosts (Kokkinos et al., 2014). Generally, *Salmonella* spp. is present in food products in very low numbers and the physiological state of cells might be weakened due to the environmental stresses (Fratamico, 2003; Velusamy et al., 2010). For these reasons, a standard culture-based method for the detection of *Salmonella* spp. (ISO 6579, 2002) includes the use of two enrichment steps followed by differential plating on selective agar to ensure the recovery of bacterial cells. Afterwards, the presumptive colonies are confirmed biochemically and serologically, which can extend the overall assay testing from days to weeks. Therefore, it is impractical to use conventional culture methods for high-throughput screening of large numbers of food samples for determining the presence or absence of *Salmonella* spp. (Liang et al., 2011).

A simple, and a cost effective novel detection method using loop mediated isothermal DNA amplification (LAMP) combined with bioluminescence detection, named as 3M™ molecular detection system (MDS), has recently been developed (3M, 2012). 3M™ molecular detection assay (MDA) is used with 3M™ MDS for qualitative analysis of pathogens in samples (Bird et al., 2013). In comparison to PCR-based detection methods, LAMP does not require costly instrumentation such as thermal cyclers and special reagents. LAMP is also characterized by higher specificity and sensitivity and it significantly shortens the time of DNA amplification due to isothermal reaction conditions (Kokkinos et al., 2014).

The evaluation of 3M™ molecular detection assay (MDA) for the detection of *Salmonella* cells has been performed only for a limited number of food products including raw ground beef and wet dog food (Bird et al., 2013), dried fruits and nuts (Yang and Benedetto, 2013), chicken nuggets, raw ground beef, raw frozen shrimps, liquid eggs, fresh spinach and pet food (Eggink, 2012). More data with various foods such as seafood, vegetables and meat products are necessary to evaluate and validate 3M MDS for food industry applications. Therefore, the main aim of this study was to evaluate the performance of 3M™ MDS for detecting healthy and thermally- or sanitizer-injured *Salmonella* cells artificially inoculated in low numbers on raw duck wings, raw mung bean sprouts and processed fishballs by comparing with a ISO standard method. Additionally, the validation study of 3M™ MDA on naturally contaminated food products was performed.

3.2 Materials and Methods

3.2.1 Bacterial cultures and preparation of inoculum

Poultry associated species: *Salmonella* Typhimurium (ATCC 14028) and *S.* Agona (ATCC BAA707), *S.* Montevideo (ATCC BAA710), *S.* Newport (ATCC 6962) and *S.* Saintpaul (ATCC 9712) were purchased from American Type Culture Collection (Manassas, VA, USA). The frozen cultures were activated in 10 ml of tryptone soya broth (TSB; Oxoid, Basingstoke, Hampshire, UK) for 24 h at 37°C. One ml of bacterial culture from respective serovars was transferred to 1.5 ml of sterile eppendorf tube, centrifuged at 3,500 g for 10 min at 4°C, washed twice with 0.1% (w/v) peptone water (PW;

Oxoid) and finally the pellet was resuspended in 1 ml of 0.1% (w/v) PW. Equal volumes of each bacterial culture were mixed to prepare the five strain cocktail (about 10^8 CFU/ml).

3.2.2 Preparation of heat- and sanitizer-injured cells.

To prepare heat-injured *Salmonella* cells, 2 ml of the 5-strain cocktail was placed in a sterile aluminum can (3.0 cm diameter and 1.2 cm height) and heated in a water bath of 60°C for 70 sec and cooled immediately in ice cubes for 1-2 min. To prepare sanitizer-injured cells, 0.1 ml of the cocktail culture was treated with 9.9 ml of 13 ppm sodium hypochlorite (4-6%; Hygold Chemical Supplies, Singapore) for 50 sec by vortexing and immediately 1 ml of mixture was transferred to 9 ml of sterile 0.1% (w/v) PW. The final concentration of free chlorine was determined using RQflex® 10 Reflectoquant® (Merck, Darmsradt, Germany) according to manufacturer's instruction.

The percentage of sublethal injury was calculated from the ratio of the numbers of colonies on xylose lysine deoxycholate agar (XLD; Oxoid) as the selective agar to tryptone soya agar (TSA; Oxoid) as the non-selective agar as follows (Uyttendaele et al., 2008):

$$\text{Sublethal injury (\%)} = \left(1 - \frac{\text{Colonies on XLD}}{\text{Colonies on TSA}}\right) \times 100$$

The final percentage of sub-lethal heat and sanitizer injuries was between 80 - 85%.

3.2.3 Inoculation of *Salmonella* cells on food samples

Raw duck wings, raw mung bean sprouts and processed fishballs were purchased from local supermarkets in Singapore and stored at 4ºC prior to use and they were screened for the absence of *Salmonella* spp. by a standard culture method (ISO 6579, 2002). For each product, 40 artificially spiked samples and corresponding 40 uninoculated naturally contaminated samples were used to evaluate the performance of 3M™ MDA. Additional 30 naturally contaminated samples were used for validation study. Prior to inoculation, duck wings and fishballs were cut with a sterile scissor and weighed to 25 g under sterile conditions. The bacterial cocktail was serially diluted with 0.1% (w/v) PW and a 10-µl aliquot of diluted culture was spot inoculated at 10 sites of the surface of weighed duck wings or fishballs to achieve 10^0 and 10^1 CFU/25 g. For mung bean sprouts, the samples of 280 g were submerged in 2 L suspension of bacterial cocktail (ca. 10^4 – 10^5 CFU/ml) in a beaker for 45 min with magnetic stirring to achieve even inoculations of 10^0 and 10^1 CFU/25 g (Neo et al., 2013). After inoculation, the bean sprouts were air dried on sterile plastic tray for 1 h in a biosafety cabinet.

Heat-injured cells were spotted on duck wings and fishballs because *Salmonella* spp present may be sublethally heat injured during plucking of duck feathers or during fishball making that involves boiling and steaming. (Zheng et al., 2013; Kok et al., 2013). Raw duck was placed in water bath between 71ºC and 77ºC for 1 to 2 minutes to soften the feathers before plucking. Bean sprouts were inoculated with sanitizer-injured cells as it is an industry practice to wash fresh produce in sanitizer to remove or inactivate any pathogen present (Sapers, 2001). As such, potentially injured pathogens that

could not be detected with a standard protocol but given a favorable condition could resuscitate and cause sickness (Baylis et al., 2000). The samples of 25 g of inoculated duck wings, bean sprouts and fishballs were transferred to stomacher bags and stored overnight in a refrigerator (at 6-7°C) to simulate supermarket conditions. A total of 330 samples were studied in this experiment including 120 artificially spiked samples, 120 uninoculated naturally contaminated samples as control and 90 naturally contaminated samples for validation. The experiment design is presented in **Figure. 3**.

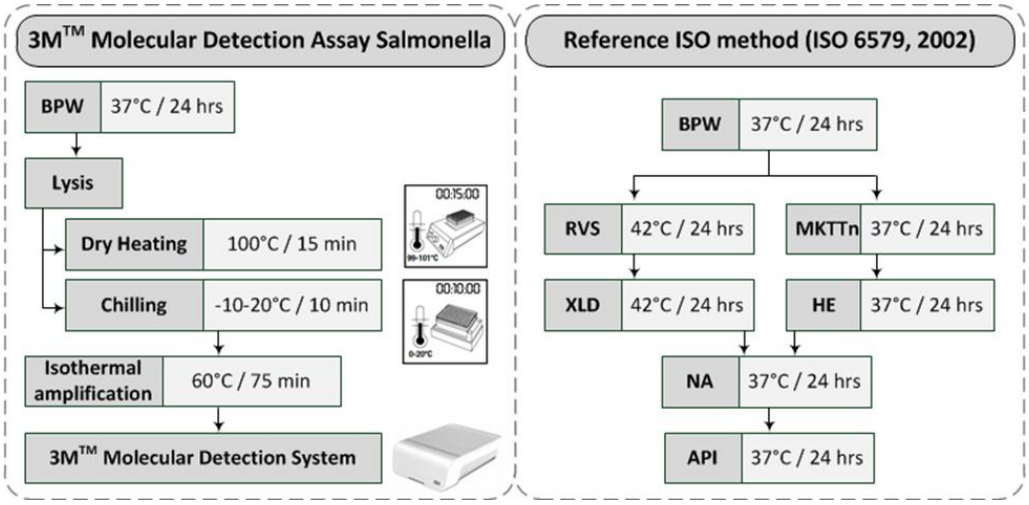

Figure 3. The experiment design of 3M™ molecular detection system (MDA) comparison with ISO methods for the detection of healthy and sub-lethally injured *Salmonella* cells inoculated in 10^0 CFU/25 g) and 10^1 CFU/25 g) on different food matrices. BPW, buffered peptone water, RVS, Rappaport–Vassiliadis medium with soya broth, MKTTn, Muller–Kauffmann tetrathionate –novobiocin broth, XLD, xylose lysine deoxycholate agar, HE, Hektoen Enteric agar, NA, nutrient agar.

3.2.4 Standard culture method

An international organization for standardization (ISO 6579, 2002) method was adapted in this study. Briefly, 225 ml of buffered peptone water (BPW; 3M, St. Paul, Minnesota, USA) was added to 25 g of inoculated and uninoculated food samples, homogenized for 2 min in a stomacher blender

(Silver Masticator, IUL Instruments GmbH, Königswinter, Germany) and subsequently incubated at 37°C for 18 – 24 h. After pre-enrichment, 0.1 and 1 ml of enrichment were transferred to 10 ml of Rappaport–Vassiliadis medium with soya broth (RVS; Oxoid) and 10 ml of Muller–Kauffmann tetrathionate – novobiocin broth (MKTTn; Oxoid), incubated at 42 and 37°C, respectively for 24 ± 3 h. A loopful of selective enrichment was streaked onto XLD agar and Hektoen Enteric agar (HE; Oxoid), respectively and incubated at 37°C for 24 ± 3 h for the isolation of presumptive colonies. The presumptive colonies were streaked onto nutrient agar (Oxoid) and incubated at 37°C for 24 ± 3 h. For the biochemical confirmation, the colony from nutrient agar was emulsified in suspension media and transferred to API 20E (BioMerieux, Chemin de l'Orme, Marcy l'Etoile, France), followed by incubating at 37°C for 24 ± 3 h. The results were interpreted by APIWEB software (BioMerieux).

3.2.5 3M™ molecular detection assay (MDA) *Salmonella*

The detection of *Salmonella* spp. by 3M™ MDA was performed following the manufacturer's manual (3M, St. Paul, Minnesota, USA) and according to Bird et al. (2013). After enrichment in BPW as described above, 20 µl of enrichment was added to lysis solution (LS) tubes. The mixtures were heated in a 3M molecular detection heat block insert (Heat Block Insert: 3M; Heater unit: Henry Troemner LLC, Thorofare, NJ, USA) at 100 ± 1°C for 15 min followed by immediate cooling at –10 to –20°C in a pre-chilled 3M molecular detection chill block (3M) for 10 min. After mixing, 20 µl of lysate was transferred into a *Salmonella* assay tube that contained assay reagents in a lyophilized form and pipetted up and down for 5 times gently to mix with the

lyophilized reagent. The tubes were placed in 3M™ MDS for the detection of *Salmonella* cells via isothermal amplification and bioluminescence detection for 75 min. All analyses included negative and reagent controls to validate the performance of MDS.

For the purpose of this investigation, for every false negative result obtained with 3M™ MDA *Salmonella*, two additional protocols for sensitivity optimization were performed. The 1st protocol was adding 20 ul of secondary enrichment - RVS to lysis solution (LS) tubes instead of BPW. For the 2nd protocol, 1 ml of BPW-enriched samples was mechanically concentrated using centrifugation and subsequently re-suspended the pellet in 20 µl of 0.1% (w/v) PW and added to lysis solution (LS) tubes.

3.2.6 Statistical analysis

Sensitivity and specificity of 3M MDA Salmonella were defined as the number of samples truly positive (T_{pos}) and truly negative (T_{neg}), respectively, compared with ISO method. The sensitivity, specificity and accuracy of 3M MDA Salmonella were calculated as follows:

$$\text{Sensitivity} = [T_{pos}/(T_{pos} + F_{neg})] \times 100$$

$$\text{Specificity} = [T_{neg}/(T_{neg} + F_{pos})] \times 100$$

$$\text{Accuracy} = [(T_{neg} + T_{pos}/(T_{pos} + T_{neg} + F_{neg} + F_{pos})] \times 100$$

where, T_{pos} and T_{neg} are the number of positive and negative samples, respectively, confirmed by both ISO and 3M MDA *Salmonella*, F_{pos} and F_{neg} are the number of positive and negative samples, respectively, confirmed by

only 3M MDA *Salmonella* (Malorny et al., 2003; Zheng et al., 2014). Kappa value of concordance, describing the statistical agreement between two detection methods was calculated as described elsewhere (Malorny et al., 2003). Kappa values were classified as follows: <0.01 indicate no concordance, 0.1- 0.4 indicate weak concordance, 0.41 - 0.60 indicate clear concordance, 0.61 - 0.80 indicate strong concordance, and 0.81 - 1.00 indicate nearly complete agreement.

3.3 Results and Discussion

It is known that processing of raw ducks involves scalding of skin to remove feather; inevitably any presence of *Salmonella* spp. externally could be injured by heat treatment (Zheng et al., 2013). Similarly, *Salmonella* cells in fishballs might be thermally injured during cooking. For mung bean sprouts, the sprouts are generally subjected to chlorinated water for removing microbial load as well as pathogenic bacteria. Thus, it is essential to evaluate conventional and rapid detection methods for injured cells since these cells could be resuscitated during storage due to the potential for improper handling and temperature abuse.

The results of this study showed that regardless of inoculum levels, all healthy and injured *Salmonella* cells on raw duck wings, raw mung bean sprouts and processed fishballs were accurately detected by 3M™ MDA with 100 % sensitivity and specificity in comparison with the ISO method, except for bean sprouts inoculated with 10^0 CFU/25 g of sanitizer-injured cells (**Table 4 and 5**). Only 2/10 inoculated bean sprout samples were confirmed as positive while 8/10 samples were not detected by 3M™ MDA after primary

enrichment in BPW (**Table 5**), resulting in lower sensitivity (20%) and Kappa value (0.2). The possible reasons for this lower sensitivity might be due to the limited selectivity of BPW enrichment broth for *Salmonella* recovery in the presence of a high number of Gram negative microflora, which was previously reported (Splittstoesser et al., 1983; Patterson et al., 1980). Microbial in bean sprouts were reported to have high counts of the family Enterobacteriaceae comprising mainly of *E.coli* (Abadias M et al., 2008). It is likely that the high background microflora could inhibit the recovery or suppress the growth of injured *Salmonella* cells during the primary enrichment step in BPW, hence, the number of *Salmonella* cells could not reach the detection limit of 3M™ MDA that was 8.8 x 10^3 CFU/ml (3M, 2012) leading false negative results. In this study, raw mung bean sprouts had the highest microbial load of 10^9 CFU/g, compared to raw duck wings and fishballs, for which 10^5 and 10^2 CFU/g, were determined, respectively (data not shown).

Table 4. Comparison of 3M™ MDA and ISO methods on the detection of healthy and sub-lethally injured *Salmonella* spp. inoculated on raw duck wings, raw mung bean sprouts and processed fishballs at an inoculum level of 10^1 CFU/25 g.

Food Matrix	Cells Type	ISO and 3M™ MDA *Salmonella*		3M™ MDA *Salmonella*		Sensitivity [%]	Specificity [%]	Accuracy [%]	Kappa
		T_{pos}	T_{neg}	F_{pos}	F_{neg}				
Duck Wing (n=20)	healthy	10	10	0	0	100	100	100	1
	heat-injured	10	10	0	0	100	100	100	1
Bean Sprouts (n=20)	healthy	10	10	0	0	100	100	100	1
	sanitizer-injured	10	10	0	0	100	100	100	1
Fishballs (n=20)	healthy	10	10	0	0	100	100	100	1
	heat-injured	10	10	0	0	100	100	100	1

Table 5. Comparison of 3M™ MDA and ISO methods on the detection of healthy and sub-lethally injured *Salmonella* spp. inoculated on raw duck wings, raw mung bean sprouts and processed fishballs at an inoculum level of 10^0 CFU/25 g.

Food Matrix	Cells Type	Enrichment[a]	ISO and 3M™ MDA *Salmonella*		3M™ MDA *Salmonella*		Sensitivity [%]	Specificity [%]	Accuracy [%]	Kappa
			T_{pos}	T_{neg}	F_{pos}	F_{neg}				
Duck Wings (n=20)	healthy	BPW	10	10	0	0	100	100	100	1
	heat-injured	BPW	10	10	0	0	100	100	100	1
	heat-injured	BPW + RVS	NT	NT	NT	NT	NT	NT	NT	NT
Bean Sprouts (n=20)	healthy	BPW	10	10	0	0	100	100	100	1
	sanitizer-injured	BPW	2	10	0	8[b]	20	100	60	0.2
	sanitizer-injured	BPW + RVS	10	10	0	0	100	100	100	1
Fishballs (n=20)	healthy	BPW	10	10	0	0	100	100	100	1
	heat-injured	BPW	10	10	0	0	100	100	100	1
	heat-injured	BPW + RVS	NT	NT	NT	NT	NT	NT	NT	NT

NT, not tested

[a] *Primary enrichment (BPW), secondary enrichment (RVS)*

[b] *False negative samples (n=8) were subjected to two additional procedures: secondary enrichment in RVS and mechanical concentration of Salmonella cells using centrifugation.*

Two additional procedures were applied to the bean sprout samples with false negative results (n=8) in order to improve the sensitivity of 3M™ MDA for sanitizer-injured *Salmonella* detection at a low inoculum level. Firstly, the secondary enrichment step in RVS was added to extend the time for the resuscitation of sanitizer-injured *Salmonella* cells. RVS broth was chosen over MKTTn as it had been reported that a higher temperature of incubation 42°C enhanced the recovery of *Salmonella* spp. and inhibited the growth of background microflora more effectively than 37ºC (Fratamico, 2003). Additionally, a trial was conducted with the objective to mechanically concentrate *Salmonella* cells using centrifugation of 1 ml of BPW-enriched samples and subsequently re-suspended the pellet in 20 µl of 0.1% (w/v) PW. Application of both procedures resulted in *Salmonella* spp. detection by 3M™ MDA reaching 100% sensitivity, indicating nearly complete concordance. These results demonstrated that more selective enrichment along with extended incubation time allowed the recovery of injured *Salmonella* cells in the presence of high background microflora. These results demonstrate that a more selective enrichment along with extended incubation time might allow the recovery of injured *Salmonella* cells in the presence of high background microflora. This observation was supported by a previous study (Zheng et al., 2013) who reported that a single enrichment step in BPW of raw duck samples was incapable of recovering 85% heat-injured *Salmonella* spp. after 24 h of incubation at 37°C. However, the urgent need of the modern industry is to shorten the overall analysis time without comprising the sensitivity of the detection assay. Therefore, the concentration of primary enrichment (BPW)

using centrifugation method could be a recommended procedure to improve 3M™ MDA sensitivity without extended analysis time.

Table 6. Validation of 3M™ MDA *Salmonella* for the detection of *Salmonella* spp. on naturally contaminated raw duck wings, raw mung bean sprouts and processed fishballs.

Food Matrix	Culture ISO method		3M™ MDA *Salmonella*		Sensitivity	Specificity	Accuracy	Kappa
	T_{pos}	T_{neg}	F_{pos}	F_{neg}	[%]	[%]	[%]	
Duck Wings (n=30)	10	18	1	1	91	95	93	1
Bean Sprouts (n=30)	2[a]	28	0	0	100	100	100	1
Fishballs (n=30)	7	23	0	0	100	100	100	1

[a] *Samples became positive after further incubation of primary enrichment and subjected to fresh secondary enrichment and selective agar.*

A total of 19 out of 90 food samples were found to be naturally contaminated with *Salmonella* spp. and detected with both methods, including 10 raw duck wings, 2 raw mung bean sprouts and 7 processed fishballs. 3M™ MDS successfully detected *Salmonella* cells on bean sprouts and processed fishballs with 100 % sensitivity and specificity, while two raw duck wing samples were detected as false positive and false negative respectively (**Table 6**).

Similar to the present study, the 3M MDA *Salmonella* has been found as a reliable and accurate method for the detection of *Salmonella* spp. in several food matrices reported. All previous studies (Bird et al., 2013; Yang and Benedetto, 2013; Eggink, 2012) reported nearly 100% specificity and sensitivity of 3M^{TM} MDA or statistically no significant difference between 3M^{TM} MDA and applied reference methods, underlining a lack of food matrix interferences on the detection of *Salmonella* spp. Only a few false positive results were reported by a multi-laboratory collaborative study (Bird et al., 2013), probably due to the cross-contamination of samples in laboratory environments from the high number of samples analyzed, including test portions inoculated at a high inoculum level. The 3M^{TM} MDA *Salmonella* was also reported to be 99% inclusivity and 100% exclusivity for the detection of 104 target *Salmonella* serotypes (except *S.* Westhampton) and 50 non-target bacterial strains (Eggink, 2012). In this study, 3M^{TM} MDA was able to detect a diversity of five *Salmonella* strains of a bacterial cocktail. However, all of the above reports focused on the detection of active and healthy bacterial cells.

The well-known advantage of conventional culture methods over molecular detection techniques is the higher detection possibility of injured or

weakened cells due to their resuscitation during the incubation in selective or semi-selective media. In this study, a single step enrichment in BPW was found not effective in the recovery of sanitizer-injured *Salmonella* cells present on bean sprout samples at an inoculum level of 10^0 CFU/25 g as opposed to thermally injured *Salmonella* cells at the same level being resuscitate to reach the detection limit of 3M™ MDA. This observation indicates that the resuscitation period for sanitizer-injured *Salmonella* cells might be longer. Similarly, Bunduki et al., (1995) also reported that the time for repair of *L. monocytogenes* was greater for sanitizer-injured cells (14 h) than for heat-injured cells (5 h) by comparing the repair curves.

Recovery rate of sanitizer injured *Salmonella* cells is dependent on the extent of injury, type of enrichment and time given to recover. It had been reported that acid-injured *Salmonella* cells recovered at a much faster rate in universal pre-enrichment broth (UPB) than in lactose broth (LB) or buffered peptone water (BPW) (Liao and Fett, 2005). Hence, it is likely that in the two naturally contaminated bean sprouts samples, injured *Salmonella* cells present grew to the detection limit of 3M™ MDA and was detected as positive. For the ISO method, it could be that resuscitation of injured *Salmonella* cells was still not enough to express their characteristics on selective plates and likely was masked by the growth of background flora. Only upon further incubation of primary enrichment (BPW) and being subjected to fresh secondary enrichment and selective agar, *Salmonella* spp. was isolated. There had been a study describing the poor performance of selective media for recovering pathogens from treated samples (Bari et al., 2003) since most selective media contain agents such as antibiotics that were designed to select for healthy

target microorganisms (Wu and Fung, 2001). This possibility coupled with the presence of high background flora is likely the cause for unsuccessful recovery of *Salmonella* cells with the ISO method in the first attempt.

3.4 Conclusion

The results of this study revealed that the 3M^{TM} MDA method performed equally effective compared with a reference ISO method for the detection of artificially inoculated healthy *Salmonella* cells and naturally contaminated raw duck wings, raw mung bean sprouts and processed fishballs within significantly shorter time (less than 26 h). However, this study suggests that in the case of sub-lethally injured cells, additional enrichment step or bacterial concentration by centrifugation should be considered to improve the sensitivity of 3M^{TM} MDA.

CHAPTER 4

COMPARISON OF 3M™ MOLECULAR DETECTION ASSAY (MDA) *LISTERIA MONOCYTOGENES* WITH ISO STANDARD METHOD FOR RAPID DETECTION OF *L.MONOCYTOGENES* ARTIFICALLY INOCULATED ON ENVIRONMENTAL SURFACES WITH NO ORGANIC LOAD AND ORGANIC LOAD

4.1 Introduction

Listeria monocytogenes is estimated to cause nearly 1,600 illnesses each year in US, resulting in more than 1,400 related hospitalizations and 250 related deaths (Scallan et al., 2011). Food contact surface contaminated with *L. monocytogenes* is a major issue since such a surface has been identified as the major source of the pathogens and keeps on contaminating the final products (Buckenhuskes and Rendlen, 2004; Verran et al., 2008).

Food contact surfaces used for the preparation of food are made mostly of stainless steel or polyethylene. Stainless steel is often applied because of its mechanical strength and abrasion resistance, while polyethylene is applied due to its flexibility and light-weight (Kusumaningrum et al., 2003). Both materials are easy to be fabricated and are long lasting. A comprehensive study conducted in a poultry processing environment for *L. monocytogenes* concluded that plastics such as those used in cutting boards have an uneven surface and would be more susceptible to pits and cuts that could harbor organic load associated with *L. monocytogenes* contamination as opposed to stainless steel surfaces that have an even surface (Chasseignaux et al., 2002).

The reason for this conclusion is that microorganisms hiding in these cuts and pits could escape cleaning and the disinfection process which could be a potential source for contamination (Hilbert et al., 2003).

To minimize cross-contamination via these food contact surfaces, it is important to establish and validate proper hygiene programs in food processing plants (Tompkin, 2002). As most pathogens are often present in low numbers on food contact surfaces, it is important to detect foodborne pathogens on the surfaces, the detection method itself should be sensitive, specific and rapid to allow corrective and preventive action to be in place promptly to contain and/or eradicate pathogen contamination if any (Garrido et al., 2013).

Although there were studies on the rapid detection of *L. monocytogenes* artificially inoculated at low levels on various food types, to my knowledge, the detection of *L. monocytogenes* artificially inoculated at low levels on environmental surfaces has not been studied. Hence, the objective of this study was to evaluate the performance of 3M™ MDA on the detection of *L. monocytogenes* on stainless steel and polyethylene by comparing with a standard ISO method. In addition, the presence of organic load was also studied to determine whether it enhanced survival of *L. monocytogenes* cells on these food contact surfaces artificially inoculated at 3 inoculum levels: 10^0, 10^1 and 10^2 CFU/100 cm^2.

4.2 Materials and methods

4.2.1 Bacterial culture

Listeria monocytogenes serovar 1/2a (ATCC BAA679), *L. monocytogenes* serovar 1/2b (ATCC BAA839) and *L. monocytogenes* serovar 4b (ATCC 13932) were purchased from American Type Culture Collection (Manassas, VA, USA). The frozen cultures were activated in 10 ml of tryptone soya broth (TSB, Oxoid, Basingstoke, Hampshire,UK) for 24 h at 37°C. One ml of cultivated bacterial culture from respective serovar was transferred to 1.5 ml of sterile eppendorf tube and centrifuged at 3,500 g for 10 min at 4°C, washed twice with 0.1% (w/v) peptone water (PW, Oxoid) and finally the pellet was resuspended in 1 ml of 0.1% (w/v) PW. Equal volumes of each bacterial culture were mixed to prepare the 3-strain cocktail (about 10^8 CFU/ml).

4.2.2 Preparation of food contact surfaces

Stainless steel (SS) and polyethylene (PE) plates were chosen as it is used extensively at food processing plants and food establishments (Norwood and Gilmour, 1999). SS plates (grade 314) were manufactured and purchased from the Physics Workshop in National University of Singapore, while PE plates were purchased from a household retail shop. Flat SS and PE plates were divided into 10 x 10 cm. SS plates were cleaned, dried, wrapped in aluminum foil and autoclaved at 121ºC for 15 min. PE plates were cleaned, dried, disinfected with 70% (v/v) ethanol and UV (254 nm) for a minimum of 30 min for sterilization.

4.2.3 Recovery of *L. monocytogenes* from the surfaces

Quality seal of 3M Enviro swab was removed, followed by removal of swab from the tube. 3M Enviro swab has a large swab head with a shaped tip to allow the maximum contact area. Swabbing was performed across the entire surface with horizontal, vertical and diagonal strokes. After swabbing, swab was replaced in its tube and enriched with media specific for ISO and MDA methods as described below.

4.2.4 Inoculation on test surfaces

The 3-strain bacterial cocktail was serially diluted with 0.1% (w/v) PW and 25 µl aliquot of each dilution was spot inoculated at 10 sites of the test surface to achieve 10^0, 10^1 and 10^2 CFU/100 cm^2, and left to air dry till visually dried in a biological safety cabinet (BSC).

As *L. monocytogenes* contamination is frequently associated with smoked salmon, hence salmon tissue was chosen as source of organic load in this study (Rørvik, 2000). For the surface with organic load, 10 g of raw salmon was placed in a stomacher bag with 90 ml of 0.1% (w/v) peptone water and homogenized for 2 min at room temperature. Subsequently, 250 µl of raw salmon homogenized suspension was added to the surface and spread evenly across the surface using a L-shape spreader, followed by drying in BSC prior to bacterial inoculation.

4.2.5 Standard culture method

An international organization for standardization (ISO 11290, 1996) method was adopted in this study. Briefly, half Fraser broth (Oxoid) as a primary enrichment was added till the swab head was completely submerged and incubated at 30 °C for 24 ± 2 h. After the primary enrichment, 0.1 ml of enrichment was transferred into Full Fraser broth (Oxoid) as a secondary enrichment and incubated for 24 – 48 h at 37°C. The presence of esculin in the secondary enrichment enabled the detection of β–D-glucosidase activity produced from *Listeria* spp. causing a blackening of the enrichment medium. A loopful of the secondary enrichment was streaked onto PALCAM and Oxford agars (Oxoid), respectively, and these plates were incubated at 37°C for 24 - 48 h for the isolation of presumptive colonies. The presumptive colonies were streaked onto nutrient agar (Oxoid) and incubated at 37ºC for 24 ± 3 h. For biochemical confirmation, the colony from NA was tested with API Listeria (BioMerieux), followed by incubating at 37ºC for 24 ± 3 h. The results were interpreted by APIWEB software (BioMerieux).

4.2.6 3M™ molecular detection assay (MDA) *Listeria monocytogenes*

Demi Fraser broth (3M™) was added till the swab head was completely submerged and incubated at 37°C for 26 - 30 h. The detection of 3M™ MDA *Listeria monocytogenes* was performed according to the manufacturer's manual as described in Chapter 3.

4.2.7 Statistical Analysis

Sensitivity and specificity of 3M^{TM} MDA *Listeria monocytogenes* were calculated as described in Chapter 3.

4.3 Results and Discussion

In this study, a 3-strain cocktail of *L. monocytogenes* isolates from different origins (animal and clinical specimens) was used in order to avoid the strain-specific effects on the detection of *L. monocytogenes*. Un-inoculated control samples on test surfaces with organic load subject to ISO and 3M^{TM} MDA methods were all negative (data not shown). Regardless of organic load, the results obtained from this study showed that detection of *L. monocytogenes* on SS and PE surfaces at inoculum levels of 10^0 and 10^2 CFU/100 cm^2 showed 100 % specificity and 100% sensitivity, respectively (**Table 7**). Both ISO and 3M^{TM} MDA methods were unable to detect *L. monocytogenes* at an inoculum level of 10^0 CFU/100 cm^2, regardless of organic load and surface type. Out of 20 swab samples detected by the ISO method, only one was positive, leading one false negative result reported by 3M^{TM} MDA. This is because the viability of *L. monocytogenes* might decrease under the dried condition. Similarly, Kang et al. (2007) reported that the population of *L. monocytogenes* significantly decreased as drying time increased for the inoculated coupons, leading to a reduction of the minimum detection limit due to loss in microbial viability during drying. At 10^2 CFU/100 cm^2, both methods enabled detection of *L. monocytogenes* in all 20 samples, indicating that the initial inoculum had a direct influence on the minimum detection limit. At a high initial inoculum,

L. monocytogenes were more likely to exist in clumps which made them more resistant to drying, hence, reducing loss of microbial viability that led to successful detection of *L. monocytogenes* in both methods.

Table 7. Comparison of 3M™ MDA and ISO methods on the detection of inoculated *Listeria monocytogenes* at inoculum levels of 10^0, 10^1 and 10^2 CFU/100 cm^2 on stainless steel (SS) and polyethylene (PE) surfaces with organic load (OL) and without (w/o) organic load (OL).

Inoculum level	Surface condition	Surface Type	Culture ISO method		3M™ MDA *L.monocytogenes*		Sensitivity	Specificity	Accuracy
			T_{pos}	T_{neg}	F_{pos}	F_{neg}	[%]	[%]	[%]
10^0	OL (w/o)	SS	0/5	5/5	0/5	0/5	NA	100	100
		PE	0/5	5/5	0/5	0/5	NA	100	100
	OL	SS	0/5	5/5	0/5	0/5	NA	100	100
		PE	0/5	4/5	0/5	1/5	0	100	80
10^1	OL (w/o)	SS	6/20	11/20	1/20	2/20	75	92	85
		PE	10/20	10/20	0/20	0/20	100	100	100
	OL	SS	8/20	10/20	2/20	0/20	100	83	90
		PE	13/20	5/20	0/20	2/20	87	100	90
10^2	OL(w/o)	SS	5/5	0/5	0/5	0/5	100	NA	100
		PE	5/5	0/5	0/5	0/5	100	NA	100
	OL	SS	5/5	0/5	0/5	0/5	100	NA	100
		PE	5/5	0/5	0/5	0/5	100	NA	100

The detection of *L. monocytogenes* at 10^1 CFU/100 cm^2 on SS had 6 and 8 confirmed positive out of 20 samples with and without organic load, respectively. In comparison, for the PE surface, 10 and 13 positives were detected under the same conditions. The previous study reported that surfaces such as SS and glass are hydrophilic materials, while PE and rubber are hydrophobic in nature, while it was observed that *L. monocytogenes* cells exhibited a higher affinity to attach to more hydrophobic materials (Stepanović et al., 2004). A theoretical premise approach on surface free energy concluded that adhesion of *L. monocytogenes* is more favorable on solid surfaces with lower energy surfaces such as polypropylene and rubber compared with glass and SS surfaces that have higher energy surfaces (Mafu et al., 1991). However, a study using scanning electron microscope techniques revealed that *L. monocytogenes* could attach to SS, PE, glass and rubber surfaces at ambient temperatures after contact times as short as 20 min or 1 h (Mafu et al., 1990).

Silva et al. (2008) observed that SS surface was one of the most hydrophobic materials with more *L. monocytogenes* attached as compared to PE material. However, a better cell viability was observed on PE surface than SS despite the least attachment of cells. Similarly, as more positive samples were reported on PE than SS in this study, it can be concluded that more cells remained viable and were able to detach more easily from surfaces to swab on PE than SS.

At an inoculum level of 10^1 CFU/100 cm^2 on SS and PE, it was observed that more positive samples for *L. monocytogenes* were detected on surfaces with organic load. This observation could be due to the availability of

nutrients reducing the loss of viability. During starvation without organic load, *L. monocytogenes* apparently increases adherence to surfaces due to changes in cell surfaces characteristics that make them more closely associated with and attached to surfaces (Kjelleberg et al., 1983). A recent study on SS with high and low organic loads reported that one log reduction was observed between 0 and 60 min drying at a high organic load, whereas a 3 log reduction was observed in the presence of a low organic load (Martinon et al., 2012), demonstrating availability of nutrients is needed to maintain cell viability on the food contact surface.

Reported sensitivity and specificity for inoculum at 10^1 CFU/100 cm^2 were more than 80%, except for SS without organic load that had a reported sensitivity and specificity of 75% and 92%, respectively. In this study, it was observed that the detection limit of both methods appeared to be drifting at an inoculum level of 10^1 CFU/100 cm^2 and became more stable at an inoculum level of 10^2 CFU/100 cm^2. It is evident that the detection limit was challenged at 10^1 CFU/100 cm^2 and was more intensified on SS without organic load as loss of microbial viability was high coupled with likely heterogeneity of sampling.

One and two samples at 10^1 CFU/100 cm^2 were reported as false positive results for SS with and without organic load, respectively, whereas no false positive result was reported for PE. Since SS were always autoclaved before use and uninoculated control samples with organic load were also negative, these results indicate that contamination from previous inoculation and the presence of *L. monocytogenes* in organic load is unlikely. It is also unlikely that the sampling tool used was inappropriate as a recent study on

environmental tools detecting low numbers of *L. monocytogenes* on different types of food contact surfaces clearly demonstrated that 3M Enviro Swab had the best recovery among the tested tools (Lahou and Uyttendaele, 2014). Hence, it is probably the heterogeneity of sampling that led to such results.

Two false negative results for 3M™ MDA were reported for SS without organic load and PE with organic load, respectively. Under the normal conditions without harsh factors, *L. monocytogenes* is already a slow growing microorganism. In this study, harsh conditions could be the drying process that is likely to make it injured or stressed, which would need longer time to resuscitate (Dupont and Augustin, 2009). Some studies demonstrated that the presence of selective agents in Fraser broth was sufficient to result in an extended lag phase period in heat-injured *Listeria* cells, leading to longer time to resuscitate (Donnelly, 2002). Due to this fact, ISO methods with extended incubation time would allow cells to easily resuscitate compared to 3M™ MDA *L. monocytogenes* with 26-30 h in single enrichment.

4.4 Conclusion

The results of this study revealed that the 3M™ MDA method performed equally effective compared with a reference ISO method at inoculum levels of 10^0 and 10^2/100 cm^2. At an inoculum level of 10^1 CFU/100 cm^2, a slight reduction in effectiveness was observed and likely due to heterogeneity in sampling and use of single enrichment in 3M™ MDA *L. monocytogenes*. More positive samples were observed for *L. monocytogenes* detected on surfaces with organic load suggesting the availability of nutrients reduced the loss of viability and enhanced survival of *L. monocytogenes* cells.

It can be concluded that *L monocytogenes* can adhered to and survived on SS and PE surfaces with and without organic load and could potentially contaminate food, especially at an inoculum level of 10^2/100 cm^2.

CHAPTER 5

OVERALL CONCLUSIONS AND FUTURE STUDY

In this study, the sensitivity and specificity of 3M™ molecular detection assays (MDA) in comparison with standard ISO methods for the detection of *Salmonella* spp. on local food matrices and *Listeria monocytogenes* in environmental samples were determined using 3M™ Molecular Detection System (MDS).

Healthy and thermally–injured *Salmonella* cells inoculated with 10^0 CFU/25 g on raw duck wings and processed fishball were detected with 3M™ MDA. However, at the same inoculum level, sanitize-injured *Salmonella* cells were undetectable in raw mung bean sprouts by 3M™ MDA. Hence, two additional procedures: a secondary enrichment in RVS and mechanical concentration of *Salmonella* cells using centrifugation were performed for bean sprouts inoculated with 10^0 CFU/25 g of sanitizer-injured *Salmonella* cells. The secondary enrichment with the extended incubation time enabled sanitizer-injured cells to reach the detection limit of 3M™ MDA, indicating that sanitizer-injured *Salmonella* spp. might need more time to recover. An alternative method was the mechanical concentration of *Salmonella* spp. by centrifugation helped to achieve the detection limit of 3M™ MDA with single enrichment. This method modification significantly improved sensitivity of the detection assay with single enrichment. In naturally contaminated bean

sprouts, *Salmonella* cells were detected by 3M™ MDA in the 1st attempt, suggesting that with proper enrichment optimization or method modification, 3M™ MDA could serve as a powerful screening tool for *Salmonella* spp. detection in food.

In the study on detection of *L. monocytogenes* inoculated on environmental surfaces with and without organic load, more positive samples were reported for organic load and PE surfaces respectively. The availability of nutrients in organic load probably enhanced survival of *L. monocytogenes* cells, while, PE surfaces were better in supporting cell viability with *L. monocytogenes* cells were able to detach more easily from surfaces to swabs.

Loop-mediated isothermal amplification (LAMP) with bioluminescence detection is a promising system due to its high specificity, efficient amplification and no requirement of expensive thermo-cycler. 3M™ MDS demonstrated rapid, accurate detection of healthy, injured *Salmonella* cells in less than 26 h and this method was validated on naturally contaminated food matrices. This study showed that the sensitivity and specificity of 3M™ MDA were dependent on pathogen inoculum levels, type of cell injury, food matrix and the material of surfaces. Therefore, further research should focus on the application and optimization of 3M™ MDA for detection of foodborne pathogens in the various food matrices of different origins and composition and different background microflora.

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