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Validation Study of a Novel Detection Kit for Rapid Detection and Quantification of Listeria Spp. in Food Samples

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By

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A single tube detection kit was designed as a rapid, easy-to use and reliable test to detect *Listeria spp.*. Various food samples (vegetables and raw catfish fillets) were used in order to validate the performance of the detection kit. *L. grayi* was detected in one ready-to-eat (RTE) vegetables with the detection kit while no *Listeria spp*. was detected using the modified FDA-BAM method. In addition, both the detection kit and modified FDA-BAM method indicated that twelve catfish fillets were *Listeria* positive. The detection kit had 100% sensitivity and specificity in less detection time (24 h) than the modified FDA-BAM method (60% specificity, >72 h). There was no difference ($P<0.05$) between the kit and the modified FDA-BAM method on MPN for *Listeria spp*.

DEDICATION

This work is dedicated to my dear parents Mingkang Jiang and Chenglin Liu.

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CHAPTER I

INTRODUCTION

Foodborne illness, remains common in the United States regardless of advances in food safety (Nyachuba, 2010). Each year, over 9 million foodborne illnesses are estimated to be caused by major pathogens (Scallan et al., 2011; Painter et al., 2013). According to the Centers for Disease Control and Prevention (CDC) estimation, 76 million foodborne illnesses, including 325,000 hospitalizations and 5,000 deaths occur in the United States each year (Nyachuba, 2010).

Listeria monocytogenes, a virulent species among 6 *Listeria* species, can cause a serious foodborne disease called listeriosis (Cartwright et al., 2013). Listeriosis is a typical foodborne illness of major public health concern due to the severity of the disease with a high mortality rate (20-30%) (Allerberger, 2003). The CDC estimates that 1,662 invasive foodborne infections with *L. monocytogenes* occur annually in the United States, causing 1,520 hospitalizations and 266 related deaths (Scallan et al., 2011). Clinical symptoms of listeriosis vary widely and often confuse with other illnesses (Ryser and Marth, 1991). The most common symptoms of listeriosis include fever, muscle aches, and vomiting. If the *Listeria* infection spreads to the nervous system, it can cause meningitis which is an infection of the brain and spinal cord that include symptoms such as headache, stiff neck, confusion, loss of balance, and convulsions (Yildiz et al., 2007). Although listeriosis remains a rare disease when compared to other foodborne illnesses

caused by other foodborne pathogens like *Norovirus*, *Salmonella* and *Clostridium perfringens*, this serious disease usually develops and results in high mortality since it primarily affects pregnant women, newborns, older adults, and people with weakened immune systems (Armstrong, 1985).

The detection for *Listeria spp* in food samples has focused mainly on DNA/PCR, immunoassays or conventional USDA or FDA methods. However, all these methods require multiple incubation and isolation steps on multi-selective media which requiring more than 72h analysis time to confirm the existence of *Listeria spp*.. In addition, since these conventional methods are technically complicated, well-trained and experienced people are required with relevant microbial knowledge and techniques.

A single-step detection system for *Listeria spp*. was designed for on-site *Listeria* testing that does not require additional machinery or equipment to read or interpret the test results. The detection kit was optimally formulated for expression of a biomarker on *Listeria spp*. as well as increased selectivity of *Listeria* without allowing the growth of background microorganisms such as Gram positive *cocci* and *Enterobacteriaceae* which could lead the false positive reactions on the selective medium (Edbery et al., 1976). The detection kit is innovative because it has included all required detection steps such as cell culturing, isolation and detection in one tube.

To validate the *Listeria* detection kit, various food samples were screened with the detection kit and compared with the official modified method of the U.S. Food and Drug Administration's Bacteriological Analytical Manual (FDA-BAM, 2011).

CHAPTER II

LITERATURE REVIEW

2.1 Classification of *Listeria*

The genus *Listeria* belongs to the Clostridium sub-branch, together with *Streptococcus*, *Staphylococcus*, *Lactobacillus*, and *Brochothrix* (Swaminathan, 2001). The genus *Listeria* contains six different species: *L.ivanovii*, *L. innocua*, *L. welshimery*, *L. seeligeri*, *L. grayi*, and *L. monocytogenes* (Kuzmanovic´ et al., 2011). This bacterium is Gram-positive, short, non-spore forming rods, facultative anaerobic, catalase-positive and oxidase-negative. At or near room temperature (below 30°C), the organism shows 'tumbling' motility when viewed with light microscopy which can aid in identifying the organism, but *Listeria* is non-motile at body temperatures (37°C) (Farber and Peterkin, 1991; Henry, 1933). Tumbling motility is best observed in tryptose broth when incubated at 20°C (Seeliger, 1961).

All *Listeria* species are morphologically similar on artificial media. When observed under a microscope, *Listeria* colonies are round, translucent with a smooth surface and entire margin, measuring 0.5-1.5mm in diameter after 24-48 h incubation. As compared to fresh cultures, old cultures, which develop after 3-5 days of incubation, are larger with 3-5 mm in diameter, and typically have a more opaque appearance (Wagner and McLauchlin, 2008; Ryser and Marth, 1991).

Within the genus *Listeria*, only *L. monocytogenes* and *L. ivanovii* are considered to be pathogenic and can lead to listeriosis. *L. monocytogenes* is primarily a human pathogen which is also capable of infecting and causing disease in a wide variety of animal species, including birds and mammals (Swaminathan, 2001); *L. ivanovii* is a pathogen of mammals, specifically ruminants, but has rarely caused listeriosis in humans (Guillet et al., 2010).

2.2 Biochemical characteristics

Listeria possesses the following biochemical characteristics: catalase +, oxidase -, urease -, and Methyl Red/ Voges-Proskauer (VP) test +/+ (Van Dissel et al., 1993). The identification of *Listeria* species is based on a limited number of biochemical markers, among which hemolysis is used to differentiate between *L. monocytogenes* and the most common nonpathogenic *Listeria* species, *L. innocua* (Seeliger and Jones, 1987; Allerberger, 2003). Hemolysis is the breakdown of red blood cells. The ability of bacterial colonies to induce hemolysis when grown on blood agar is used to classify certain microorganisms (Ray and Ryan, 2004). Biochemical tests that are useful for discriminating between the *Listeria* species are acid production from D-Xylose, L-Rhamnose, a-Methl-D-mannoside, and D-Mannitol. Only *L. monocytotenes*, *L. seeligeri*, and *L. ivanovii* are hemolytic (table 2.1) (Allerberger, 2003). In order to improve the assessment of hemolysis, the CAMP (Christie, Atkins, and Munch-Petersen) test is recommended to differentiate species of *Listeria* (Seeliger and Jones, 1987; Rocourt et al., 1983). This test is used for the presumptive identification of Group B *Streptococcus* (*Streptococcus agalactiae*). This is the only *beta*-hemolytic *Streptococcus* which yields a positive CAMP test. To perform the test, streak a β-hemolytic *staphylococcus aureus* and

a *rhodococcus equi* culture in parallel and diametrically opposite each other on a sheep blood agar plate. *Listeria* test cultures are then streaked at right angles to the two other cultures. After 24-48 hours of incubation at 35 C, β-hemolysis by *L. ivanovii* is enhanced near the *Rhodococcus* streak, whereas β-hemolysis by *L. seeligeri* and *L. monocytogenes* is enhanced near the *S. aureus* streak. The other species remain nonhemolytic (Table 2.2) (Seeliger and Jones, 1987; Hitchins and Jinneman, 2011).

2.3 Growth and survival characteristics

Generally, it is hypothesized that other *Listeria spp.* show similar resistance to environmental stress (temperature, acid, salt, ete.) to that observed from *L. monocytogenes*. Because data available on stress resistance or survival characteristics of other *Listeria spp.* are limited (Antoniollo et al., 2003).

2.3.1 Temperature

Under laboratory conditions, *L. monocytogenes* showed growth at temperature between 30 and 45 °C (Reha et al., 2009). The optimal growth was reported at temperature between 30 and 37 °C (Petran and Zottola, 1989); while any temperature above 37 °C imparts stress (Hansen and Riemann, 1963). The minimum growth temperature (MGT) of *Listeria monocytogenes* has been reported at 1°C (Seeliger and Jones, 1987). However, Walker et al. (1990) reported that three *L. monocytogenes* strains exhibited generation times of 62 to 131 hours in chicken broth and pasteurized milk, respectively, during extended incubation at – 0.1 to – 0.4 °C (Walker et al., 1990). Even though *L. monocytogenes* is usually unable to grow below 0.5 °C, this pathogen can readily survive at much lower temperature. Unlike most foodborne bacteria, *Listeria* can

grow in the refrigerator; it survives well for several weeks at -18°C in various food substrates (Golden et al., 1988) and grows more rapidly at refrigerator temperature above 4 °C (FDA, 2013a). Furthermore, there may be some survival under frozen temperature between -18°C to -198°C for one month (El-Kest and Marth, 1991). For ready-to-eat foods, *Listeria* will grow more as storage time increased. In addition, *Listeria* will spread from one food to another through spills in the refrigerator (FDA, 2013a).

2.3.2 pH

L. monocytogenes was reported to grow at pH range from 5.6 to 9.6, based on the 9th edition of Bergey's Manual of Systematic Bacteriology (Seeliger and Jones, 1987). Petran and Zottola (1989) determined that optimal growth occurred at neutral to slightly alkaline pH values. Seeliger (1961) reported that *L. monocytogenes* failed to grow in dextrose broth at pH<5.6 after incubation at 37°C for 2-3 days, which is regarded as the minimum pH for *L. monocytogenes* growth. However, additional studies have indicated that *L. monocytogenes* can grow at a lower pH in laboratory media. Farber et al. (1992) stated that *L. monocytogenes* can grow at a minimum pH value of 4.2-4.3 at 30°C (Farber et al., 1992). Tienungoon et al. (2000) reported that similar pH values allowed the growth of *L. monocytogenes* (4.23-4.25, none specified temperature) for two *L. monocytogenes* strains. Vermeulen et al. (2007) tested the growth of 26 *L. monocytogenes* strains at different pH values and water activitys (Aw). They found that the minimum pH that most strains could survive was between 4.1-4.4 (using HCl as the acidulant) at a water activity (Aw) of 0.995 (non-adjusted brain heart infusion broth) and no acetic acid added, after 30 days incubation at 30°C (Vermeulen et al., 2007).

2.3.3 Water activity

The optimal water activity (Aw) for the growth of *L. monocytogenes* is ≥0.97 (Petran and Zottola, 1989). However, unlike most other spoilage organisms, it has a rather unique ability to multiply at lower water activities. Petran and Zottola (1989) demonstrated that *L. monocytogenes* could grow at as low as 0.92 aw, using Trypticase Soy Broth (TSB) containing 39.4% sucrose during incubation at 30 °C for 24 h. In addition, according to data from New Zealand Food Safety Authority (NZFSA) in 2001, the minimal Aw for the growth of *Listeria* is at 0.92 (New Zealand Food Safety Authority [NZFSA], 2001). However, Daza et al. (1991) found that two strains of *L. monocytogenes*, Scott A (serotype 4b) and Brie 1 (serotype) 1b), grew well in glycerol-supplemented TSB at a 0.90 Aw under 30 °C incubation. Farber et al. (1992) tested five strains of *L. monocytogenes* in their study. They reported that three of five strains of *L. monocytogenes* capable of growing in BHI broth that was adjusted with glycerol to a water activity value of 0.90 at 30 °C (Farber et al., 1992).

So far, no research has indicated that *L. monocytogenes* appears to grow at Aw < 0.90. However, this bacterium was reported to survive at lower water activity, particularly under refrigeration. Johnson et al. (1998) reported that *L. monocytogenes* is capable of surviving at an Aw of 0.79-0.86 in fermented hard salami at 4C for at least 84 days (Johnson et al., 1988). In addition, Baranenkov (1969) reported that *L. monocytogenes* survived on the surface of intact eggs that were stored at 5 °C for 90 days. Therefore it is evident that *L. monocytogenes* is remarkable resistant to drying.

2.3.4 Salt

L. monocytogenes is remarkably tolerant of high salt concentrations (McClure et al., 1989). Historically, *L. monocytogenes* has been isolated from lots of salt containing food such as cheese, salted fish, cooked ham and other cured meat (Rocourt, 1994; Greenwood et al, 1991). In addition, it has been documented that *Listeria* was isolated at a level of 10^6 CFU/g from mushroom at a salt concentration of 7.5%, which had been stored for 5 months (Junttila and Brander, 1989). NaCl and Potassium chloride (KCl) are commonly used as food preservative and increase shelf-life by reducing water activity (Bidlas and Lambert, 2008). The effects of various concentrations of NaCl and KCl on the growth of *L. monocytogenes* were evaluated by Zarei et al (2012). In the study, they found that *L. monocytogenes* can grow in the presence of 1, 3, 5, 7, and 9 % NaCl. As concentration increased, the length lag phase (Zarei et al., 2012). In addition, *L. monocytogenes* can grow at a concentration of 11 % KCl, but is not able to grow in NaCl with the same concentration. Furthermore, It was observed that this pathogen tolerates KCl better than NaCl in broth (Zarei et al., 2012).

According to the 9th edition of Bergey's Manual of Systematic Bacteriology, *L. monocytogenes* can grow in nutrient broth with up to 10% NaCl (Seeliger and Jones, 1987). Shahamat et al. reported that *L. monocytogenes* could survive in medium containing 10.5% NaCl at 37 °C (Shahamat et al., 1980), while Seeliger and Welshimer demonstrated that this pathogen could survive for 8 weeks at 4°C in 20% NaCl (Seeliger and Welshimer, 1974).

2.4 Incidence in food sample

2.4.1 Seafood

Listeria spp. is ubiquitous in aquatic environments and is present in many aquatic creatures including fish, oysters, shrimp and crab (Hartemink and Georgsson, 1991). The first case of listeriosis linked to consumption of fish or seafood was reported in 1989 when a 54-year-old Italian woman contracted listeric meningitis for after consuming steamed fish in which *L. monocytogenes* was later isolated (Facinelli et al., 1989). Concern about the potential hazard of *Listeria* contaminated seafood began in the United States when *L. monocytogenes* was isolated from frozen cooked crabmeat (Anonymous, 1987).

Chen et al. (2010) reported the incidence of *Listeria spp*. in whole raw catfish, catfish fillets, and processing environments from two catfish processing facilities. The study showed that 53.3 and 18.4% of 212 catfish samples collected in August 2008 were positive for *Listeria spp*. and *L. monocytogenes*, respectively. Of 209 samples collected in August 2009, 43% were positive for *Listeria spp.*, and 12.4% of total were positive for *L.monocytogenes* (Rodas-Suárez et al., 2006). Rodas-Suarez et al. (2006) tested 66 fish, 66 oysters, and 144 estuarine water samples collected from June 2001 to May 2002 in Veracruz, Mexico. The authors found that *Listeria spp*. were found in 22.7 and 30.5% of fish and estuarine water samples, respectively, with *L. monocytogenes* present in 4.5% of the 66 fish samples and 8.3% of the 144 water samples; while they were not recovered in oyster samples.

2.4.2 Vegetables

Even though the incidence of *L. monocytogenes* in vegetables is generally lower than that of milk, dairy and processed meat products, they are also important vehicles of *Listeria* transmission, especially in minimally processed (fresh, fresh-cut vegetables under modified atmosphere packaging (MAP) or frozen) and directly purchased and eaten by consumers (Chen et al., 2006). Minimally processed vegetables are often used as ingredients in salads, which may have long refrigerated shelf lives, with risk of increasing of the *L. monocytogenes* population (Aguado et al., 2004). Moreno et al. (2012) sampled for *L. monocytogenes* from 191 vegetable samples (fresh, frozen and fresh-cut under MAP). In this study, *L. monocytogenes* was isolated from eight products (4.19% of the examined samples). Among the positive results, 8.33% were obtained from frozen food (two spinach, one broccoli and one vegetable stir-fry samples); 4.28% were obtained from fresh-cut vegetables under MAP (one isolate from spinach and two from broccoli); and 1.37 % were obtained from a fresh sample of spinach.

Several surveys have demonstrated that the prevalance of *L. monocytogenes* in fresh vegetables (Bae et al., 2011; Ding et al., 2013). This bacteria has been isolated from fresh cabbage, celery, carrots, lettuce, cucumber, onion, potatoes, tomato and fennel (Beuchat, 1996). Jeyaletchumi et al. (2011) reported the following incidence of *Listeria spp.* in fresh vegetables, cabbages (30%), cucumber (20%), yardlong beans (10%) and carrots (10%). A large number of outbreaks have been attributed to the consumption of fresh vegetables including raw celery, tomatoes, lettuce, cabbage and coleslaw, which have been reported in the United State (Faber and Peterkin, 1991). In addition, leafy

vegetables are regarded as the second most common vehicle of foodborne illness in the United States.

2.4.3 Ready-to-eat (RTE) foods

RTE foods refer to a range of preprocessed fish, meat, and vegetables that can be consumed without further cooking or handling (Liu, 2008). The RTE food is usually stored in a refrigerator at low temperature. However, research indicates that *Listeria* can grow under at a wide range of temperatures. As discussed in previously in this chapter, it could survive for several weeks at -18 °C in various food substrates (Golden et al., 1988). Thus, RTE foods readily support the growth of *Listeria*. Zhou and Jiao (2006) examined 844 RTE food samples from retail markets in China. Twenty-one of these samples were positive for *L. monocytogenes*. Jamali et al. (2013) evaluated 396 RTE food samples (including salads, vegetables, chicken and chicken products, egg and egg products, beef and beef products, sea food and sea food products, and lunch box) for the presence of *Listeria spp.* and *L. monocytogenes*. In the study, *Listeria spp*. was detected in 71 (17.9%) samples in which 45 (11.4%) were positive for *L. monocytogenes*. Another study screened 40 deli meat and 40 deli fish samples collected from 17 stores in British Columbia. *Listeria spp*. was only obtained from fish samples (20%); 5% had *L.innocua*, 5% harboured *L. monocytogenes* and 10% contained *L. welshimeri* (Kovačević et al, 2012).

2.4.4 Meat and meat products

Many studies from different countries have shown that a variety of meat and meat products have been associated with contamination of *Listeria spp*. (Inoue et al., 2000;

Baek et al., 2000; Capita et al., 2001; Soultos et al., 2003; Ochiai et al., 2010). For example, *Listeria spp*. has been isolated from poultry, red meat and meat products in many countries such as New Zealand (Hudson et al., 1992), Australia (Ibrahim et al., 1991), and Japan (Ryu et al., 1992). In 1992, an outbreak in France of listeriosis was caused by contaminated pork tongue and affected 279 patients took (Jacquet et al., 1995). In the United States, approximately 100 cases of listeriosis were attributed to the consumption of *L. monocytogenes* contaminated hot dogs (Evans et al., 2004). El-Malek et al. (2010) who examined 100 meat samples between January and July 2009 reported that *Listeria spp.* were detected in 8 (32%) minced frozen beef samples, 8 (32%) luncheon meat samples, 13 (52%) frozen chicken legs samples and 14 (56%) frozen chicken fillet samples. Kalender (2012) isolated *Listeria spp*. from 180 ground beef and 180 chicken meat samples. Among the screened samples, *L. monocytogenes* was isolated from 7.2% of ground beef samples and 17.8% of chicken samples. *L. innocua* was detected in 15.5% and 36.7% ground beef and chicken meat samples, respectively. *L. welshimeri* was detected in 6.1% and 5.5% ground beef and chicken meat samples, respectively. In addition, *L. seeligeri* and *L. murrayi* was detected in 4.4% and 1.1% of chicken samples, respectively. Doijad et al. (2010) evaluated a total of 109 meat (50 beef, 52 pork sausages and 7 pork) for the presence of *Listeria*. Of 25 recovered isolates, seven (6.42%) were *L. monocytogenes*, and one (0.9%) was *L. ivanvovii*. Other isolates were *L. innocua* (4), *L. welshimeri* (2), *L. seeligeri* (10) and *L.grayi* (1).

2.5 Detection of *Listeria spp*

2.5.1 Isolation method

Several official, standard methods are currently designed for enriching and isolating *Listeria spp*. Conventional and commonly used methods include the United States Department of Agriculture – Food Safety and Inspection Service (USDA-FSIS) method (USDA-FSIS, 2002; USDA-FSIS, 1999), the United States Food and Drug Administration (FDA) method (Hitchins and Jinneman, 2011), the ISO 11290 Standard method (International Organization for Standardization [IOS], 2004), and the Association of Official Analytical Chemists (AOAC) official method (AOAC, 2000). one method might be more suitable than others, depending on the type, source and nature of the sample that is tested. FDA is the most common method in the United States that can be used with almost any food samples, while the ISO method is most commonly used in European countries and is also designed for any food product (Gorski, 2008). The USDA-FSIS method is recommended for meat, poultry, egg and egg products (USDA-FSIS, 1999). The AOAC method is designed for different kinds of food samples, especially dairy products and seafoods (AOAC, 2000).

The FDA method uses a single enrichment broth, Buffered *Listeria* Enrichment Broth (BLEB) and requires a pre-enrichment step which is intended for the recovery of injured cells (Bhat et al., 2012). This step is carried out at 30 °C for 4 hours without any supplementation. After pre-enrichment, selective agents are added into the broth to enhance the selectivity (Hitchins and Jinneman, 2011). Pimaricin (natamycin) is preferred selective substitute if cycloheximide is not available, and is much safer to use than cycloheximide (Johansson et al., 1995). In the USDA-FSIS, AOAC and ISO methods,

samples are directly processed into selective enrichment broth. Both the USDA-FSIS method (USDA-FSIS) and the ISO (Gorski, 2008) method use two enrichment steps. The USDA-FSIS uses the University of Vermont medium 1 (UVM 1) in primary enrichment, while ISO is enriched in Half Fraser broth. Both methods use Fraser broth that containing the selective compounds at full concentration for the secondary enrichment. For the AOAC method, TSB with acriflavine, nalidixic acid and cycloheximide are used for selective enrichment. Also, UVM 1, half Fraser broth, and Fraser broth contain acriflavine and nalidixic acid as selective compounds. Nalidixic acid is effective against both Gram-positive and Gram-negative bacteria. It inhibits the DNA synthesis of cells and is often combined with other inhibitors. Several studies have reported that nalidixic acid has no effect on *Listeria spp*. growth in culture (Jacobsen, 1999; Beuer et al., 1996), while acriflavine at high concentrations inhibited the growth of *Listeria spp*. (Jacobsen, 1999; Rodriguez et al., 1984). Samples are enriched at 30 °C, 35 °C or 37 °C for 22 – 72 hours, depending on the method specifications. After 4 hours pre-enrichment in the FDA method, BLEB culture is streaked onto one of the following selective isolation agars: PALCAM, OXA, MOX or LPM with esculin and iron added. Typical colonies of *Listeria spp*. are small, black and surrounded with a black halo. All *Listeria spp*. can hydrolyze esculin so that it forms esculetin. Esculetin reacts with Fe^{3+} which causes the formation of a black precipitate on the media (Siragusa et al., 1990). In addition, the FDA method and ISO method call for additional chromogenic agar such as Biosynth Chromogenic Medium (BCM), agar *Listeria* according to Ottaviani and Agosti (ALOA), and CHROMagar *Listeria*. These chromogenic agars differentiate *L. monocytogenes* or *L. ivanovii* colonies of other nonpathogenic *Listeria spp*. and reduce the problem of masking

L. monocytogenes by *L. innocua* (USDA-FSIS, 2002; Restaino et al., 1999; Greenwood et al., 2005). The ISO method prefers to use ALOA since its formulation is published. Typical colonies of *L. monocytogenes* in ALOA agar are green-blue, surrounded with an opaque halo (IOS, 2004). The coloration of colonies results from β-glucosidase activity of the bacteria.

In addition, the lecithin which is present in the agar is hydrolysed by the phospholipase enzyme produced by pathogenic *Listeria spp*., but not other *Listeria spp*.. Hydrolysis results in the production of an opaque halo around the colonies (Ottaviani et al., 1997). Another type of selective chromogenic medium, Rapid *L.mono* agar (Bio-Rad Laboratories, Inc) is allowed for use by the FDA and AOAC. On this agar, *L. monocytogenes* and *L. ivanovii* form blue colonies due to the activity of phospholipase, while nonpathogenic *Listeria spp*. produce white colonies. In addition, *L. monocytogenes* is distinguished from *L. ivanovii* based on the fermentation of xylose. *L. monocytogenes* is unable to ferment xylose, while *L. ivanovii* uses phospholipase C to ferment xylose, which results in the formation of a yellow halo around the colony (Greenwood et al., 2005).

After incubation, at least five suspicious colonies from the selective medium should be streaked onto non-selective medium. The minimum colonies are five because more than one *Listeria* species may be isolated from the same sample (USDA-FSIS, 2002; Ikeh et al., 2010). Trypticase soy agar (TSA) is the most frequently non-selective agar. *Listeria spp*. show colorless colonies on this non-selective agar after either 30 °C or 35 °C or 37 °C incubation, depending on the method. The colonies from non-selective

agar should be verified by additional confirmation testing (FDA-BAM, 2013; Beumer, et al., 1996).

The detection processes of some standard methods are shown in the Figure 2.1.

2.5.2 Identification

Suspicious *Listeria spp*. colonies from the selective agar plates are needed for further confirmation. The confirmation methods include conventional biochemical identification and multiplex-PCR (m-PCR).

2.5.2.1 Conventional biochemical identification

The CAMP test was validated by the standard methods include FDA, USDA-FSIS, ISO and AOAC methods. As described previously in the biochemical characteristics part, this method's detection mechanism is based on β-hemolysis (Seeliger and Jones, 1987; Rocourt et al., 1983). This method consists of streaking a β-haemolytic *Staphylococcus aureus* and *Rhodococcus equi* in single straight lines in parallel, on a sheep blood agar plate. A positive reaction is constitutive of an enhanced zone of β-hemolysis, at the intersection of the test/control and indicator strains after incubation at 35-37 °C for 24-48 h. (Miller et al., 2009). *L. monocytogenes* is positive with the *S. aureus* streak but negative with *R. equi*, while *L. ivanovii* shows the reverse result (Quinn et al., 1999).

Sugar utilization patterns are also available to differentiate *Listeria spp*.. Suspicious colonies from the tryptic soy broth yeast extract (TSBYE) culture are inoculated into the purple carbohydrate fermentation broth tubes containing dextrose, esculin, maltose, rhamnose, mannitol and xylose, and then incubated at 35 °C for up to 7

days (Hitchins and Jinneman, 2011). Positive results are indicated by acid production without gas. All *Listeria spp*. utilize dextrose, esculin, and maltose, while only some *Listeria spp*. utilize mannitol, rhamnose and xylose (Gawade et al., 2010; Hitchins and Jinneman, 2011). With the exception of *Listeria grayi*, *Listeria* species should be negative for mannitol (Jeyaletchumi et al., 2010) (Table2.3).

2.5.2.2 Multiplex-PCR (m-PCR)

There are several approaches to nucleic acid amplification. Polymerase chain reaction (PCR) was the first and remains the most widely used technique in both research and clinical laboratories. This simple method is based on the amplification of specific sequences of target DNA from the indicator organism to an amount that can be viewed by human eyes with detection devices (Jeyaletchumi et al., 2010).

Multiplex PCR (m-PCR) is a way to amplify two or more amplicons in a single PCR reaction. (Xu et al., 2012). Bubert, et al. (1999) developed the method with five different primers that allows the specific detection and differentiation of *Listeria spp*. by amplifying a shared *iap* gene with a single multiplex-PCR. *Iap* gene encodes the major extracellular protein p60 (Kohler et al., 1990), which is common to all *Listeria spp*. and has been shown to be an essential murein hydrolase that is required for septum separation in cell division (Wuenscher et al., 1993). In the study of Bubert et al. (1999), one primer was derived from the conserved 3' end and is specific for all *Listeria spp*.; the other four primers are specific for *L. monocytogenes*, *L. innocua*, *L. grayi*, or the three grouped species *L. ivanovii*, *L. seeligeri*, and *L. welshimeri*.

Although multiplex PCR has many advantages, it has several disadvantages which include low amplification efficiency, self-inhibition among different sets of

primers, and no identical efficiency on different templates which inhibits its further development and broad application (Xu et al., 2012). In order to overcome the disadvantage of conventional m-PCR, the Universal primer-multiplex-polymerase chain reaction (UP-M-PCR) was developed (Zhang et al., 2011). Up to now, UP-M-PCR has been used to detect *L.monocytogenes, Escherichia coli*, and Salmonella *spp*. in food samples at the same time (Yuan et al., 2009). This universal adapter was designed in the 5' end of each specific primer pair that matches the specific DNA sequences and also used as the universal primer (UP) (Xu et al., 2012). Yuan et al. (2009) tested 36 food samples and reported a relative accuracy of 91.8% when compared with traditional microbiological analysis. They also presented that this method could serve as a rapid screening method for pathogen detection and can be applied for the detection of target genes in dead pathogenic cells.

2.5.2.3 Enumeration

Standard protocols require the enumeration of *L. monocytogenes* in food samples. Both FDA and FSIS have a "zero tolerance" policy for *L. monocytogenes* in ready-to-eat foods (FDA, 2013a). According to the standard protocols, enumeration of *Listeria spp*. is carried out using the MPN technique, is a serial dilution technique that measures the concentration of a target microbe in a sample with an estimate, that is particularly useful when small concentration of microbes are present in the sample (<100/g) (Blodgett, 2010).

For *Listeria* enumeration in food samples, each dilution of serial aliquots or dilutions is placed into 3 or 5 tubes of appropriate enrichment broth for either three- or five-tube method. The inoculated tubes are then incubated at 30 °C for 24h. An aliquot of

0.1 ml is then transferred to other tubes containing 10 ml of Fraser broth and incubated at 35 °C for 48h. In order to confirm the MNP of *Listeria spp*., an aliquot of dark Fraser is streaked onto a selective medium such as MOX. The same procedure is then used that is also used for its detection. The MPN estimation is obtained from the number of tubes with *Listeria* confirmed ("true" MPN) and from the number of dark Fraser broth tubes ("predictive" MPN) (Loura et al., 2004). The number of positive MPN tubes is compared to a table (Table 2.4) that uses statistical analysis to determine the level of contamination of the original sample (Gorski, 2008).

Alternatively, other methods include MPN filter, Filter/colony-lift, and DNA probe colony hybridization, were validated by AOAC and peer reviewed by FDA. (Hitchins and Jinneman, 2011). The first method is useful for all *Listeria spp*. (Entis and Lerner, 2000), while the other two methods are specific to *L. monocytogenes* (Carroll et al., 2000; Datta et al., 1988; Datta et al., 1993).

2.5.2.4 Commercially available test kit

Alternative methods include commercially available test kits that are listed in Table 2.5 include the Gene Trak *Listeria* Assay, *Listeria* Tek, TECRA *Listeria* Visual Immunoassay, Assurance *Listeria* EIA, VIP *Listeria*, and VIDAS *Listeria*, to assist in the identification of *Listeria spp*. These test kits were approved by the AOAC International Official Methods protocol.

2.5.2.4.1 GENE-TRAK *Listeria* assay

The GENE-TRAK *Listeria* ASSAY is a colorimetric DNA hybridization method for the detection of *Listeria* sequences that has been validated by the AOAC as method

993.09 (AOAC, 2000). This method is mainly used with dairy products, meats and seafood. Since this method may have false-positives, it must be confirmed by standard cultural methods (Curiale et al., 1994). The procedure requires sample enrichment and an overall average testing time for 2.5 days. This assay is specific for *L. monocytogenes* directly, thus reducing analytical time. However, this method requires highly trained person and strict controls for the lysis and heating steps (Curiale et al., 1994; Garrido and Otwell).

2.5.2.4.2 Colorimetric monoclonal enzyme-linked immunosorbent assay (*Listeria*-Tek)

The *Listeria*-Tek is intended for the detection of *Listeria spp*. in dairy products, seafood and meat. The test uses 2 monoclonal antibodies that are (MAbs) specific for *Listeria* in an enzyme-linked immunosorbent assay (ELISA) format. However this test is not confirmatory for *L. monocytogenes*, because the MAbs which used in the test may cross react with other *Listeria spp* (AOAC, 2000). The ELISA test is safely performed on the open bench of the laboratory since the live cultures are not necessary. So this method does not need special licenses or reserved laboratory space. In addition, it is easy to perform hundreds of assays per day if necessary, and a printed data sheet is available for records (Mattingly et al., 1988).

2.5.2.4.3 Colorimetric polyclonal enzyme immunoassay screening method (TECRA *Listeria* Visual Immunoassay [TLVIA])

The TLVIA, AOAC official method 2002.09, is used for the detection of *Listeria spp*. in raw meats, fresh vegetables, processed meats, seafood, dairy foods, fruits and fruit juices. This version was optimized with enrichment protocols (AOAC, 2003) compared

to the AOAC official method 995.22 which is not available in raw ground meat (AOAC, 2000). The positive enrichment cultures must be inoculated onto the selective media, and the suspected colonies should be confirmed under the FDA or USDA-FSIS method (Knight et al., 1996).

2.5.2.4.4 Assurance polyclonal enzyme immunoassay method

The Assurance *Listeria* Enzye Immunoassay, the AOAC official method 996.14, can be used to detect all *Listeria spp*. in dairy foods, red meats, poultry products, seafood, bone meal, fruits, vegetables, nutmeats, pasta, chocolate, cheese, environmental surfaces, and eggs. This method indicates *Listeria spp*. by using proprietary antibodies bound to microwell plates, with a high specificity for *L. monocytogenes* and related *Listeria spp*. antigens (AOAC, 2003). Feldsine et al. (1997) evaluated 1764 food samples and controls by the EIA method and by either the FDA-BAM or the USDA culture method for detecting *Listeria spp*. During this study, 492 samples were positive and 947 were negative for both methods. One hundred fifty nine samples were positive by culture method but negative by the EIA, and 188 samples that were negative by culture method but positive by the EIA. Twenty-two samples were negative by EIA and by culture method but confirmed positive when Assurance selective enrichment broths were subcultured to selective agar (Feldsine et al., 1997).

2.5.2.4.5 Visual Immunoprecipitate assay (VIP)

The VIP assay is AOAC official method 997.03 that can be used for the detection of *Listeria spp*. in dairy foods, red meats, poultry and poultry products, eggs, seafood, vegetables, fruits, pasta, chocolate, nutmeats, environmental surfaces and bone meal

(AOAC, 2003). This test is required to be performed with an enriched culture of test samples. Suspicious positive results should be confirmed by further identification as described under the FDA method (Feldsine et al., 1997). This method is based on binding specific *L. monocytogenes* antigens in an antigen-antibody-chromogen complex. This complex flows across a lateral flow membrane and is subsequently bound by antibody immobilised on a membrane. When *Listeria* is present in the sample, a detection-line will form in the viewing window. An internal control is present to allow the operator to determine if the test has been performed correctly (AOAC, 2003). Feldsine et al. (1997) indicated that VIP method is faster for presumptive response which takes about 52 hrs compared to conventional method.

2.5.2.4.6 Enzyme-linked Immunofluorescent assay (ELFA) Vidas Lis assay screening method

The VIDAS LSX enzyme-linked immunocluorescent assay (ELFA), AOAC official method 999.06, is used for screening *Listeria spp*. in vegetables, dairy products, seafood, poultry and raw meats (AOAC, 2000). The principle of this method is related to solid phase receptacle (SPR®). The internal surface of the SPR® is pre-coated during kit production with anti-*Listeria* antibodies. Anti-*Listeria* antibodies which coating the interior of the SPR® will bind to *Listeria* antigens that are present in the sample. Unbound sample material is then washed away. Antibodies conjugated are cycled in and out of the SPR® and will bind with the *Listeria* antigen-antibody complexes. Unbound conjugate is also washed away by further steps. 4-methyl-umbelliferyl-phosphate, a fluorescent substrate, is also cycled in and out of the SPR®. The conjugate enzyme

catalyzes the hydrolysis of this substrate into a fluorescent product; the fluorescence is measured at 450 nm (Ottawa, 2012).

Table 2.1 Biochemical differentiation of species in the genus *Listeria*

	L. monocytogenes	L. seeligeri	L. ivanovii	L. innocua	L. welshimeri	L. grayi
D-Xylose	-	+	+	-	+	-
L-Rhamnose	+	-	-	v	V	V
a-Methl-D-mannoside	+	-	-	+	+	+
D-Mannitol	-	-	-	-	-	+

Note: +: positive; -: negative; V: variable

Table 2.2 CAMP test hemolytic enhancement of *Listeria* species

	Hemolysis enhancement with	
	Staphylococcus aureus (S)	*Rhodococcus equi* (R)
L monocytogenes	+	-*
L. ivanovii	-	+
L. innocua	-	-
L. welshimeri	-	-
L. seeligeri	+	-

Note: * Rare strains are S+ and R+. The R+ reaction is less pronounced than that of *L. ivanovii*.

Table 2.3 Carbohydrate utilization for *Listeria spp.*

Acid production from	L. monocytogenes	L. seeligeri	L. ivanovii	L. innocua	L. welshimeri	L. grayi
Dextrose	+	+	+	+	+	+
Esculin	+	+	+	+	+	+
Maltose	+	+	+	+	+	+
D-Xylose	-	+	+	-	+	-
L-Rhamnose	+	-	-	v	v	v
a-Methl-D-mannoside	+	-	-	+	+	+
D-Mannitol	-	-	-	-	-	+

Note: +: positive; -: negative; v: variable

Table 2.4 Three-tube Most Probable Number (MPN) table

No. of Tubes Positive in:			MPN in the inoculum of the middle set of tubes
First Set	Middle Set	Last Set	
0	0	0	<0.03
0	0	1	0.03
0	0	2	0.06
0	0	3	0.09
0	1	0	0.03
0	1	1	0.061
0	1	2	0.092
0	1	3	0.12
0	2	0	0.062
0	2	1	0.093
0	2	2	0.12
0	2	3	0.16
0	3	0	0.094
0	3	1	0.13
0	3	2	0.16
0	3	3	0.19
1	0	0	0.036
1	0	1	0.072
1	0	2	0.11
1	0	3	0.15
1	1	0	0.073
1	1	1	0.11
1	1	2	0.15
1	1	3	0.19
1	2	0	0.11
1	2	1	0.15
1	2	2	0.2
1	2	3	0.24
1	3	0	0.16
1	3	1	0.2
1	3	2	0.24
1	3	3	0.29
2	0	0	0.091
2	0	1	0.14
2	0	2	0.2
2	0	3	0.26

Table 2.4 (Continued)

2	1	0	0.15
2	1	1	0.2
2	1	2	0.27
2	1	3	0.34
2	2	0	0.21
2	2	1	0.28
2	2	2	0.35
2	2	3	0.42
2	3	0	0.29
2	3	1	0.36
2	3	2	0.44
2	3	3	0.53
3	0	0	0.23
3	0	1	0.39
3	0	2	0.64
3	0	3	0.95
3	1	0	0.43
3	1	1	0.75
3	1	2	1.2
3	1	3	1.6
3	2	0	0.93
3	2	1	1.5
3	2	2	2.1
3	2	3	2.9
3	3	0	2.4
3	3	1	4.6
3	3	2	11
3	3	3	>24

Table 2.5 Selected commercial test kits approved by AOAC International Official Methods protocol for rapid *Listeria* screening and confirmation

Test	ID level	Principle	Approx. test time	Main use
Gene Trak *Listeria* Assay	*Listeria spp.*	Nucleic acid hybridization probe	50 hours	Screening
Listeria Tek	*Listeria spp.*	ELISA	50 hours	Screening
TECRA *Listeria* Visual Immunoassay (TLVIA)	*Listeria spp.*	ELISA	50 hours	Screening
Assurance *Listeria* EIA	*Listeria spp.*	ELISA	50 hours	Screening
VIP *Listeria*	*Listeria spp.*	Immunochromatography	50 hours	Screening
VIDAS *Listeria*	*Listeria spp.*	ELISA	50 hours	Screening

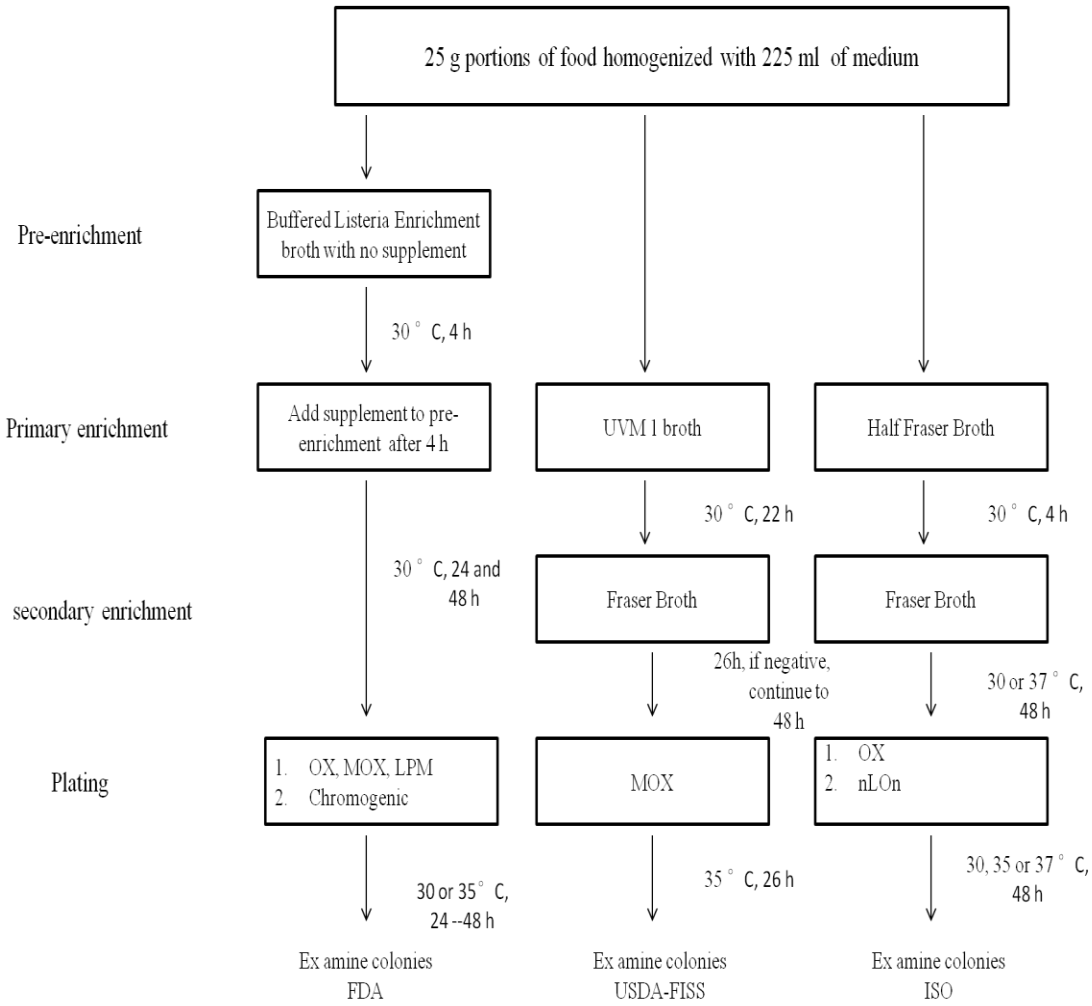

Figure 2.1 Schematics of common standardized enrichment and isolation protocols for *Listeria spp*.

(FDA method, USDA-FSIS method, and ISO method)

CHAPTER III

VALIDATION OF THE DETECTION KIT ON *LISTERIA SPP*. WITH FRESH VEGETABLES

3.1 Abstract

This research was conducted to validate the performance of a recently developed *Listeria* detection kit with various vegetable products including ready-to-eat salads purchased from local markets and restaurants. In order to verify the accuracy of the test kit, samples were also tested in correlation with the conventional method according to FDA-BAM (2011). A total of 35 vegetable samples, 10 bagged salad samples, 10 fresh leafy vegetable samples and 15 ready-to-eat salad samples were evaluated. All positive isolated cells were confirmed using multiplex- PCR analysis. No *Listeria spp*. were found in the bagged salad or fresh leafy vegetable samples. *Listeria grayi* was detected in one restaurant sample by the detection kit (24 h) but not recovered with the modified FDA-BAM method (48-72h). Among all tested samples (n=35), no false positives occurred with the detection kit, but the modified FDA-BAM method had a high false positive rate (40%) on selective media. These results suggest that the detection kit offers higher specificity than the modified FDA-BAM method. In addition, the sensitivity of the detection kits is single cell.

Keywords: *Listeria spp.*, *Listeria* detection kit, vegetable, salad, detection.

3.2 Introduction

Listeria species are ubiquitous that are adaptable to the environment, animals and plants (Rahimi et al., 2012). Salads, a mixture of vegetables, are a RTE dish made of heterogeneous ingredients that are served chilled or at a moderate temperature. With the development of people's lifestyles in recent years, there has been a growing trend towards the consumption of bagged salads which are minimally processed (Santos et al., 2012). Also, as RTE foods, they are usually consumed in restaurants and directly served to consumers. Fresh vegetables, the main components of salads, are considered as essential components of human diets (Adadias et al., 2008). Members of the genus *Listeria*, including *Listeria monocytogenes* are ubiquitous in the agricultural environment (Jeyaletchumi et al., 2011). *Listeria* contamination of vegetables may occur at farm environment such as though soil (Weiss and Seeliger, 1975), water and sewage (Watkins and Sleath, 1981), or unsanitized vehicles and facilities (Beuchat, 1996). In addition, during cutting, grinding, and other processing steps, the structure of vegetables is broken so that some liquid is released, which serves as a good culture medium for the growth of microorganisms (Brackett, 1987).

Between February 2011 and February, 2013, thirteen recalls (FDA inspected foods) were caused by *L. monocytogenes* which related to vegetables or salad products (Table 3.1) (FDA, 2013b). Obviously, vegetables and vegetable products are important vehicle for the transmission of *Listeria*.

The objective of this study was to validate an easy-to-use single tube detection kit for *Listeria spp*. in vegetable and vegetable products.

3.3 Materials and methods

3.3.1 Sample preparation

Different vegetable samples were collected from different places (Table 3.2) in Starkville, MS between February 2013 and April 2013. Samples were detected in the same day as they were purchased from supermarkets or restaurants. Before detection, samples were stored in the refrigerator at 4 °C.

3.3.2 Preparation for the single tube detection kit

Due to the proprietary nature of the kit developed, some of the details in this manuscript have been omitted. Two gels with formulation A and B, and one liquid culture C were prepared by autoclaving at 121 °C for 15 minutes. After cooling below to 50 °C, supplement D was added into formulation A. The formulation A with supplement D was placed into each 18mm glass tube and allowed to cool and solidify. After solidifying formulation A, 1 ml formulation B was poured into the tubes and solidified.

3.3.3 Validation of the single tube detection kit with vegetable tissues

Twenty five grams from each sample were aseptically placed in a sterile stomacher bag (Difco, Fisher Scientific) with 225 ml liquid culture C and rinsed for 60 sec. A 10 ml rinsed sample was transferred to the detection kit. Three kits were used for each sample (Table 3.3). As a positive control, one detection kit was inoculated with one loop overnight suspension culture of *L. monocytogenes* (ATCC LM 19114) for each testing sample (Table 3.4). All detection kits were incubated at 37 °C. After 24h incubation, aliquots from each detection kit were streaked onto Oxford Medium Base agar plates (Difco, Fisher Scientific) containing Modified Oxford Antibiotic Supplement

(Difco, Fisher Scientific) and incubated for 24-48 h at 37 °C. Black colonies that appeared on the plates were considered to be presumptive *Listeria spp*. Suspected colonies were selected from each plate and further confirmed by multiplex-PCR.

Cells of *Listeria spp*. were also collected and enriched from the same tissue samples according to the modified FDA-BAM method (2011) with some modifications. Briefly, twenty-five grams from each sample was aseptically placed in a sterile bag with 225 ml *Listeria* Enrichment Broth (LEB) (Difco, Fisher Scientific) and rinsed for 60 sec. All collected samples were incubated at 37 °C for 24h. Aliquots of enriched cultures were then streaked onto Oxford Medium Base agar plates containing Modified Oxford Antibiotic Supplement and incubated for 24-48 h at 37 °C. Black colonies that appeared on the plates were considered to be presumptive *Listeria spp*. Suspected colonies were selected from each plate and further identified by multiplex-PCR.

The process for the detection of *Listeria spp*. with both the detection kit and FDA-BAM method for vegetable and salad samples is shown in Figure 3.1.

3.3.4 Multiplex-PCR identification of *Listeria spp.*

According to the FDA-BAM (2011), suspected colonies on the Oxford Medium Base agar plates were selected and identified by multiplex PCR. Suspected colonies were suspended with 50 µl distilled water. The suspension was boiled for 5 min and then centrifuged at 10000 x g for 2 min. The supernatant was used as DNA template for multiplex-PCR. The reaction mixture (25 µl) contained 2X GoTaq Green Master Mix (Promega, USA), 5.5 µl deionized distilled water, 1µl of four species-specific forward primers (MonoA, Ino2, MugraI, and Siwi2) and one conserved reverse primer (Lis1B) (Bubert et al.), and 2 µl of template DNA. The primers that were used in the multiplex-

PCR are listed in Table 3.5. The amplifications were performed in a thermocycler (Eppendorf, New York, NY, USA). The cycling started with an initial denaturation at 94 °C for 3 min followed by 30 cycles of a 94 °C denaturation for 30 s, 55 °C annealing for 30 s and 72 °C elongation for 20 s. the mixture was then subjected to a final extension at 72 °C for 5 min. The PCR products were separated by electrophoresis on a 2.0% agarose gel with 5 µl ethidium bromide, and photographed by a BioDoc-it^{M} Imaging System (UVP, Upland, CA, USA).

3.3.5 Sensitivity test for the detection kit without food tissue

In order to determine the detection limit of the detection kit, three different *Listeria* strains (ATCC LI 19119, ATCC LM 19114, and ATCC LM 7694) were conducted by serial dilution from 10^{-1} to 10^{-8}. Dilutions at 10^{-6}, 10^{-7} and 10^{-8} were used to assess the sensitivity test. One ml diluent from each dilution was transferred onto one 3M ^{TM} Petrifilm ^{TM} Aerobic Count Plate (U.S. AOAC®), and 1 ml diluent from the same dilution was transfer to one detection kit. All plates and detection kits were incubated at 37°C for 24 h. Three replications of each strain were conducted in this study.

3.3.6 Sensitivity and specificity

The sensitivity and specificity were calculated to show the reliability of the detection methods. The sensitivity measures the proportion of actual positives while the specificity measures the proportion of correct negatives (FSIS, 2010). The formulations are expressed as follows:

Sensitivity = number of true positives/number of positives

Specificity = number of true negatives/number of negatives

3.4 Results and discussion

A black precipitate of the bottom of the detection kit while black colonies on the Oxford Medium Base agar plates were regarded as positive for *Listeria spp*.(Figure 3.2). Figure 3.3 A and Figure 3.3 B presented the examples of positive, false positive and negative results in the Oxford Medium Base agar plate, respectively. All the results were confirmed by multiplex-PCR.

Among the 35 samples, no *Listeria spp*. were detected using the modified FDA-BAM method within 48-72h while one case of *L. grayi* (one tube out of three tested tubes) was isolated using the detection kit in 24h (Table 3.3). The positive *L. grayi* sample (Figure 3.4) was confirmed by multiplex-PCR (Figure 3.5).

In this study, it was not possible to calculate sensitivity since no true positive results were found with the modified FDA-BAM method, while the detection kit indicated 100% sensitivity (one *L. grayi* occurrence) (Table 3.6). For the specificity, the detection kit showed higher specificity (100%, no false positives) while Oxford Medium Base agar plate in the FDA-BAM method only had 60% specificity with 40% false positives (Table 3.6). In addition, the sensitivity test (Table 3.7) which demonstrated the detection limit for the detection kit showed that this detection kit is effective at detecting a single cell. All inoculated detection kits had positive results (Table 3.4).

The standard *Listeria* isolation method takes longere than 72h which includes required multiple enrichments with multiple selective mediums (FDA-BAM, 2011). In addition, the standard method causes a high false positive rate (40% in this study). This high false positive rate (low specificity) is due to esculin hydrolysis of various microorganisms such as Gram positive *cocci* and *Enterobacteriaceae* (Edbery et al.,

1976). However, compared to the standard method, the single tube *Listeria* detection kit showed 100% specificity on vegetables due to the high selectivity of novel components and optimized culturing conditions. The detection process only took 24 hours without any false positives detected in this study.

Table 3.1 Recalls that were caused by L. monocytogenes which related to vegetables or salad products between February 2011 and February 2013

Date	Brand	Product description	Company
02/28/2011	Tfarms, Raleys, Signature Café	Broccoli items	Taylor Farms Pacific
06/23/2011	Dole	Salad	Dole Fresh Vegetables
01/01/2012	Let's grow healthy together!, Green valley food corp., Broccosprouts	Sprouts	Green valley food corp
01/03/2012	Let's grow healthy together, green valley food corp, broccosprouts	Sprouts	Green valley food corp.
06/25/2012	Fresh Selections, Marketside	Bagged Salads	Dole Fresh Vegetables, Inc.
06/29/2012	Dole Hearts of Romaine	Bagged Salad	Dole Fresh Vegetables, Inc.
07/27/2012	Cool creations and Fiesta garden fresh salsa	Specific packages of products containing onions	Cool Creations, LLC
07/28/2012	Delish, Marketside, Raley's and various brands	Various products containing yellow onions	GH Foods CA, LLC
08/23/2012	Dole	Bagged Salad	Dole Fresh Vegetables
10/17/2012	Dole	Salad	Dole Fresh Vegetables
12/13/2012	Taylor Farms	Romaine Lettuce	Taylor Farms Retail, Inc.
02/01/2013	Sprouters northwest and lifeforce	Sprouts, wheatgrass, and pea shoots	Sprouters northwest
02/27/2013	Sprouters Northwest, LifeForce and Brassica	Sprout products, wheatgrass and pea shoots	Sprouters Northwest

Table 3.2 Sampling site, type and component of vegetables

Sample NO.	Sample Source	Sample type	Sample components
1	Supermarket	Bagged salad	Mixed [a]
2	Supermarket	Bagged salad	Spinach
3	Supermarket	Bagged salad	Mixed [b]
4	Supermarket	Bagged salad	Mixed [c]
5	Supermarket	Bagged salad	Icebery
6	Supermarket	Bagged salad	Mixed [a]
7	Supermarket	Bagged salad	Spinach
8	Supermarket	Bagged salad	Mixed [b]
9	Supermarket	Bagged salad	Mixed [c]
10	Supermarket	Bagged salad	Icebery
11	Supermarket	Leafy vegetable	Cilantro
12	Supermarket	Leafy vegetable	Lettuce
13	Supermarket	Leafy vegetable	Savoy
14	Supermarket	Leafy vegetable	Red cabbage
15	Supermarket	Leafy vegetable	Turnip greens
16	Supermarket	Leafy vegetable	Cilantro
17	Supermarket	Leafy vegetable	Lettuce
18	Supermarket	Leafy vegetable	Savoy
19	Supermarket	Leafy vegetable	Red cabbage
20	Supermarket	Leafy vegetable	Turnip greens
21	Restaurant A	RTE-salad	NA
22	Restaurant B	RTE-salad	NA
23	Restaurant C	RTE-salad	NA
24	Restaurant D	RTE-salad	NA
25	Restaurant E	RTE-salad	NA
26	Restaurant A	RTE-salad	NA
27	Restaurant B	RTE-salad	NA
28	Restaurant C	RTE-salad	NA
29	Restaurant D	RTE-salad	NA
30	Restaurant E	RTE-salad	NA
31	Restaurant A	RTE-salad	NA
32	Restaurant B	RTE-salad	NA
33	Restaurant C	RTE-salad	NA
34	Restaurant D	RTE-salad	NA
35	Restaurant E	RTE-salad	NA

Note: [a] includes romaine, icebery, carrots, pea pods, red cabbage, and readishes. [b] includes baby lettuces, baby greens, endive, carrots, radicchio, broccoli, and red cabbage. [c] includes broccoli, carrots, and red cabbage.

Table 3.3 *Listeria spp*. detection and PCR confirmation result with the detection kit and the modified FDA-BAM method

Sample No.	Detection kit method				Modified FDA-BAM Method		
	Kit No.	Kit Result	Plate result	PCR	Plate No.	Plate result	PCR
	1--1	-	-	NA	1--1	+	-
1	1--2	-	-	NA	1--2	+	-
	1--3	-	-	NA	1--3	+	-
	2--1	-	-	NA	2--1	+	-
2	2--2	-	-	NA	2--2	+	-
	2--3	-	-	NA	2--3	+	-
	3--1	-	-	NA	3--1	+	-
3	3--2	-	-	NA	3--2	+	-
	3--3	-	-	NA	3--3	+	-
	4--1	-	-	NA	4--1	+	-
4	4--2	-	-	NA	4--2	+	-
	4--3	-	-	NA	4--3	+	-
	5--1	-	-	NA	5--1	-	NA
5	5--2	-	-	NA	5--2	-	NA
	5--3	-	-	NA	5--3	-	NA
	6--1	-	-	NA	6--1	-	NA
6	6--2	-	-	NA	6--2	-	NA
	6--3	-	-	NA	6--3	-	NA
	7--1	-	-	NA	7--1	+	-
7	7--2	-	-	NA	7--2	+	-
	7--3	-	-	NA	7--3	+	-
	8--1	-	-	NA	8--1	+	-
8	8--2	-	-	NA	8--2	+	-
	8--3	-	-	NA	8--3	+	-
	9--1	-	-	NA	9--1	-	NA
9	9--2	-	-	NA	9--2	-	NA
	9--3	-	-	NA	9--3	-	NA
	10--1	-	-	NA	10--1	-	NA
10	10--2	-	-	NA	10--2	-	NA
	10--3	-	-	NA	10--3	-	NA
	11--1	-	-	NA	11--1	+	-
11	11--2	-	-	NA	11--2	+	-
	11--3	-	-	NA	11--3	+	-
	12--1	-	-	NA	12--1	-	NA
12	12--2	-	-	NA	12--2	-	NA
	12--3	-	-	NA	12--3	-	NA
	13--1	-	-	NA	13--1	+	-
13	13--2	-	-	NA	13--2	+	-
	13--3	-	-	NA	13--3	+	-

Table 3.3 (Continued)

	14—1	-	-	NA	14--1	-	NA
14	14—2	-	-	NA	14--2	-	NA
	14—3	-	-	NA	14--3	-	NA
	15—1	-	-	NA	15--1	+	-
15	15—2	-	-	NA	15--2	+	-
	15—3	-	-	NA	15--3	+	-
	16—1	-	-	NA	16--1	+	-
16	16—2	-	-	NA	16--2	+	-
	16—3	-	-	NA	16--3	+	-
	17—1	-	-	NA	17--1	-	NA
17	17—2	-	-	NA	17--2	-	NA
	17—3	-	-	NA	17--3	-	NA
	18--1	-	-	NA	18--1	-	NA
18	18--2	-	-	NA	18--2	-	NA
	18--3	-	-	NA	18--3	-	NA
	19--1	-	-	NA	19--1	-	NA
19	19--2	-	-	NA	19--2	-	NA
	19--3	-	-	NA	19--3	-	NA
	20--1	-	-	NA	20--1	+	-
20	20--2	-	-	NA	20--2	+	-
	20--3	-	-	NA	20--3	+	-
	21--1	+	-	*L.grayi*	21--1	-	NA
21	21--2	-	-	NA	21--2	-	NA
	21--3	-	-	NA	21--3	-	NA
	22--1	-	-	NA	22--1	-	NA
22	22--2	-	-	NA	22--2	-	NA
	22--3	-	-	NA	22--3	-	NA
	23--1	-	-	NA	23--1	-	NA
23	23--2	-	-	NA	23--2	-	NA
	23--3	-	-	NA	23--3	-	NA
	24--1	-	-	NA	24--1	-	NA
24	24--2	-	-	NA	24--2	-	NA
	24--3	-	-	NA	24--3	-	NA
	25--1	-	-	NA	25--1	-	NA
25	25--2	-	-	NA	25--2	-	NA
	25--3	-	-	NA	25--3	-	NA
	26--1	-	-	NA	26--1	-	NA
26	26--2	-	-	NA	26--2	-	NA
	26--3	-	-	NA	26--3	-	NA
	27--1	-	-	NA	27--1	-	NA
27	27--2	-	-	NA	27--2	-	NA
	27--3	-	-	NA	27--3	-	NA

Table 3.3 (Continued)

	28--1	-	-	NA	28--1	-	NA
28	28--2	-	-	NA	28--2	-	NA
	28--3	-	-	NA	28--3	-	NA
	29--1	-	-	NA	29--1	-	NA
29	29--2	-	-	NA	29--2	-	NA
	29--3	-	-	NA	29--3	-	NA
	30--1	-	-	NA	30--1	+	-
30	30--2	-	-	NA	30--2	+	-
	30--3	-	-	NA	30--3	+	-
	31--1	-	-	NA	31--1	+	-
31	31--2	-	-	NA	31--2	+	-
	31--3	-	-	NA	31--3	+	-
	32--1	-	-	NA	32--1	+	-
32	32--2	-	-	NA	32--2	+	-
	32--3	-	-	NA	32--3	+	-
	33--1	-	-	NA	33--1	-	NA
33	33--2	-	-	NA	33--2	-	NA
	33--3	-	-	NA	33--3	-	NA
	34--1	-	-	NA	34--1	+	-
34	34--2	-	-	NA	34--2	+	-
	34--3	-	-	NA	34--3	+	-
	35--1	-	-	NA	35--1	-	NA
35	35--2	-	-	NA	35--2	-	NA
	35--3	-	-	NA	35--3	-	NA

Note: NA = Not available
-: Negative result
+: Positive result

Table 3.4 Inoculation result with *Listeria* strain (ATCC LM 19114) on vegetable and salad samples for detection kit, incubating at 37°C for 24h

Sample NO.	Kit result
1	+
2	+
3	+
4	+
5	+
6	+
7	+
8	+
9	+
10	+
11	+
12	+
13	+
14	+
15	+
16	+
17	+
18	+
19	+
20	+
21	+
22	+
23	+
24	+
25	+
26	+
27	+
28	+
29	+
30	+
31	+
32	+
33	+
34	+
35	+

Table 3.5 Forward and reverse primers used in the multiplex-PCR for *Listeria spp.*

Name	Type	Primer (5'-3')	Target *Listeria spp.*
MonoA	Forward	CAAACTGCTAACACAGCTACT	*L. monocytogenes*
Ino2	Forward	ACTAGCACTCCAGTTGTTAAAC	*L. innocua*
MugraI	Forward	CCAGCAGTTTCTAAACCTGCT	*L. grayi*
Siwi2	Forward	TAACTGAGGTAFCGAGCGAA	*L. seeligeri, L. ivanovii,* & *L. welshimeri*
Lis1 B	Reverse	TTATACGCGACCGAAGCCAAC	All *Listeria spp.*

Table 3.6 Specificity and sensitivity of the detection kit and modified FDA-BAM method for vegetables

	Detection kit	Modified FDA-BAM method
False positive	0	40%
False negative	0	0
Specificity	100%	60%
Sensitivity	100%	NA

Note: NA= not available.
Sensitivity = number of true positives/number of positives
Specificity = number of true negatives/number of negatives

Table 3.7 Sensitivity test for detection kit with three different *Listeria spp.* (ATCC LI 19119, ATCC LM 19114, and ATCC LM 7694)

Dilution	Rep	ATCC LI19119		ATCC LM19114		ATCC LM7694	
		No. of cells	Kit result	No. of cells	Kit result	No. of cells	Kit result
10^{-6}	1	TNTC	+	TNTC	+	TNTC	+
	2	TNTC	+	TNTC	+	TNTC	+
	3	TNTC	+	TNTC	+	TNTC	+
	Average	TNTC	/	TNTC	/	TNTC	/
10^{-7}	1	95	+	16	+	93	+
	2	97	+	19	+	93	+
	3	93	+	13	+	94	+
	Average	95	/	16	/	93	/
10^{-8}	1	11	+	1	+	6	+
	2	12	+	1	+	8	+
	3	12	+	2	+	5	+
	Average	12	/	1	/	6	/

Note: TNTC= Too Numerous To Count

+: Positive result.

/: Tube result is not available.

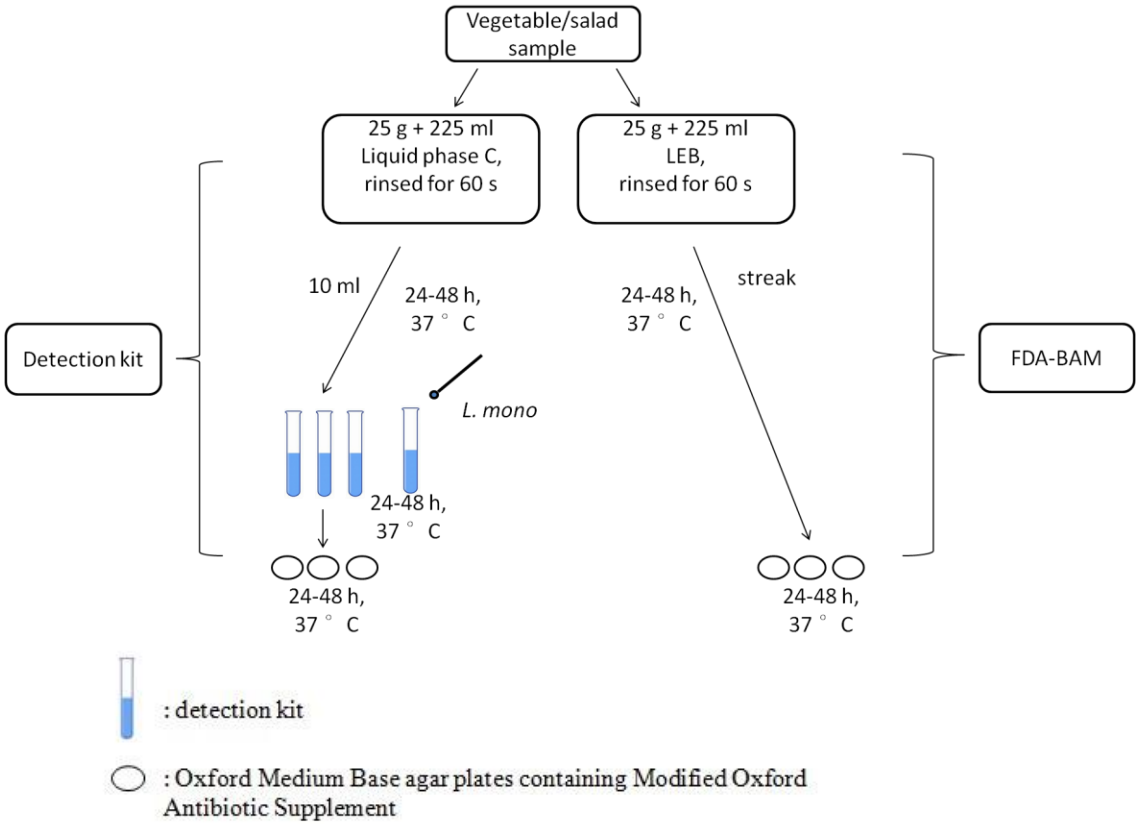

Figure 3.1 Schematics for the detection of *Listeria spp*. with the detection kit and modified FDA-BAM method in vegetables

Note: The suspicious colonies on the Oxford Medium Base agar plates were confirmed by multiplex PCR.

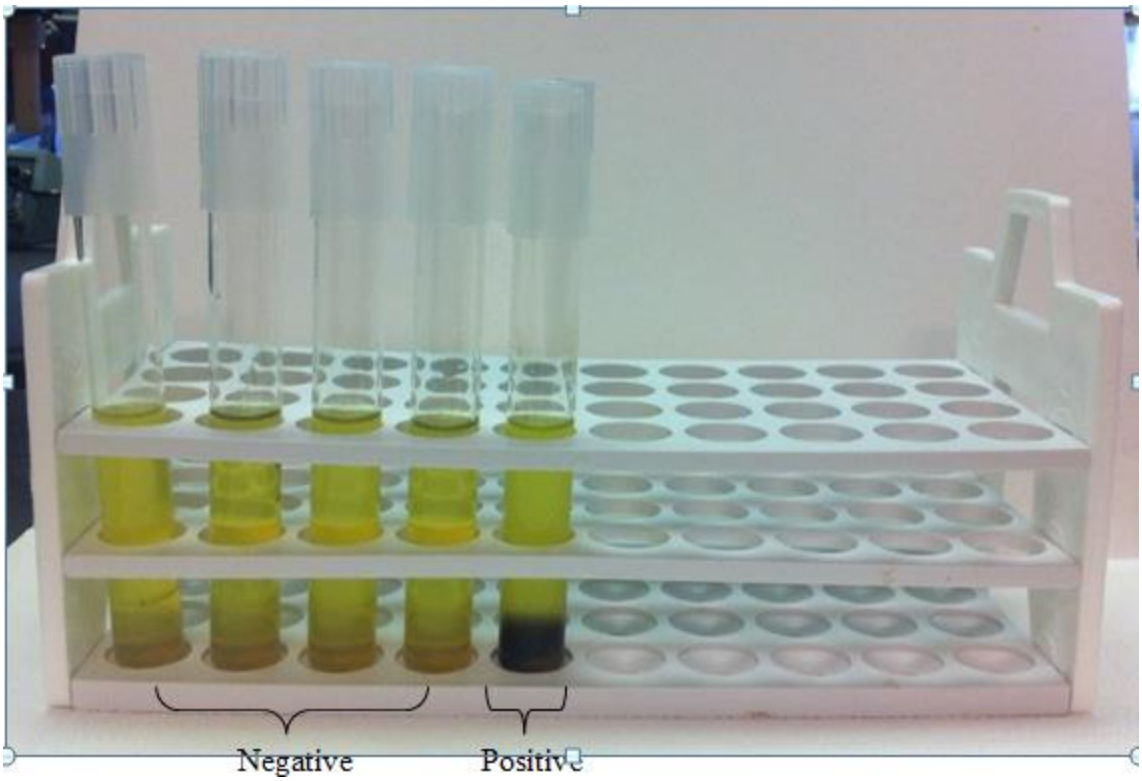

Figure 3.2 Positive and negative results of the detection kits

Note: From left to right, the first four kits without any color changes at the bottom showed the negative results. The last kit with black color at the bottom showed a positive result.

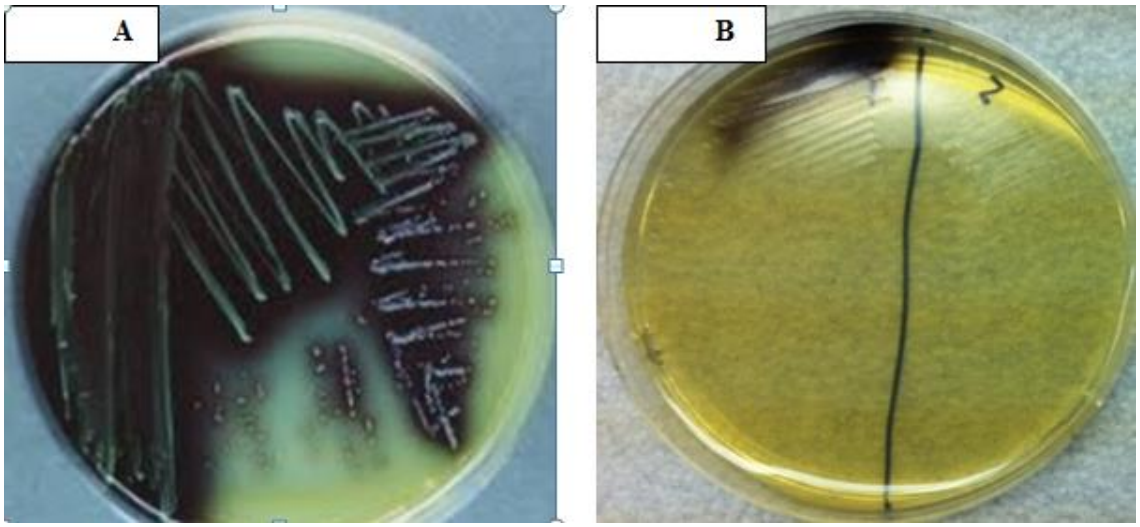

Figure 3.3 Example of positive (A), false positive and negative (B) results on Oxford Medium Base agar plates

Note: The *Listeria* strain ATCC LM 19114 was streaked on the Oxford Medium Base agar plate (A). The left part in B presented false positive result while the right part on the same plate showed negative result.

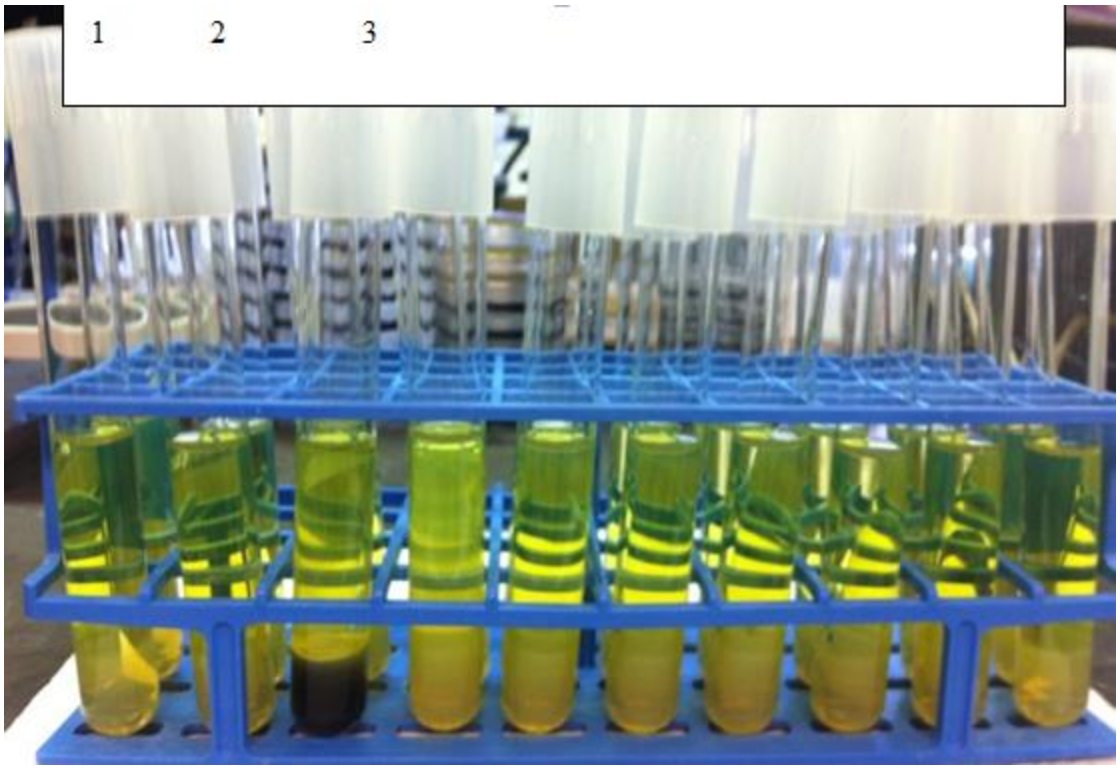

Figure 3.4 Illustration of *Listeria spp*. detection on vegetables with the detection kits

Note: From left to the right in the first lane, the NO. 3 detection kit was showed as positive result (black color at the bottom) and confirmed as *L. grayi* by multiplex-PCR. Other kits without color change at the bottom were regarded as negative.

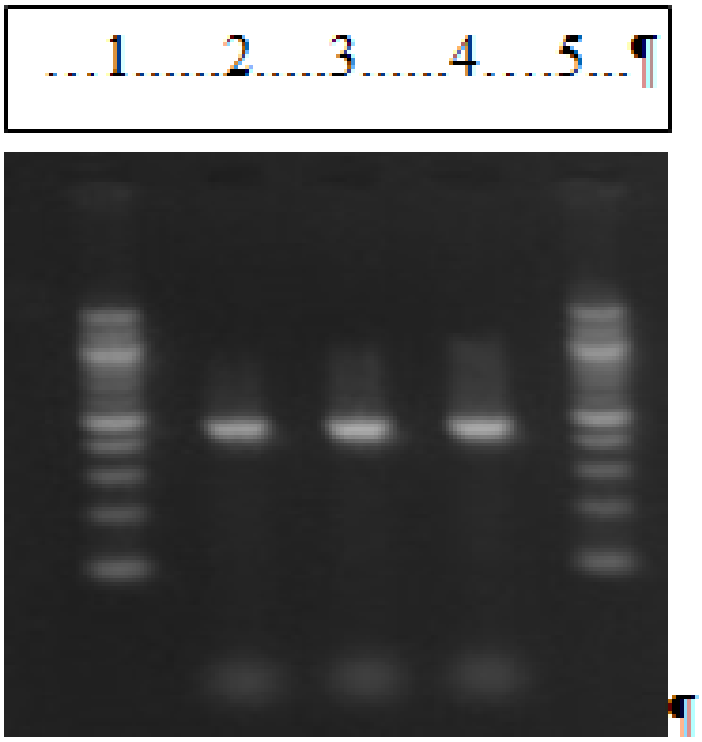

Figure 3.5 Agarose gel image of multiplex-PCR

Note: 2 to 4 bands2 to 4 bands yielded around 0.48 kb were regarded as L. grayi, bands 1 and 5 were DNA ladders.

CHAPTER IV

DETECTION AND QUANITIFICATION OF *LISTERIA* CELLS IN RAW CATFISH FILLETS USING A NOVEL SINGLE *LISTERIA* DETECTION KIT

4.1 Abstract

The study of this chapter aimed to validate and quantify *Listeria* cells naturally contaminated in raw catfish fillets using the *Listeria* detection kit. A total of 12 catfish fillets were used to validate the detection kit in correlation with the modified FDA-BAM method (2011). Both methods showed 100% *Listeria* positives in all screened samples (n=12). Quantification study of cell numbers in raw catfish fillets showed that there were no significant difference ($P<0.05$) between two methods.

Key words: *Listeria spp.*, *Listeria* detection kit, catfish fillet, detection, enumeration

4.2 Introduction

L. monocytogenes and other *Listeria spp.* have been isolated from catfish and various non-catfish seafoods (Chou and Wang, 2006).

Catfish is a lean and highly nutritious food commodity with widely consumer acceptance in the United States. According to the National Agricultural Statistics Service (2011), the overall sales for catfish grower in 2010 was reported as 403 million U.S. dollars, in which Alabama, Arkansas, Mississippi, and Texas accounted for 94% of the

overall sales (Desai et. al, 2012). Chou et al. (2006) presented that *L. monocytogenes* dominated *Listeria* isolations from channel catfish filets, accounting from 25% to 47% of the strains obtained. Other *Listeria spp.* showed in Chou's study included *L. welshimeri*, *L. innocua*, *L. ivanovii*, *L. grayi*, and *L. seeligeri*. Even though *L. monocytogenes* has been isolated from catfish fillets (Chou et al., 2006; Chou and Wang, 2006), no outbreaks have been linked directly to the ingestion of contaminated catfish products. However, limited information is available regarding the occurrence of *L. monocytogenes* and other *Listeria spp.* isolates from catfish products.

Enumeration of *Listeria* in positive samples is required by FDA-BAM method (2011). Standard methods enumerate cells by colony count on selective agar in conjunction MPN method using selective enrichment with subsequent plating on selective agar. However, this process usually takes more than 3 days by using multi-selective media. Our single tube detection kit was also designed for the enumeration of *Listeria spp* in conjunction with MPN method. Results were available to be read within 24 hours.

The aim of this study was to develop an easy to use single tube detection kit for catfish fillets. The test kit was validated with raw catfish samples to compare with the modified method according to the modified FDA-BAM (2011). This detection kit was also tested for the quantification of *Listeria spp.*. The MPN method according to the FDA-BAM (2011) was used to validate sensitivity of the detection kit.

4.3 Materials and methods

4.3.1 Sample preparation

Twelve raw catfish fillets were collected from three different local supermarkets.

4.3.2 Preparation for the single tube detection kit

The preparation for detection kit was described in the chapter 3.3.2.

4.3.3 Validation of the single tube detection kit

A whole raw catfish fillet was placed in a sterile bag and blended for 2 min (consider this as an original sample) in a stomacher (Tekmar Company, Cincinnati, OH, USA). Twenty-five gram of the blended sample was aseptically transferred to another sterile bag with 225 ml liquid culture C and blended for 60 sec in stomacher. A 10 ml suspension was transferred to the detection kit. Three detection kits of each sample were conducted. All the detection kits were incubated at 37 °C. After 24h incubation, aliquots from each detection kit were streaked onto Oxford Medium Base agar plates (Difco, Fisher Scientific) containing Modified Oxford Antibiotic Supplement (Difco, Fisher Scientific) and incubated for 24-48h at 37 °C. The detection kit with black color on the bottom was regarded as positive result. Black colonies appeared on the plates were considered to be *Listeria spp*. Suspected colonies were selected from each plate and further identified by multiplex-PCR.

Listeria. *Spp* were also isolated from the same sample using the FDA-BAM (2011) with some modification. Briefly, 25 grams from the blended original sample was aseptically placed in a sterile bag with 225 ml LEB and blended for 60 sec in stomacher. All collected samples were incubated at 37 °C for 24h. Aliquots of enriched cultures were then streaked onto the Oxford Medium base gar plates containing Modified Oxford Antibiotic Supplement and incubated for 24-48 h at 37 °C. Black colonies appeared on the plates were considered to be *Listeria spp*. Suspected colonies were selected from each plate and further identified by multiplex-PCR.

The detailed process for both detection methods was described in the Figure 4.1.

4.3.4 MPN Enumeration

One gram of the blended catfish fillet was aseptically transferred to three detection tubes (detection kit MPN enumeration) to make 10^0 dilution. At the same time, another 1 gram catfish fillet from the same blended sample was transferred to sterile 18mm tube with 9 ml LEB (FDA-BAM MPN enumeration) (FDA-BAM, 2011). Then, aseptically transferred 25gram catfish fillet from the same blended sample to a sterile bag with 225 ml Phosphate Buffered Saline (PBS, pH 7.2) was stomached in stomacher (Tekmar Company, Cincinnati, OH, USA) for 60 sec to make 1: 10 dilution. Decimal dilutions were prepared with sterile PBS until 10^{-3} diluent. All diluents were vortexed for 7 sec. One milliliter of each dilution was transferred to three detection kits and three LEB tubes. All kits and tubes were incubated at 37 °C for 24 hours. After incubation, the detection kits with color change in bottom agar were regarded as positive result. In order to confirm the result, aliquots from each detection kit were streaked onto Oxford Medium base plates containing Modified Oxford Antibiotic Supplement. Black colonies appeared on the plates were considered to be *Listeria spp*. For the FDA-BAM MPN method, aliquots from each tube were also streaked onto Oxford Medium base plates containing Modified Oxford Antibiotic Supplement. All the plates from detection kits and FDA-BAM method were incubated at 37 C for 24-48 hours. Black colonies on the plate after incubation were considered as *Listeria spp.*. Suspected colonies were selected from each plate and further identified by multiplex-PCR.

The detailed enumeration process of *Listeria spp*. with detection kit and modified FDA-BAM method in catfish fillet samples was shown in the Figure 4.2.

4.3.5 Mutiplex-PCR detection for *Listeria spp.* in catfish fillet

The multiplex-PCR detection process for *Listeria spp.* in catfish fillet samples was described in chapter 3.3.4.

4.3.6 Statistical analysis

The sensitivity and specificity were used to show reliability of the detection kit. The sensitivity measures the proportion of actual positive while the specificity measures the proportion of correctly negatives (FSIS, 2010). The formulation is expressed as follows:

Sensitivity = number of true positives/number of positives

Specificity = number of true negatives/number of negatives

A completely randomized design was used to see difference between the kit and FDA-BAM MPN enumeration. Data were analyzed with SAS software (SAS Institute, 9.3 Version). When differences ($P<0.05$) existed between MPN numbers of the detection kit method and modified FDA-BAM method, means were separated by using Fisher's protected Least Significant Difference.

4.4 Results and discussion

Detection results of *Listeria spp.* in raw catfish fillets with both detection methods were shown in Table 4.1. All tested catfish fillets (n=12) showed *Listeria* positives on both methods which had 100% specificity (Table 4.2). Confirmed multiplex-PCR result was showed in the Figure 4.3.

Enumeration for *Listeria spp.* in catfish fillets by using both MPN detection kit and FDA-BAM methods was shown in the Table 4.3. Difference ($P<0.05$) between both

MPN methods on enumeration was analyzed by SAS software with the paired t test (Table 4.4). According to the result from Table 4.4, there is no significant difference ($P<0.05$) between both MPN numbers of the detection kit and modified FDA-BAM method. The Positive MPN enumeration results of the detection kits were showed in the Figure 4.4.

The *Listeria* detection kit on raw catfish fillets (n=12) showed 100% sensitivity in 24 h. Even through the FDA-BAM method also showed 100% sensitivity, this conventional method required longer detection time (more than 72 hours) with multi-incubation steps. In addition, unlike other commercial test kits that were talked in the literature review, this detection kit with MPN method is also available for detection and quantification at the same time in 24 h.

Table 4.1 Detection of *Listeria spp.* with the detection kit and modified FDA-BAM method in raw catfish fillets

Sample NO.	Source	Detection kit method			FDA-BAM method		
		Kit NO.	Result	PCR	Plate NO.	Result	PCR
1	Market A	1--1	+	+	+	+	+
		1--2	+	+	+	+	+
		1--3	+	+	+	+	+
2		2--1	+	+	+	+	+
		2--2	+	+	+	+	+
		2--3	+	+	+	+	+
3		3--1	+	+	+	+	+
		3--2	+	+	+	+	+
		3--3	+	+	+	+	+
4		4--1	+	+	+	+	+
		4--2	+	+	+	+	+
		4--3	+	+	+	+	+
5	Market B	5--1	+	+	+	+	+
		5--2	+	+	+	+	+
		5--3	+	+	+	+	+
6		6--1	+	+	+	+	+
		6--2	+	+	+	+	+
		6--3	+	+	+	+	+
7		7--1	+	+	+	+	+
		7--2	+	+	+	+	+
		7--3	+	+	+	+	+
8		8--1	+	+	+	+	+
		8--2	+	+	+	+	+
		8--3	+	+	+	+	+
9	Market C	9--1	+	+	+	+	+
		9--2	+	+	+	+	+
		9--3	+	+	+	+	+
10		10--1	+	+	+	+	+
		10--2	+	+	+	+	+
		10--3	+	+	+	+	+
11		11--1	+	+	+	+	+
		11--2	+	+	+	+	+
		11--3	+	+	+	+	+
12		12--1	+	+	+	+	+
		12--2	+	+	+	+	+
		12--3	+	+	+	+	+

Table 4.2 Specificity and sensitivity of the detection kit and modified FDA-BAM method on catfish fillets

	Detection kit	Modified FDA-BAM
Positive rate	100%	100%
Sensitivity	100%	100%
Specificity	NA	NA

Note: Sensitivity = number of true positives/number of positives
Specificity = number of true negatives/number of negatives

Table 4.3 Enumeration for *Listeria spp.* by using MPN method with the detection kit and modified FDA-BAM method on catfish fillets

Sample NO.		Detection kit MPN					FDA-BAM MPN				
		1 d	2 d	3 d	4 d	CFU/g	1 d	2 d	3 d	4 d	CFU/g
1		3	0	0	0	2.3	3	1	0	0	4.3
2		3	0	0	0	2.3	3	0	0	0	2.3
3	NO.	3	0	0	0	2.3	3	1	0	0	4.3
4	of	3	0	0	0	2.3	2	1	0	0	1.5
5	+	3	0	0	0	2.3	3	0	0	0	2.3
6	result	3	0	0	0	2.3	2	1	0	0	1.5
7		3	0	0	0	2.3	3	0	0	0	2.3
8		3	0	0	0	2.3	2	1	0	0	1.5
9		3	0	0	0	2.3	3	0	0	0	2.3
10		3	0	0	0	2.3	3	0	0	0	2.3
11		3	0	0	0	2.3	3	0	0	0	2.3
12		3	0	0	0	2.3	3	0	0	0	2.3

Note: The result for CUF/ml column was for undiluted samples. 1 d, 2 d, 3 d, and 4 d represent 10^0, 10^{-1}, 10^{-2}, 10^{-3} dilution, respectively.

Table 4.4 Statistical result: difference between the detection kit and modified FDA-BAM method on enumeration

Mean	95% CL Mean	Std Dev	95% CL Std Dev	Mean	95% CL Mean	DF	t Value	Pr > |t|
0.1333	-0.4635	0.7302	0.9394	0.1333	-0.4635	11	0.49	0.6326

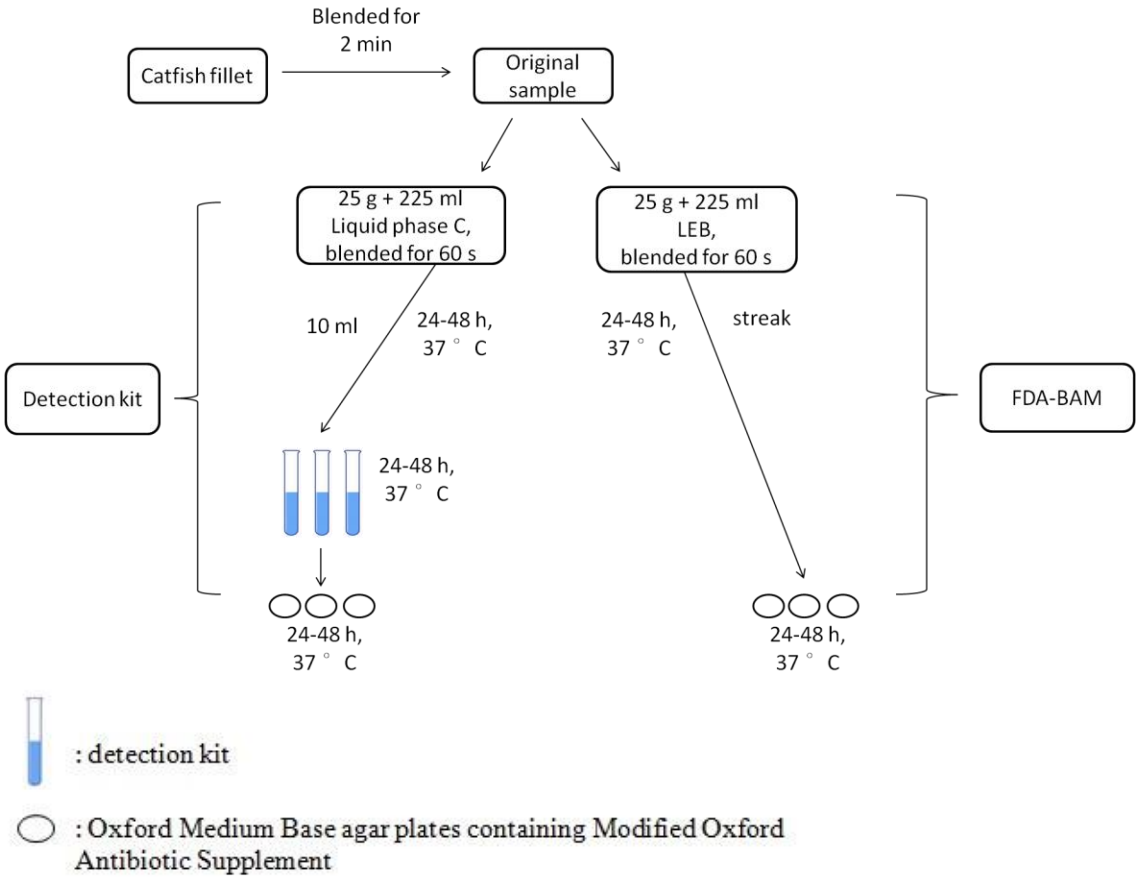

Figure 4.1 Schematics for the detection of *Listeria spp*. with the detection kit and modified FDA-BAM method in raw catfish fillets

Note: The agar plates were confirmed by multiplex PCR detection

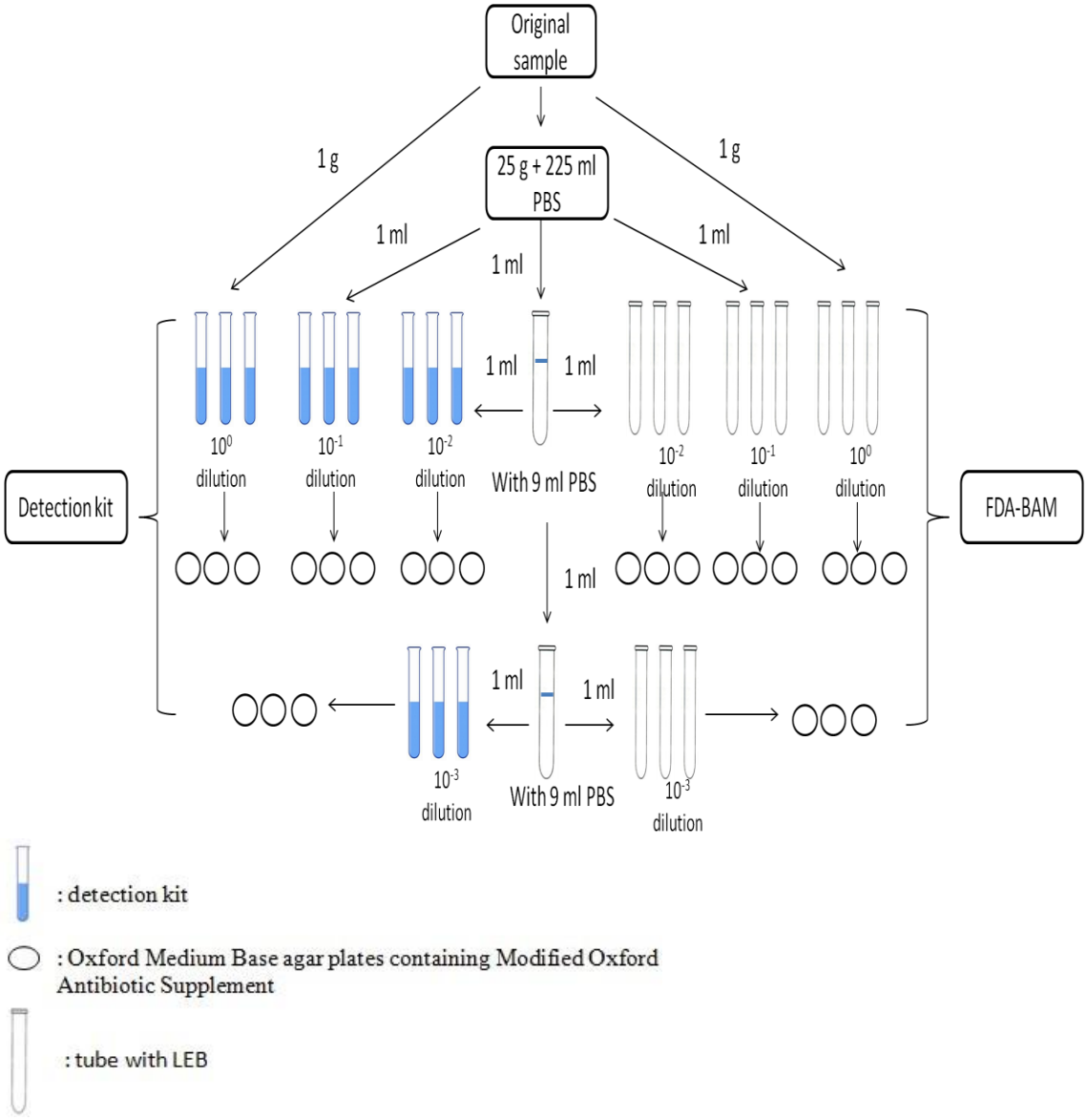

Figure 4.2 Schematics for MPN enumeration of *Listeria spp*. with detection kit and modified FDA-BAM method in raw catfish fillets

Note: All the detection kits and tubes were incubated for 24h at 37 °C. All the agar plates were incubated for 24-48h at 37 °C and confirmed by multiplex PCR detection

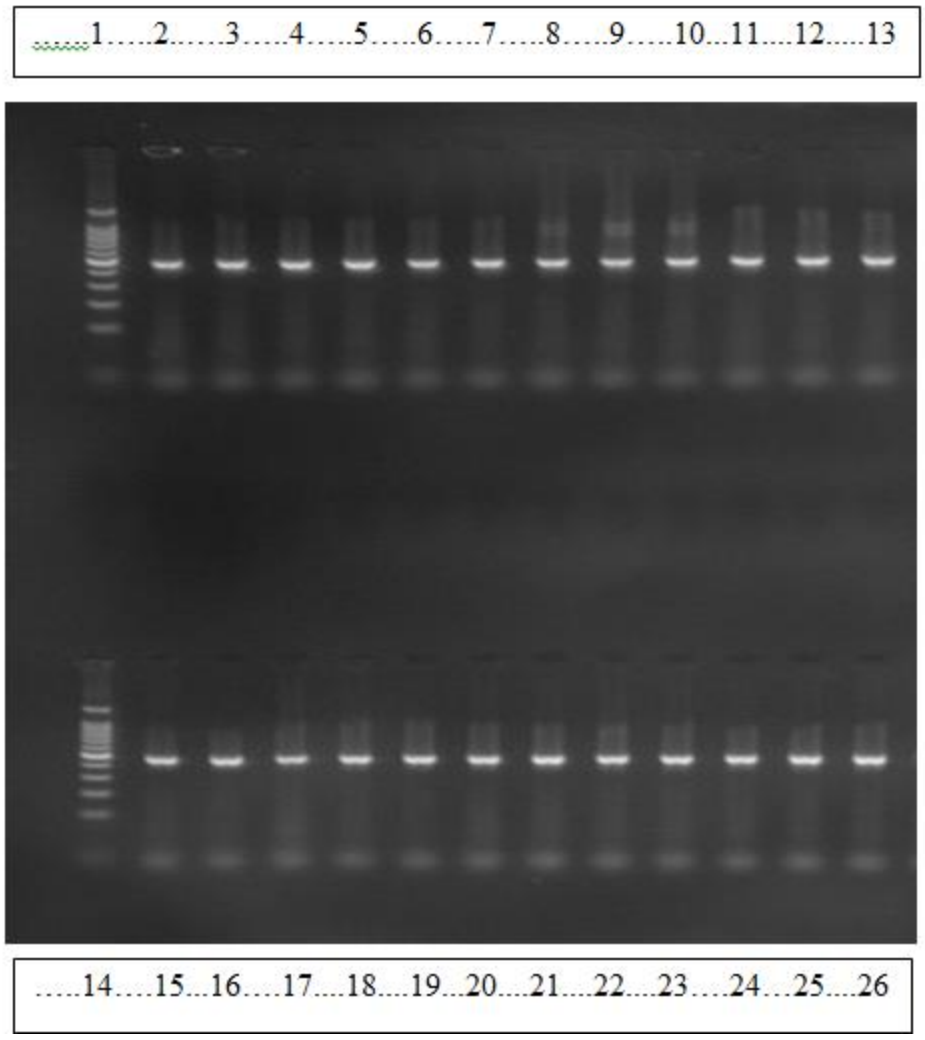

Figure 4.3 Agarose gel image of multiplex-PCR for four catfish fillets

Note: The first line was the result for detection kits and the second line was the result for modified FDA-BAM method. Bands 2-4 and 15-17 were the results for sample NO. 1, bands 5-7 and 18-20 were the results for sample NO. 2, bands 8-10 and 21-23 were the results for the sample NO. 3, bands 11-13 and 24-26 were the results for the sample NO. 4. Bands 1 and 14 were DNA ladders. Bands 2-13 and 14-26 yielded around 0.48 kb were regarded as *L. grayi.*

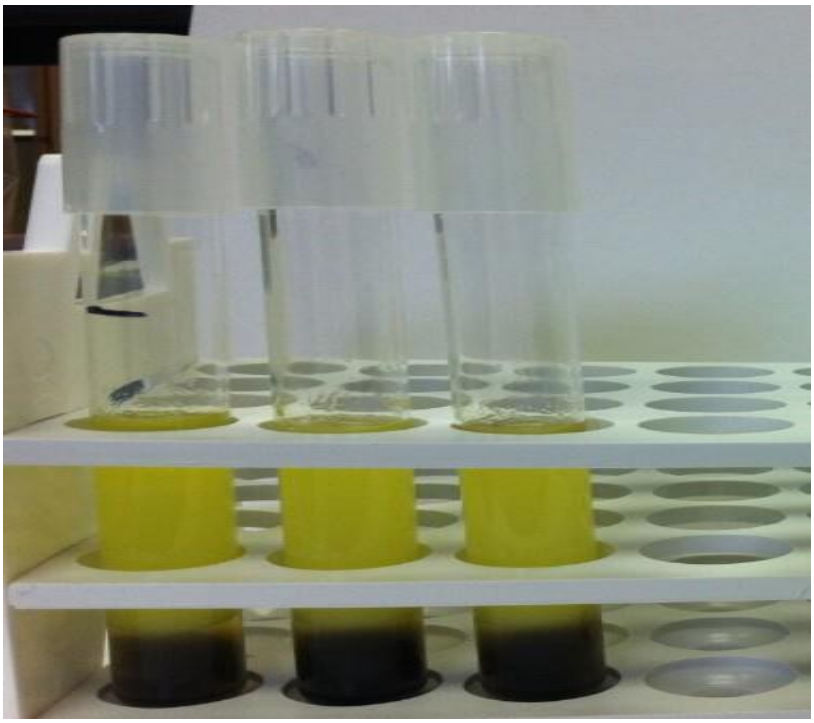

Figure 4.4 MPN enumeration results with detection kits on the catfish fillets

CHAPTER V

SUMMARY AND CONCLUSION

Current detection of *Listeria spp.* is still based on the conventional standard methods which required multi-enrichment steps with the various selective medium. This process always takes more than 72h and causes a high false positive rate due to poor selectivity of the selective media such as Oxford agar. However, the novel detection kit showed no false positive rate and high sensitivity with 24 h detection time. Sensitivity and specificity can be used for indicators to measure reliability of the test kit. According to the Table 5.1, the detection kit showed both 100% sensitivity on catfish fillet and vegetable/salad and 100% specificity (vegetable/salad). Even though the modified FDA-BAM method in this study also presented 100% sensitivity on catfish fillets, specificity on vegetables was only 60% with modified FDA-BAM method.

The single tube *Listeria* detection kit is also designed as a field applicable tool with minimum instrument such as a portable incubator. Besides the detection, the detection kit can be applied for cell enumeration with MPN method (Table 5.2). According to the statistical analysis, there is no significant difference ($P<0.05$) on the MPN enumeration between the detection kit and modified FDA-BAM study in this study.

In conclusion, the singe tube *Listeria* detection kit was designed for not only detecting *Listeria spp*. but also enumerating cells contaminated in various food samples.

Table 5.1 Comparison between the detection kit and modified FDA-BAM method

Test method	ID level	Principle	Sensitivity	Specificity	Approx. test time
Detection kit	*Listeria spp.*	Single tube detection	100%[a]	100%[b]	24 hours
Modified FDA-BAM	*Listeria spp.*	multiple selective steps with multiple selective mediums	100%[c]	60%[d]	72 hours

Note: 100% sensitivity for vegetable/salad and catfish fillet samples with detection kit.
100% specificity for vegetable/salad samples with detection kit.
100% sensitivity for catfish fillet samples with modified FDA-BAM method.
60% specificity for vegetable/salad sample with modified FDA-BAM method.

Table 5.2 Comparison between the commercially test kits and single tube detection kit

Test	ID level	Principle	Approx. test time	Main use
Gene Trak *Listeria* Assay	*Listeria spp.*	Nucleic acid hybridization probe	50 hours	Screening
Listeria Tek	*Listeria spp.*	ELISA	50 hours	Screening
TECRA *Listeria* Visual Immunoassay (TLVIA)	*Listeria spp.*	ELISA	50 hours	Screening
Assurance *Listeria* EIA	*Listeria spp.*	ELISA	50 hours	Screening
VIP *Listeria*	*Listeria spp.*	Immunochromatography	50 hours	Screening
VIDAS *Listeria*	*Listeria spp.*	ELISA	50 hours	Screening
Detection kit	*Listeria spp.*	Single tube detection with novel components	24 hours	Screening & Enumeration

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