THESIS

DEVELOPMENT OF SINGLE PRIMER-BASED POLYMERASE CHAIN REACTION COMBINED WITH ETHYDIUM BROMIDE MONOAZIDE FOR RELIABLE DETECTION OF VIABLE *SALMONELLA* SPP. IN FOODS

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THESIS

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SOITHONG SAIYUDTHONG

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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The main objective of this research was to develop the single primer-based random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) combined with ethydium bromide monoazide (EMA) for reliable detection of only viable *Salmonella* spp. in food samples. The results indicated that RAPD-PCR using 20-mer oligonucleotide primer (primer 3) could produce the specific 770-bp DNA of all 80 *Salmonella* strains. No 770-bp DNA band was amplified from any DNA sample of 20 non-*Salmonella* bacteria. The DNA band had high specificity and consistency for the detection of various *Salmonella* serotypes. For the stress conditions, this method could detect stressed *Salmonella* cells from various treatments, *in vitro* and processed foods such as chilled, frozen and fermented pork and chicken Nham with the same positive signal of 770-bp DNA band as the non-stressed cells. In addition, primer 3-based RAPD-PCR combined with ethidium bromide monoazide (EMA-RAPD-PCR) was developed for the discrimination of viable *Salmonella* cells from dead cells. The optimum amount of EMA, light exposure time and distance were 3 μg/ml, 5 min and 20 cm, respectively. The detection limit was not less than 1.3×10^3 viable cells and EMA-RAPD-PCR could inhibit the DNA amplification of 1.3×10^6 dead cells. The obtained results of the detection of *Salmonella* contaminated in all tested chicken products from fresh market and supermarket by EMA-RAPD-PCR were identical to the conventional method. The developed EMA-RAPD-PCR had potential to detect only viable *Salmonella* contaminated in the food samples providing the advantages of rapidity, reliability, high sensitivity and specificity.

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Soithong Saiyudthong
May 2013

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LIST OF ABBREVIATIONS

bp	=	Base pair
°C	=	Degree celcius
CFU/g	=	Colony forming unit/gram
CFU/ml	=	Colony forming unit/milliliter
DNA	=	Deoxyribonucleic acid
dNTP	=	Deoxynucleotide triphosphate
g	=	Gram
h	=	Hour
kb	=	Kilobase
l	=	Liter
Mb	=	Megabase
min	=	Minute
ml	=	Milliliter
mM	=	Millimolar
µl	=	Microliter
ng	=	Nanogram
nm	=	Nanometer
nM	=	Nanomolar
ppm	=	Part per million
rpm	=	Revolutions per minute
rRNA	=	Ribosomal ribonucleic acid
s	=	Second
UV	=	Ultraviolet
v/cm	=	Volt/centrimetre
v/v	=	Volume by volume
W	=	Watt
w/v	=	Weight by volume

DEVELOPMENT OF SINGLE PRIMER-BASED POLYMERASE CHAIN REACTION COMBINED WITH ETHYDIUM BROMIDE MONOAZIDE FOR RELIABLE DETECTION OF VIABLE *SALMONELLA* SPP. IN FOODS

INTRODUCTION

Salmonella enterica is one of the major foodborne pathogenic bacteria causing human gastroenteritis outbreaks worldwide (Cardinale *et al.,* 2005). Animal meats and their products, particularly eggs are considered as the major sources of transmission to human. Sanitary barriers on exports demand the control of contamination of chicken meat by such pathogenic bacterium. In recent years, according to the *Salmonella* contaminated food recalls, *Salmonella* was found in eggs, ground beef, ground tuna and peanut butter (Ellingson *et al*., 2004; WHO, 2009). Furthermore, the incidence of foodborne outbreaks caused by the *Salmonella* contamination of fresh fruits and vegetables has increased and become a great concern in industrialized countries. Outbreaks of salmonellosis have been linked to a wide variety of fresh fruits and vegetables including apple, cantaloupe, alfalfa sprout, mango, lettuce, cilantro, unpasteurized orange juice, tomato, melon, celery and parsley (Pui *et al*., 2011). Up to present, the problems of various food products contaminated with *Salmonella* spp. have been reported worldwide even in the developed contry such as, USA.

For the epidermiological data of salmonellosis, typhoid cases are stable with low numbers in developed countries, but nontyphoidal salmonellosis has increased worldwide. Typhoid fever usually causes mortality in 5 to 30% of typhoid-infected individual in the developing world. The World Health Organization (WHO) estimates 16 to 17 million cases occur annually, resulting in about 600,000 deaths. The mortality rates differ from region to region, but can be as high as 5 to 7% despite the use of appropriate antibiotic treatment. On the other hand, nontyphoidal cases account for 1.3 billion cases with 3 million deaths. In USA, approximately 2 to 4 million

cases of *Salmonella* gastroenteritis occur with about 500 deaths per year. A more accurate figure of salmonellosis is difficult to determine because normally only large outbreaks are investigated whereas sporadic cases are under-reported. Data on salmonellosis are scarce in many countries of Asia, Africa and South and Central America where only 1 to 10% of cases are reported (Pui *et al*., 2011). In Thailand, *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis have been the predominant serovars isolated from cases of food poisoning. WHO National *Salmonella* and *Shigella* Center reported the incidence of *S.* Enteritidis, which had dramatically increased since 1990. The average numbers of *S.* Enteritidis isolated from human patient samples were 0.70 ± 0.41% of the total *Salmonella* reported during 1972-1989 and then increased to 1.33, 2.98, 9.54 and 16.98% in 1990, 1991, 1992 and January-June 1993, respectively (Bangtrakulnonth *et al.*, 1993). During 2000-2003, the epidemiology of *Salmonella* spp. from chicken was processed. The numbers of positive samples of *Salmonella* in the chicken products from the farm, slaughterhouse and market were 4, 9 and 57%, respectively (Padungtod and Kaneene, 2006). Recently, the prevalences of foodborne pathogens in open markets and supermarkets studied from June 2006 to July 2007 were reported (Minami *et al*., 2010). *Salmonella* spp. were found in 22 of 61 (36%) open market samples and in 12 of 75 (16%) samples from supermarkets which were 57, 12, 24 and 0% from chicken, pork, beef and shrimp respectively. According to the importance of *Salmonella* as the causative agent of diarrheal disease, many methods have been used to trace the contaminated sources of the outbreaks for prevention and control of *Salmonella* infection (Helmuth and Schroeter, 1994; Jasson *et al*., 2010). Most microbiological laboratories isolate *Salmonella* spp. by various standard testing methods (Van der Zee, 1994), which are high sensitive in the specific detection of the bacteria as low as 1 CFU/ml. Nevertheless, such methods are relatively expensive, time consuming, and laborious. The testing procedure normally takes up to 7 days using pre-enrichment broth, selective enrichment broth and selective plating media for presumptive result of *Salmonella* spp., followed by biochemical confirmation and specific serological tests for the purpose of identification (Candrian, 1995; Omiccioli *et al*., 2009). Over the past decades, polymerase chain reaction (PCR) technique has been developed to improve the specificity and reduce the detection time (Jasson *et al*., 2010; Munoz *et*

al., 2010). However, standard PCR technique still has one crucial disadvantage that it cannot distinguish between DNAs originated from viable and dead cells. Therefore, PCR can have false positive from dead cell DNA. The presence of dead cells limits the use of PCR for microbiological detection of food samples. Many researchers have developed various techniques in order to overcome such PCR limitation. Mustapha and Li (2006) suggested the method for the presence of RNA from target microorganisms was reverse transcription PCR (RT-PCR). This technique is believed to be an effective method in order to indicate the presence of viable cells because of its relative instability after cell death. However, the researchers reported pure RNA extraction is difficult and troublesome procedure, especially when applied with food samples due to the presence of various inhibitors. In addition, pure RNA is difficult to isolate in the presence of high populations of dead cells and often contaminated with DNA from dead cells (Wang and Mustapha, 2010). Recently, a photoreactive nucleic acid dye, either ethidium bromide monoazide (EMA) or propidium monoazide (PMA) has been successfully used as a DNA-binding dye to inhibit the amplification of the dead cell DNA in the PCR reaction (Nogva *et al*., 2003; Rudi *et al*., 2005b; Pan and Breidt, 2007; Cawthorn and Witthuhn, 2008; Bae and Wuertz, 2009; Delgado–Viscogliosi *et al*., 2009; Nocker and Camper, 2009; Agusti *et al*., 2010; Wang and Mustapha, 2010; Soejima *et al*., 2011). However, the PCR combinded with nucleic acid dye still need crucial consideration such as, required high throughput technology (real-time PCR) with cost consumption, and affected by disinfection and environment conditions.

This research aimed to develop the efficiency of primer 3-based random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) combined with EMA for reliable detection of only viable *Salmonella* spp. in food samples. The specific objectives were divided into 3 parts: to study the consistency of primer 3-based RAPD-PCR for detection of various *Salmonella* serotypes, to study the efficiency of primer 3-based RAPD-PCR for detection of stressed *Salmonella* spp. in various stress treatments *in vitro* and food process models and to develop primer 3-based RAPD-PCR combined with EMA to discriminate viable *Salmonella* cells from dead cells for *Salmonella* detection in foods.

OBJECTIVES

The main objective of this research was to develop primer 3-based random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) combined with EMA for reliable detection of only viable *Salmonella* spp. in food samples. The results are divided into 3 parts with the specific objectives as follows.

The objective of part 1 was to study the consistency of primer 3-based RAPD-PCR for detection of various *Salmonella* serotypes.

The objective of part 2 was to study the efficiency of primer 3-based RAPD-PCR for detection of *Salmonella* spp. in various stress treatments *in vitro* and food process models.

The objective of part 3 was to develop primer 3-based RAPD-PCR combined with EMA involving cultering step for detection of only viable *Salmonella* spp. in foods.

LITERATURE REVIEW

1. Taxonomy of *Salmonella* spp.

Salmonella is a facultative anaerobe, gram negative flagellated rod-shaped bacterium which is about 2.0-3.0 × 0.4-0.6 μm in size (Yousef and Carlstrom, 2003; Montville and Matthews, 2008). It is among the most commonly isolated foodborne pathogens associated with animal meat, egg, fresh fruits and vegetables.

1.1 Classification and nomenclatures

Historically *Salmonella* had been named based on the original places of isolation such as *S.* London and *S.* Indiana. This nomenclature system was replaced by the classification based on the susceptibility of isolates to different selected bacteriophages which is also known as phage typing (Bhunia, 2008). Phage typing is generally employed when the origin and characteristic of an outbreak must be determined by differentiating the isolates of the same serotype. It is very reproducible when international standard sets of typing phages are used. More than 200 definitive phage types (DT) have been reported so far. For example, *S.* Typhimurium DT104 designates a particular phage type for Typhimurium isolates (Hanes, 2003; Andrews and Baumler, 2005). Epidemiologic classification of *Salmonella* is based on the host preferences. The first group includes host-restricted serotypes that infect only humans such as *S.* Typhi. The second group includes host-adapted serotypes which are associated with one host species but can cause disease in other hosts serotypes such as *S.* Pullorum in avian. The third group includes the remaining serotypes. Typically, *S.* Enteritidis, *S.* Typhimurium and *S.* Heidelberg are the three most frequent serotypes recovered from humans each year (Gray and Fedorka-Cray, 2002; Boyen *et al*., 2008). Kauffmann-White scheme classifies *Salmonella* according to three major antigenic determinants composed of flagellar H antigens, somatic O antigens and virulence (Vi) capsular K antigens. This was adopted by the International Association of Microbiologists in 1934. Agglutination by antibodies specific for the various O antigens is employed to group salmonellae into the 6 serogroups: A, B, C1, C2, D and

E. For instance, *S.* Paratyphi A, B, C and *S.* Typhi express O antigens of serogroups A, B, C1 and D, respectively. More than 99% of *Salmonella* strains causing human infections belong to *Salmonella enterica* subspecies *enterica* (*S. enterica* subspecies *enterica*). Although not common, cross-reactivity between O antigens of *Salmonella* and other genera of *Enterobacteriaceae* do occur. Therefore, further classification of serotypes is based on the antigenicity of the flagellar H antigens which are highly specific for *Salmonella*. In brief, O antigens are lipopolysaccharide (LPS) of the outer bacterial membrane. They are heat stable, resistant to alcohol and dilute acids. H antigens are heat-labile proteins associated with the peritrichous flagella and can be expressed in one of two phases. The phase 1 H antigens are specific and associated with the immunological identity of the particular serovars whereas phase 2 antigens are non-specific antigens containing different antigenic subunit proteins which can be shared by many serovars. K antigens which are heat-sensitive carbohydrates are produced by *Salmonella* serovars that express a surface-bound polysaccharide capsular antigen (Hu and Kopecko, 2003; Yousef and Carlstrom, 2003). Bacteria can be classified based on phylogeny. A phylogenetic tree can be derived from the comparison with 16S rRNA or other gene sequences. There are 2463 *Salmonella* serotypes which are now placed under 2 species due to the difference in 16S rRNA sequence analysis: *S. enterica* (2443 serotypes) and *S. bongori* (20 serotypes). The system is currently used by World Health Organization (WHO) Collaborating Centre, Centers for Disease Control and Prevention (CDC) and some other organizations. *S. enterica* is further divided into six subspecies, which are designated by roman numerals. *S. enterica* subspecies I are mainly isolated from warm-blooded animals and accounts for more than 99% of clinical isolates whereas remaining subspecies and *S. bongori* are mainly isolated from cold-blooded animals and account for less than 1% of clinical isolates. As an example, the Kauffmann species *S.* Typhimurium is now designated as *S. enterica* subspecies I serotype Typhimurium. Under the modern nomenclature system, the subspecies information is often omitted and culture is called *S. enterica* serotype Typhimurium and in subsequent appearance, it is written as *S.* Typhimurium. This system of nomenclature is used nowadays to bring uniformity in reporting (Andrews and Baumler, 2005; Parry, 2006; Bhunia, 2008).

1.2 Characteristic

Salmonellae are non-fastidious as they can multiply under various environmental conditions outside the living hosts. They do not require sodium chloride for growth, but can grow in the presence of 0.4 to 4%. Most *Salmonella* serotypes grow at temperature range of 5 to 47°C with optimum temperature of 35 to 37°C but some can grow at temperature as low as 2 to 4°C or as high as 54°C (Gray and Fedorka-Cray, 2002). They are sensitive to heat and often killed at temperature of 70°C or above. Salmonellae grow in a pH range of 4 to 9 with the optimum between 6.5 and 7.5. They require high water activity (a_w) between 0.99 and 0.94 (pure water a_w=1.0) yet can survive at a_w <0.2 such as in dried foods. Complete inhibition of growth occurs at temperatures <5°C, pH <3.8 or water activity <0.94 (Hanes, 2003; Bhunia, 2008).

2. Clinical manifestation and pathogenesis

In human disease, the clinical pattern of salmonellosis can be divided into four disease patterns namely enteric fever, gastroenteritis, bacteremia and chronic carrier state.

2.1 Enteric fever

S. Typhi causes typhoid fever whereas *S.* Paratyphi A, B and C cause paratyphoid fever with symptoms which are milder and a mortality rate that is lower for the latter. Both serotypes are solely human pathogens. Infection typically occurs due to ingestion of food or water contaminated with human waste. In recent years, antibiotic-resistant strains have been isolated in most endemic areas, particularly Southeast Asia, India, Pakistan and Middle East. Roughly 10% of patients may relapse, die or encounter serious complications such as typhoid encephalopathy, gastrointestinal bleeding and intestinal perforation. Relapse is the most common occurrence probably due to persisting organisms within reticuloendothelial system (RES). Typhoid encephalopathy, often accompanied by shock, is associated with

high mortality. Slight gastrointestinal bleeding can be resolved without blood transfusion but in 1 to 2% of cases can be fatal if a large vessel is involved. Intestinal perforation may present with abdominal pain, rising pulse and falling blood pressure in sick people. Hence, it is very serious in 1 to 3% of hospitalized patients (Bell and Kyriakides, 2002; Hu and Kopecko, 2003; Parry, 2006).

2.2 Gastroenteritis

Nontyphoidal salmonellosis or enterocolitis is caused by at least 150 *Salmonella* serotypes with *S.* Typhimurium and *S.* Enteritidis being the most common serotypes in the United States. Infection always occurs via ingestion of water or food contaminated with animal waste rather than human waste. The emergence of multidrug-resistant *S.* Typhimurium DT104 has been associated with outbreaks related to beef contamination and resulted in hospitalization rates twice than that of other foodborne salmonellosis (Gray and Fedorka-Cray, 2002; Yousef and Carlstrom, 2003). Ciprofloxacin is often administered at the first sign of severe gastroenteritis whereas ceftriaxone is given to children with systemic salmonellosis. In production animals like swine, treatment is usually contraindicated but, when necessary, can be given via injection with several treatment alternatives based on considerations such as withdrawal time. Antibiotic treatment is usually not advised except for rare cases because it can prolong the presence of bacteria in the stool (Gray and Fedorka-Cray, 2002; Yousef and Carlstrom, 2003).

2.3 Bacteremia

About 8% of the untreated cases of salmonellosis result in bacteremia. Bacteremia is a serious condition in which bacteria enter the bloodstream after passing through the intestinal barrier. It has been associated with highly invasive serotypes like Cholearaesuis or Dublin. Bacteremia caused by *Salmonella* should be taken into account in cases of fever of unknown origin. Patients with bacteremia and other complications should be treated with antibiotics (Hanes, 2003).

2.4 Chronic carrier state

Salmonellosis can be spread by chronic carriers who potentially infect many individuals, especially those who work in food-related industries. Factors contributing to the chronic carrier state have not been fully explained. On average, nontyphoidal serotypes persist in the gastrointestinal tract from 6 weeks to 3 months, depending on the serotypes. Only about 0.1% of nontyphoidal *Salmonella* cases are shed in stool samples for periods exceeding 1 year. About 2 to 5% of untreated typhoid infections result in a chronic carrier state. Up to 10% of untreated convalescent typhoid cases will excrete *S.* Typhi in feces for 1 to 3 months and between 1 and 4% become chronic carriers excreting the microorganism for more than one year (Parry, 2006).

The pathogenesis of Salmonellosis includes several syndromes (gastroenteritis, enteric fevers, septicemia, focal infections, and an asymptomatic carrier state). Particular serovars show a strong propensity to produce a particular syndrome (*S.* Typhi, *S.* Paratyphi-A, and *S.* Schottmuelleri produce enteric fever; *S.* Choleraesuis produces septicemia or focal infections; *S.* Typhimurium and *S.* Enteritidis produce gastroenteritis).

Most non-typhoidal salmonellae enter the body when contaminated food is ingested (Figure 1). After ingestion, the organisms colonize the ileum and colon, invade the intestinal epithelium, and proliferate within the epithelium and lymphoid follicles. The mechanism by which salmonellae invade the epithelium is partially understood and involves an initial binding to specific receptors on the epithelial cell surface followed by invasion. After invading the intestine, most salmonellae induce an acute inflammatory response, which can cause ulceration. They may elaborate cytotoxins that inhibit protein synthesis. Only strains that penetrate the intestinal mucosa are associated with the appearance of an acute inflammatory reaction and diarrhea; the diarrhea is due to secretion of fluid and electrolytes by the small and large intestines. Invasion of the intestinal mucosa is followed by activation of mucosal adenylate cyclase; the resultant increase in cyclic AMP induces secretion.

The mechanism by which adenylate cyclase is stimulated is not understood; it may involve local production of prostaglandins or other components of the inflammatory reaction. In addition, *Salmonella* strains elaborate one or more enterotoxin-like substances which may stimulate intestinal secretion. However, the precise role of these toxins in the pathogenesis of *Salmonella* enterocolitis and diarrhea has not been established (Giannella, 1981).

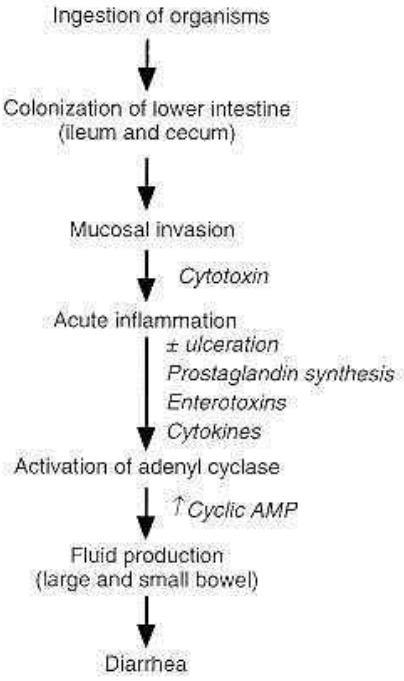

Figure 1 Scheme of the pathogenesis of *Salmonella* enterocolitis and diarrhea.

Source: Giannella (1981)

Person-to-person spread of salmonellae also occurs. To be fully pathogenic, salmonellae must possess a variety of attributes called virulence factors. These include the ability to invade cells, a complete lipopolysaccharide coat, the ability to replicate intracellularly, and possibly the elaboration of toxin. Invasion occurs by the organism inducing the enterocyte membrane to undergo "ruffling" and thereby to stimulate pinocytosis of the organisms (Figure 2). Invasion is dependent on rearrangement of the cell cytoskeleton and probably involves increases in cellular

inositol phosphate and calcium. Attachment and invasion are under distinct genetic control and involve multiple genes in both chromosomes and plasmids.

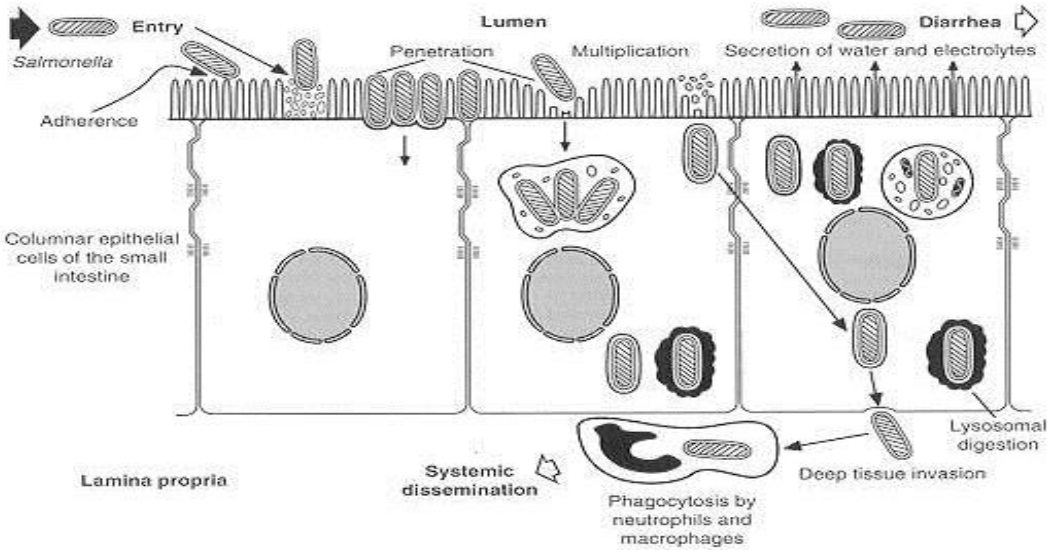

Figure 2 Invasion of intestinal mucosa by *Salmonella*.

Source: Giannella (1981)

After invading the epithelium, salmonellae multiply intracellularly and then spread to mesenteric lymph nodes and throughout the body via the systemic circulation; they are taken up by the reticuloendothelial cells. The reticuloendothelial system confines and controls spread of the organism. However, depending on the serotype and the effectiveness of the host defenses against that serotype, some organisms may infect the liver, spleen, gallbladder, bones, meninges, and other organs (Figure 3).

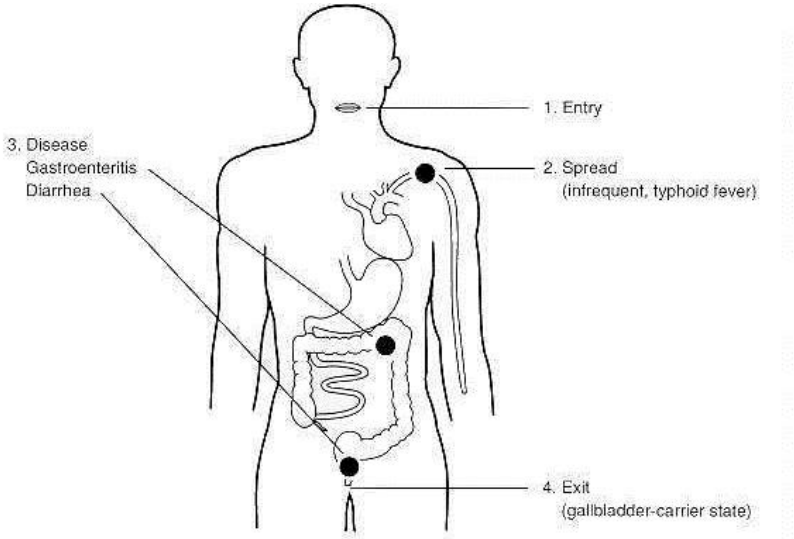

Figure 3 Pathogenesis of salmonellosis in various organs.

Source: Giannella (1981)

Most serovars are killed promptly in extraintestinal sites, and the most common human *Salmonella* infection, gastroenteritis remains confined to the intestine. Only strains that penetrate the intestinal mucosa are associated with the appearance of an acute inflammatory reaction and diarrhea (Figure 4).

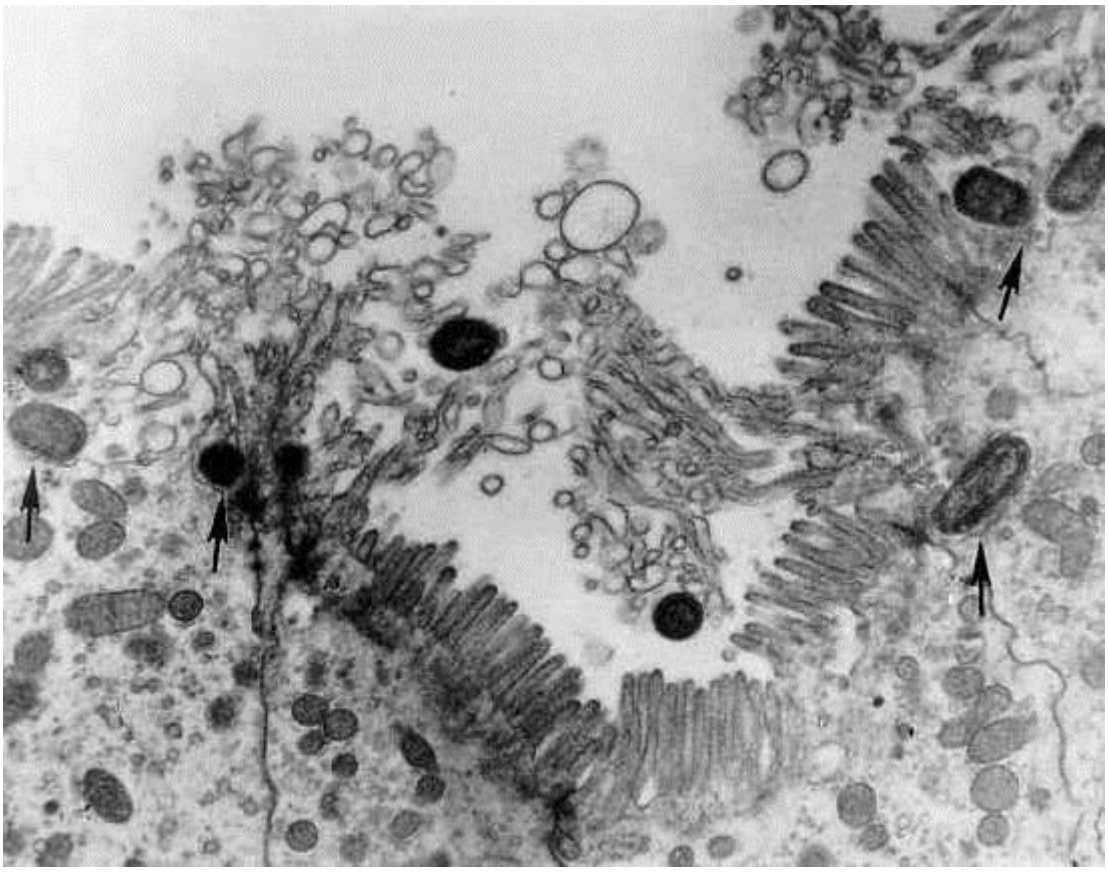

Figure 4 Electron photomicrograph demonstrating invasion of guinea pig ileal epithelial cells by *S.* Typhimurium (Arrows point to invading *Salmonella* organisms).

Source: Giannella (1981)

For the virulence properties, *Salmonella* strains possess an invasion gene (*inv*A), which encodes proteins for adherence and invasion. Therefore, *Salmonella* can penetrate the gut lumen into the epithelium cells of host small intestine (Galan *et al.*, 1992). Upon internalization, *Salmonella* enters enterocytes, M cells, and dendritic cells in the intestinal epithelium and subsequently reaches to the submucosa by resident macrophages. Immediately, *Salmonella* spreads through the blood stream and accumulates in mesenteric lymph nodes and spleen, causing inflammation which leads to salmonellosis (Salcedo *et al.*, 2001). *Salmonella* can also produce enterotoxins and cytotoxins in the host intestinal tracts, but these toxins seem to have

only minor effects on the infection (Jay *et al.*, 2005). Therefore, *Salmonella* causes typical foodborne infection rather than intoxication.

3. Epidemiology and transmission of salmonellosis

3.1 Epidemiology

Typhoid cases are stable with low numbers in developed countries, but nontyphoidal salmonellosis has increased worldwide. Some of the incidence, notification and isolation rate of salmonellosis in different part of the world is shown in Figure 5.

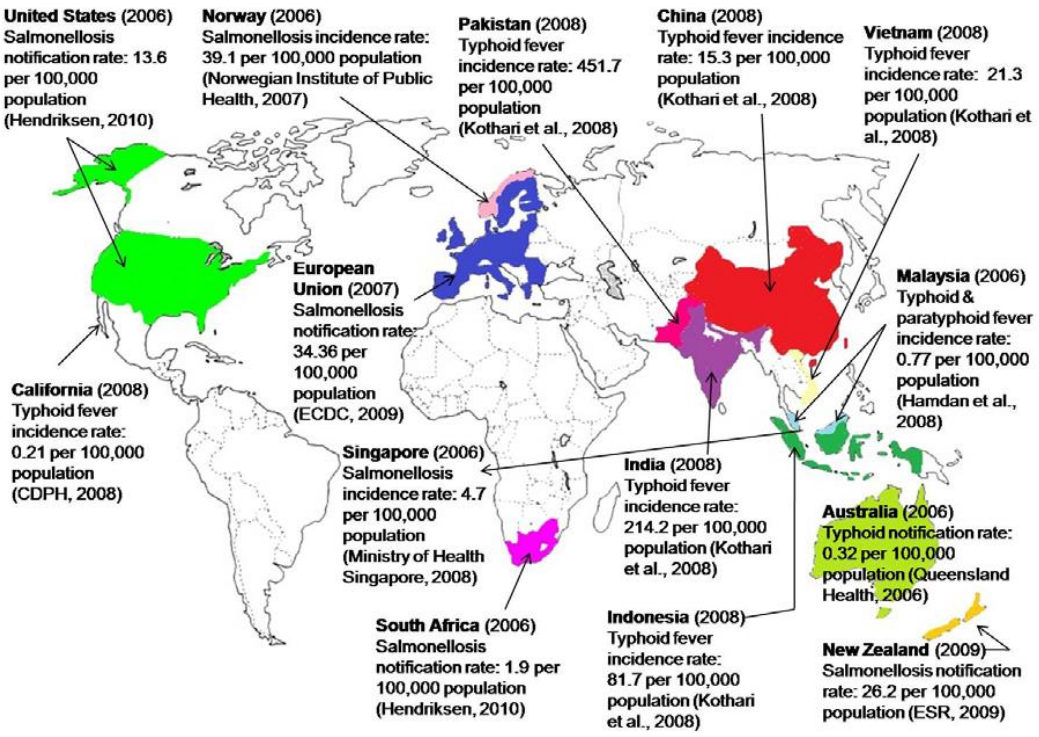

Figure 5 Some of the incidence and notification rate of enteric fever and salmonellosis in different parts of the world.

Source: Pui *et al*. (2011)

Typhoid fever usually causes mortality in 5 to 30% of typhoid-infected individual in the developing world. The World Health Organization (WHO) estimates 16 to 17 million cases occur annually, resulting in about 600,000 deaths. The mortality rates differ from region to region, but can be as high as 5 to 7% despite the use of appropriate antibiotic treatment. Typhoid fever is endemic throughout Africa and Asia as well as persists in the Middle East, some eastern and southern European countries and central and South America. In the USA and most of Europe, typhoid is predominantly a disease of the returning traveler. Typhoid incidence in endemic areas is typically low in the first few years of life, peaking in school-aged children and young adults and then falling in middle age. Most infections occur in childhood especially in Mekong Delta region of Vietnam and are recognizable although often mild. The most famous outbreak of enteric fever is Typhoid Mary. Mary Mallon, a New York City hired household cook, transmitted typhoid fever to at least 22 individuals causing 3 deaths between 1900 and 1907. After being apprehended by public health officials in 1907, she was isolated for 3 years. Even though, she was released with the stipulation that she had never cooked again, she broke the promise and consequently caused at least 25 more cases of typhoid fever at Manhattan maternity hospital when she was employed as a cook in 1915. She was finally isolated until her death in 1938 (Parry, 2006).

On the other hand, nontyphoidal cases account for 1.3 billion cases with 3 million deaths. In the United States, approximately 2 to 4 million cases of *Salmonella* gastroenteritis occur with about 500 deaths per year. A more accurate figure of salmonellosis is difficult to determine because normally only large outbreaks are investigated whereas sporadic cases are under-reported. Data on salmonellosis are scarce in many countries of Asia, Africa and South and Central America where only 1 to 10% of cases are reported (Portillo, 2000; Wong *et al*., 2002; Hanes, 2003; Hu and Kopecko, 2003). *S.* Enteritidis is one of the predominant serotype caused salmonellosis outbreaks worldwild.

In the USA, *S.* Enteritidis steadily increased in frequency from being the sixth most common serotype in 1963 to becoming the most frequently reported

serotype in 1990. Epidemics in the USA are marked by regional differences. *S.* Enteritidis emerged in 1979 in New England and the Mid-Atlantic regions. In the early 1990s, while *S.* Enteritidis rates of infection in the Northeast began to decline, the *S.* Enteritidis epidemic expanded to the Pacific region. Nationwide, the number of isolated *S.* Enteritidis increased from 1.0 per 100,000 of the population in 1974 to 3.8 per 100,000 of the population in 1994 and declined to 2.0 per 100,000 of the population in 1998 until 2001 (WHO, 2005).

In England and Wales, there were 200 reported human cases in 1966, which rose to 10,000 in 1981, and peaked at 33,000 in 1997 (more than 70% of human cases of salmonellosis). Despite a subsequent decline in its incidence, *S.* Enteritidis continues to be the most frequently isolated *Salmonella* serotype in the United Kingdom with 16,465 cases in 2001 (Cogan and Humphrey, 2003).

In France, *S.* Enteritidis has become the most common isolated serotype in 1993. The incidence of *S.* Enteritidis human isolates increased exponentially from 1987 to peak at 6,500 in 1994 and 1997 and subsequently declined to 4,500 cases in 1999. Similar trends have also been reported from other countries in South America and Europe (Cogan and Humphrey, 2003; Rabsch *et al*., 2001).

In Thailand, *S.* Enteritidis has also been the predominant serovar isolated from cases of food poisoning. WHO National *Salmonella* and *Shigella* Center reported the incidence of *S.* Enteritidis which has been dramatically increased since 1990. The average *S.* Enteritidis isolates from human patient samples were 0.70±0.41% of the total reported *Salmonella* isolates during 1972-1989 and increased to 1.33, 2.98, 9.54 and 16.98% in 1990, 1991, 1992 and January-June 1993, respectively. The available data had suggested it is related to the increasing of *S.* Enteritidis isolates from chicken and chicken meat rather than eggs and other sources (Bangtrakulnonth *et al.,* 1993). Sakai and Chalermchaikit (1996) reported that the percentages of *S.* Enteritidis isolated from human patients and animal samples during 1972-1989 were only 0.59% and 0.32%, respectively. Furthermore, *S.* Enteritidis

isolates from human foods and chicken meat in Thailand were also increased from 1990 to 1993. So the *S.* Enteritidis isolates have emerged in Thailand since 1990.

3.2 Transmission vehicles

Salmonella is widely distributed in nature and they survive well in a variety of foods. Poultry, eggs and dairy products are the most common vehicles of salmonellosis. In recent years, fresh produce like fruits and vegetables have gained concern as vehicles of transmission where contamination can occur at multiple steps along the food chain (Bouchrif *et al*., 2009). First, environment contaminated with *Salmonella* serves as the infection source because *Salmonella* can survive in the environment for a long time. After that, *Salmonella* is transmitted to vectors such as rats, flies and birds where *Salmonella* can be shed in their faeces for weeks and even months. Following the direct transmission, moving animals such as swines, cows and chickens act as the important risk factor for infection. These animal reservoirs are infected orally because *Salmonella* normally originates from the contaminated environment and also contaminated feed. Human get infected when eating the food or drinking the water that is contaminated with *Salmonella* through animal reservoirs. However, *S.* Typhi and *S.* Paratyphi A do not have animal reservoir; therefore, infection can be happened by eating the improperly handled food by infected individuals (Newell *et al*., 2010). Besides, transmission of *Salmonella* to the food processing plants and equipments for food preparation are also of great importance. Once carried by vectors or transferred to food, consumption by human can result in the risk of salmonellosis. The *Salmonella* cells can attach to food contact surfaces such as plastic cutting board which may develop into biofilm once attached and hence cause cross-contamination. Consequently, *Salmonella* can enter the food chain at any point from livestock feed, through food manufacturing, processing and retailing as well as catering and food preparation in the home (Wong *et al*., 2002; Omwandho and Kubota, 2010). The infectious dose of *Salmonella* depends upon the serovar, bacteria strain, growth condition and host susceptibility. On the other hand, host factors controlling susceptibility to infection include the condition of the intestinal tract, age and underlying illnesses or immune deficiencies. The infectious dose of *Salmonella*

is broad varying from 1 to 10^9 CFU/g. However, single-food-source outbreaks indicate that as little as 1 to 10 cells can cause salmonellosis with more susceptibility to infection. (Yousef and Carlstrom, 2003; Bhunia, 2008).

3.3 Antibiotic resistant *Salmonella*

The resistance of *Salmonella* to a single antibiotic was first reported in the early 1960s (Montville and Matthews, 2008). Since then, the isolation frequency of *Salmonella* strains resistant to one or more antibiotics have increased in the Saudi Arabia, United States, United Kingdom and other countries of the world. This is due to the increased and uncontrolled use as well as easy accessibility to antibiotics in many countries of the world (Grob *et al*., 1998; Yoke-Kqueen *et al*., 2007). Emerging resistance in *S.* Typhi has been described especially in Africa and Asia and the appearance of *S.* Typhimurium DT104 in the late 1980s raised main public health concern, thereby threatening the lives of infected individuals (Grob *et al*., 1998; Montville and Matthews, 2008). Van *et al*. (2007) stated that multi-resistance occurred in *Salmonella* serotypes including Albany, Anatum, Havana, London and Typhimurium. The resistance towards the traditional first-line antibiotics such as ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole define multidrug resistance (MDR) in *S. enterica* (Crump and Mintz, 2010). This is of great concern because majority infections with MDR *Salmonella* are acquired through the consumption of contaminated foods of animal origin such as swines and chicken eggs Asai *et al*. (2010) mentioned that cephalosporinand fluoroquinone-resistant strains of *S.* Choleraesuis have been identified in swines in Taiwan and Thailand. Apart from that, antibiogram testing by Singh *et al.* (2010) revealed *Salmonella* isolates from chicken eggs in marketing channels and poultry farms in North India were resistant to bacitracin, colistin and polymyxin-B. Due to the use of antibiotics for the promotion of growth and prevention of disease in food animals, there is an increase of human salmonellosis cases caused by foodborne MDR *Salmonella* nowadays (Yang *et al*., 2010). This indiscriminate and injudicious use of antibiotics in any setting especially in food animals worldwide should be monitored to reduce the transfer risk of MDR *Salmonella* to humans (Zhao *et al*., 2003). Finally, there is a need of continuous

surveillance and sharing of antimicrobial susceptibility data for *Salmonella* among countries worldwide (de Oliveira *et al*., 2010) to ensure the effectiveness of control programmes. In addition, the studies of genetic and/or biochemical analysis have shown that of the nearly dozen aux mutations described so far most are in genes involved in cell wall synthesis (murE, pbp2, glmM, glnR, femA/B, llm, etc.) or in complex regulatory functions (sigma B), suggesting that optimal expression of resistance may involve the co-operative functioning of a number of genes in cell wall metabolism as well as stress response. Stresses typically elicit protective and/or adaptive responses that serve to enhance bacterial survivability. Because they impact upon many of the same cellular components and processes that are targeted by antimicrobials, adaptive stress responses can influence antimicrobial susceptibility. In targeting and interfering with key cellular processes, antimicrobials themselves are 'stressors' to which protective stress responses have also evolved. Cellular responses to nutrient limitation (nutrient stress), oxidative and nitrosative stress, cell envelope damage (envelope stress), antimicrobial exposure and other growth-compromising stresses, have all been linked to the development of antimicrobial resistance in Gram-negative bacteria - resulting from the stimulation of protective changes to cell physiology, activation of resistance mechanisms, promotion of resistant lifestyles (biofilms), and induction of resistance mutations. The stressed organisms can contaminate along food chain and environment which cause harm to consumer (Poole, 2012).

4. Foodborne *Salmonella* illnesses and outbreaks

According to CDC's FoodNet report, in 2009 *Salmonella* was responsible for 7,039 cases of laboratory confirmed foodborne infections in 10 states, accounting for more than 40% of the total laboratory-confirmed infections (CDC, 2010a). Food-related salmonellosis is mostly associated with the consumption of poultry, undercooked meat or ground beef, dairy products, eggs, and fresh produce (Bouchrif *et al*.,2009). Although poultry is historically regarded as the major cause of *Salmonella*-implicated outbreaks (Cox, 2002; Bhunia, 2008; Cox and Pavic, 2010; Newell *et al*., 2010). Furthermore, in recent years, an increasing number of

Salmonella-related outbreaks linked to fresh produce have been observed in part due to the increasing consumption of produce (Harrison, 1992; Delarocque, 1998; Mead *et al.*, 1999; Harris *et al.*, 2003). A variety of produce items including melons, tomatoes, sprouts, spinaches, and peppers have been implicated in multiple *Salmonella* outbreaks (CDC, 2005; CDC, 2008; CDC, 2009; Schnirring, 2007; Hanning *et al.*, 2009; USDA-ERS, 2009). *Salmonella* outbreaks caused by various foods in USA during 1999-2010 were shown in Table 1.

Table 1 *Salmonella* outbreaks caused by various foods in USA during 1999-2010.

Year	Food type	No. Cases	Organism
1999	Produce	121	*Salmonella* spp.
2001	Produce	80	*Salmonella* spp.
2002-2003	Produce	31	*Salmonella* spp.
2002-2003	Poultry	29	*Salmonella* spp.
2008	Fresh peppers, raw tomatoes	1,442	*S.* Saintpaul
2008-2009	Peanut butter, peanut palse	529	*S.* Typhimurium
2009-2010	Salami	272	*S.* Muenchen

Source: CDC (2010a, b)

Not all 2,500 *Salmonella* serotypes are created equal with regards to causing human infections. According to CDC's FoodNet report (CDC, 2010a), Enteritidis, Typhimurium, Newport, and Javiana are the top 4 most common serotypes to cause *Salmonella* foodborne diseases in 10 states of the USA in 2009, accounting for 55.9% of the total *Salmonella* infections. Other serotypes including Heidelberg, Montevideo, I 4,[5],12:i:-, Muenchen, Saintpaul, Oranienburg, etc. (Table 2) have also been associated with food-linked infections in human.

Table 2 Most common *Salmonella* serotypes causing salmonellosis in 2009.

Rank	*Salmonella* serotype	The number of infections reported
1	Enteritidis	1,226
2	Typhimurium	1,024
3	Javiana	772
4	Newport	544
5	Heidelberg	230
6	Montevideo	206
7	I 4, [5], 12:i:-	197
8	Muenchen	170
9	Saintpaul	157
10	Oranienburg	154

Source: CDC (2010a)

5. Detection and identification of *Salmonella* spp.

Routine detection of salmonellae involves a sequence of pre-enrichment, enrichment, selective enrichment, selective-differential plating, isolation and identification (ICMSF, 1978; ICMSF, 1996; ISO, 2002; FDA/ BAM, 2007).

Pre-enrichment may or may not be done, depending on the degree of likelihood that injured cells are present in the food. It is used when testing heated, frozen and dried foods, and foods in which cells are expected to be present in low numbers. It reduces any effect the food itself may have on the selectivity of the enrichment broth. Pre-enrichment liquid media are non-inhibitory and permit the growth of indigenous flora as well as salmonellae. Foods of high or low pH may require neutralization of the pre-enrichment broth after the food has been suspended.

Slow rehydration of some dried foods can increase the sensitivity of *Salmonella* detection.

Selective enrichment is designed to inhibit the growth of other organisms while allowing salmonellae to grow. This is accomplished by the use of inhibitory chemicals (dyes, tetrathionate, selenite), temperature and duration of incubation, permitting the proportion of salmonellae relative to other microorganisms. Incubation times are usually 16-24 h. Normally, incubation temperatures range from 35 to 43°C; incubation at 41-43°C often results in increased detection of salmonellae. Multiple enrichment media and time and temperature incubation routines are recommended.

The presence of salmonellae is determined by plating samples of enrichment broths on selective plating media. Commonly used selective plating media include brilliant green with or without sulphadiazine or sulphapyridine, xylose lysine deoxycholate, bismuth sulphite, Hektoen enteric agar, MacConkey, deoxycholate citrate and *Salmonella-Shigella* agars. Two or more of these should be used in standard cultural methods, in view of the physiological diversity of the *Salmonella* group and the mixed flora present in foods.

Further identification can be done by biochemical testing and serological test of pure cultures, which can be time-consuming (Brenner,1984; Ewing, 1986). Lysine, urease and indole tests are frequently used for initial screening. Commercial kits such as Micro ID, Minitek, API 20E, Enterotube II and Vitek for identifying biochemical characteristics are available.

The conventional methods include International Commission on Microbiological Specifications for Foods (ICMSF, 1996), International Organization for Standardization (ISO 6579: 2002) (ISO, 2002) and Food and Drug Administration's Bacteriological Analytical Manual (FDA/ BAM, 2007) are shown as Figure 6, 7 and 8.

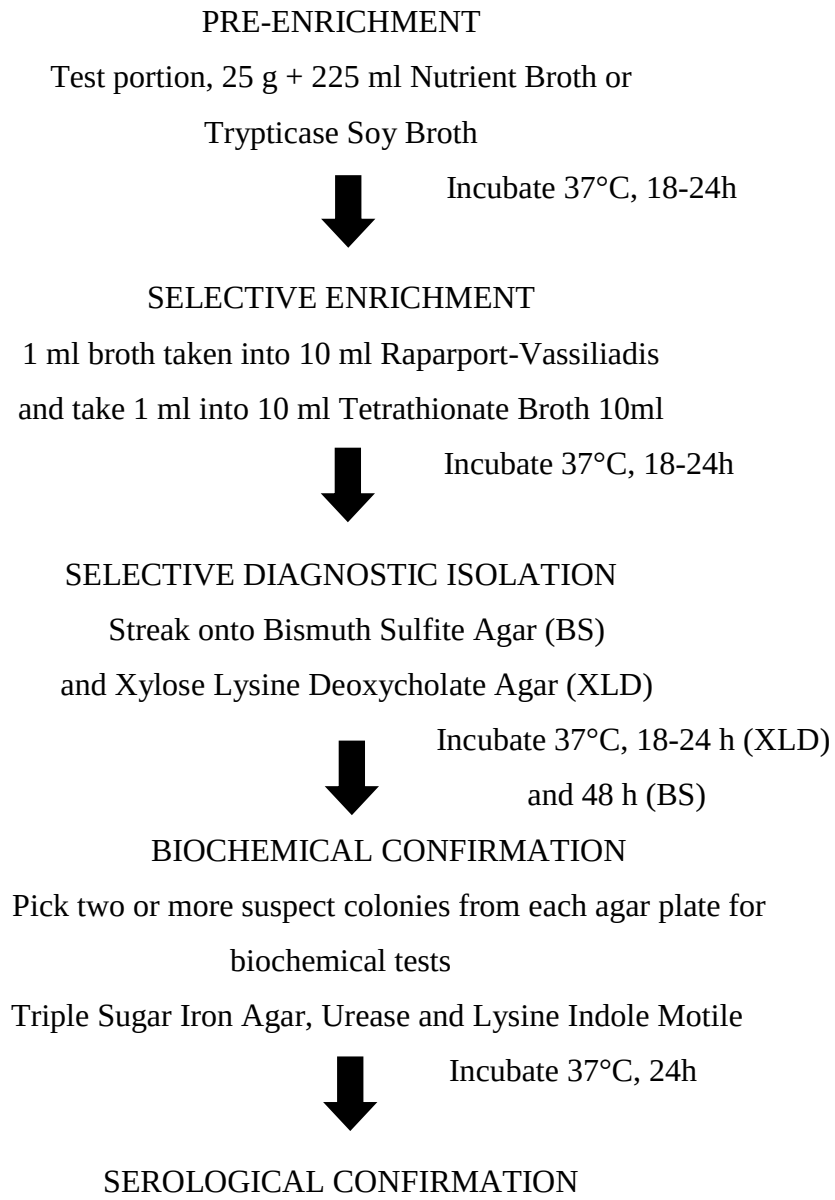

Figure 6 ISO 6579: 2002 *Salmonella* isolation procedure**.**

Source: ISO (2002)

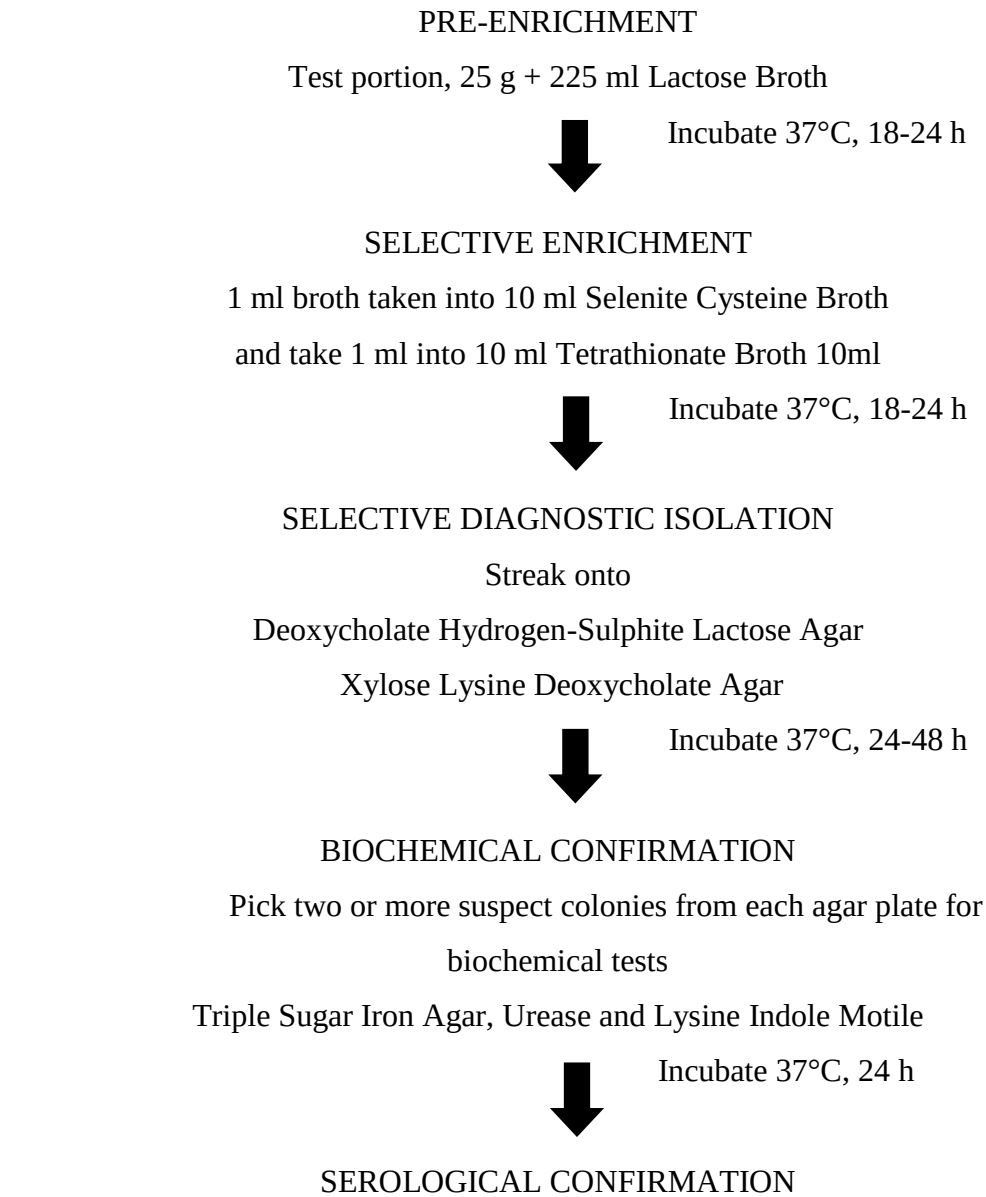

Figure 7 FDA/ BAM *Salmonella* isolation procedure.

Source: FDA/ BAM (2007)

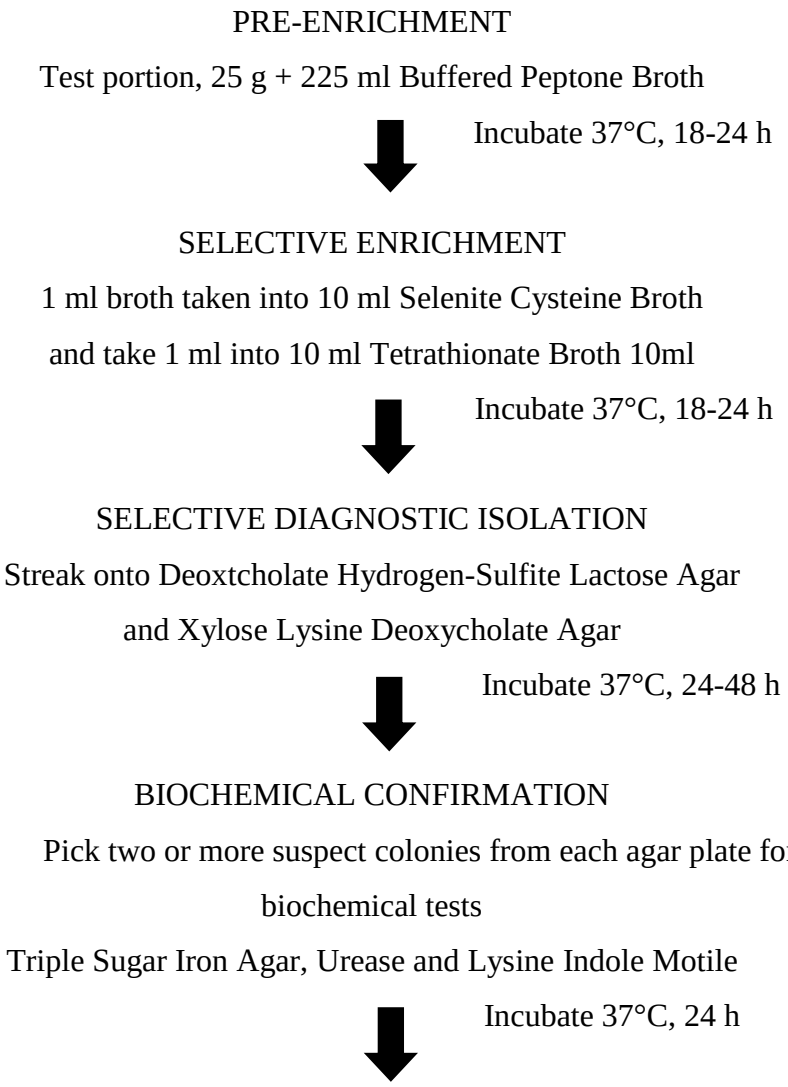

Figure 8 ICMSF *Salmonella* isolation procedure.

Source: ICMSF (1996)

The analysis of multiple bacterial isolates by phenotypic or genotypic methods can be used to identify characteristics within a particular species. Due to the problems in the use of phenotype based methods which are increasingly challenged by the use of DNA-based methods. In the last few years, innovative molecular techniques have provided powerful tools for direct DNA analysis which offers many

advantages. One of the most important advantages is that since DNA can always be extracted from bacteria, all bacteria should be typeable. Another is that the discriminatory power of DNA-based methods is greater than that of phenotypic procedures (Bush *et al*., 1999). A listing of some of the characteristics of bacterial typing systems can be seen in Table 3.

Because *Salmonella* cause major food poisoning cases and outbreaks in the world. The detection, identification and also molecular characterization of *Salmonella* are very important for human and animal health and food industry. For these reasons, salmonellae strains isolated from patients of food poisoning cases and suspected animals are used in detection, identification and molecular characterization studies both with phenotypic and DNA-based genotypic methods.

Table 3 Characteristics of bacterial typing systems.

Typing System	Proportion of Strain Type	Reproducibility	Discriminatory Power	Ease of Interpretation	Ease of Performance
Phenotypic Methods					
Biotyping	All	Poor to fair	Poor	Excellent	Excellent
Serotyping	Most	Good	Fair	Good to excellent	Fair to good
Phage Typing	Variable	Fair	Fair	Fair to good	Poor to fair
Genotyping Methods					
Plasmid Fingerprinting	Variable	Fair to good	Good	Good	Fair to good

Table 3 (continued)

Typing System	Proportion of Strain Type	Reproducibility	Discriminatory Power	Ease of Interpretation	Ease of Performance
Restriction Endonuclease	All	Very good	Good	Poor	Excellent
Pulsed-Field Analysis Gel Electrophoresis	All	Excellent	Excellent	Excellent	Fair to good
PCR-RFLP	All	Excellent	Good	Excellent	Very good to excellent
RAPD-PCR	All	Good	Very good to excellent	Very good	Very good to excellent

Source: Busch and Nitschko (1999)

5.1 Phenotypic methods

Phenotypic methods are those that characterize the products of gene expression in order to differentiate strains. Properties such as biochemical profiles, bacteriophage types, antigen present on the cell's surface, and antimicrobial susceptibility profiles all are examples of phenotypic properties that can be determined in the laboratory, methods such as biotyping, serotyping, phage-typing, antibiotic susceptibility, immunologic methods etc. (Tenover *et al*., 1997).

There are problems with many of these methods because of the need for specialized reagents, only a few reference labs around the world can reliably carry them out. Other problems with some phenotypic methods include non-typeability of strains, non-reproducibility, and lack of discriminatory power (Tenover *et al*., 1997).

5.1.1 Biotyping

Frequently, initial differentiation within a newly-delineated species is achieved by examining the cultural and biochemical characteristics of a large collection of individual strains belonging to the species. Such characteristics may include colonial morphology, growth requirements, fermentation ability, carbon source utilization and antibiotic resistance.

In theory, such properties are easy to determine, but in practice the determination of biotypes may not always be straightforward. Differences in colonial morphology are often extremely subtle and, therefore, can be rather subjective (Towner, 1993) that is the discriminatory power of biotyping is low. Biotyping, like most phenotypic methods, has only modest reproducibility, because microorganisms can alter unpredict the expression of many cellular products (Tenover *et al*., 1997).

5.1.2 Serotyping

Serotyping is one of the oldest typing procedures, but particularly when used in conjunction with other typing methods, still represents an important tool for routine identification and typing of many microbial species. Based on reactions with specific antisera (such as antisomatic, antiflagellar and anticapsular) raised according to the antigenic structure of microbes in each group, the method has been developed in detailed for bacteria belonging to the Enterobacteriaceae such as *Salmonella* and *Shigella* (Ewing, 1986).

Serotyping of *Salmonella* is the most common method used to differentiate strains, which are epidemiologically the smallest bacterial units from which isolates share the same phenotypic and genotypic traits. Serotyping separates strains based on their somatic (O), capsular (Vi), and flagellar (H) antigens into distinct serotypes. Classically, serotyping was used to divide *Salmonella* into distinct species based on their serotypes, giving rise to one species per serotype. Of the two

species of *Salmonella*, *S. enterica* and *S. bongori*, over 99% of serotypes, are grouped into the species *S. enterica*, and nearly 60% of them belong to the subspecies *enterica.*

To perform serotyping, the suspension of a *Salmonella* isolate is mixed and incubated with a panel of antisera that recognize specific O and H epitopes. The agglutination profiles generated are used to determine the particular serotype of the isolate being tested. A comprehensive serotyping scheme used to determine the particular serotype is the Kauffmann-White identification system, which has been extensively used by many public health organizations. Because of its wide acceptance as a method to differentiate *Salmonella* strains, serotyping is an important tool in public health. However, maintaining stocks of typing sera (including the >2,200 antisera required for definitive *Salmonella* typing) is a major limitation of this method. That is the main disadvantages seem to be associated with problems in antisera production and standardization of methodology (Yan *et al*., 2004).

5.1.3 Phage typing

Bacteriophages (phages) are viruses capable of infecting bacteria, leading in some cases to lysis of bacterial cell and release of further infective phage particles, while in other cases, the phage can persist as a relatively stable prophage within the bacterial cell. Phage typing is a method for bacterial strain identification that is based upon sensitivity to defined collections of bacteriophages which have been selected to provide the maximum sensitivity for differentiating strains within a particular species. Typing may be ‘direct’, i.e. based on direct sensitivity to either unadapted or adapted phages, or ‘indirect’, i.e. based on detection and identification of phages present as prophages in bacteria.

Phage typing remains the major typing method for *Staphylococcus aureus* (*S. aureus*), and is also particularly useful for subdividing serotypes of *Pseudomanas aeruginosa* (*P. aeruginosa*) and *Salmonella/Shigella* spp. (Towner, 1993). Different bacteriophages are able to selectively infect *Salmonella* isolates due

to differences in the phage and phage receptor present on the surface of the bacterium. Phage typing has been used to describe pandemic clones of *Salmonella*, such as *S.* Typhimurium definitive type 104 (DT 104) that causes severe gastrointestinal illness and is typically resistant to multiple antimicrobials (Randall *et al*., 2001).

It can be concluded that phage typing is a highly sensitive, but technically demanding method of typing that requires the maintenance of multiple biologically active phage stocks, it is usually performed only by public health and reference laboratories.

5.1.4 Antibiotic susceptibility

The antimicrobial susceptibility (antibiogram) is analyzed by the growth of an isolate in the presence of given antibiotic. Antimicrobial susceptibility patterns also have relatively poor discriminatory power, because antimicrobial resistance is under tremendous selective pressure in healthcare institutions and often is associated with mobile genetic elements (transposons and plasmids). Changes in antibiograms also may reflect spontaneous point mutations, such as seen with fluoroquinolones. Thus, isolates that are epidemiologically related and otherwise genetically indistinguishable may manifest different antimicrobial susceptibilities due to acquisition of new genetic material over time or loss of plasmids. Conversely, unrelated isolates may have indistinguishable resistance profiles, which may represent acquisition of the same plasmid by multiple species (Tenover *et al*., 1997).

5.1.5 Immunological methods

5.1.5.1 Immunoblotting

Immunoblotting is used for strain differentiation of a variety of medically important microorganisms. This technique involves the electrophoretic transfer of proteins or Lypopolysaccharide (LPS) molecules which are separated by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) from

polyacrylamide nitrocellulose or nylon membrane. Membranes are then incubated with a polyclonal serum or monoclonal antibody, after which antibody binding to antigens is detected by incubation with tagged antibody conjugate and an appropriate detection system using radiolabels. After the development of blots to visualize the antigenic profile, membranes are washed and dried. They are then photographed to provide a permanent record.

Immunoblotting is been employed to investigate the validity of serogroups of some gram-negative bacteria established with conventional typing sera. For example, LPS from isolates of *Pasteyrella haemolytica* assigned originally to different serotypes showed very similar immunoblot profiles, supporting results of earlier studies which suggested that the assigned serotypes were artefactual (Rimler *et al*., 1990).

Immunoblotting is not ideal method for some applications for antigenic analysis of microorganisms. In particular, some epitopes on protein antigens that may be of potential use for typing may be destroyed during sample preparation for SDS-PAGE. This method also have some technical disadvantages which are associated with poor transfer and distortion of profiles because of trapping of air bubbles within the blotting sandwich, or because of incomplete equilibration of the gel with the transfer buffer before blotting (Towner, 1993).

5.1.5.2 Enzyme-linked immunosorbent assay (ELISA)

Since the analysis of large numbers of strains and antibodies by immunoblotting have some problems, ELISA techniques can be used to overcome some of these problems.

Close examination and comparison show that all *Salmonella* ELISA share many common features. Kit assays require enrichment steps to resuscitate injured cells and to selectively amplified *Salmonella*. The enrichment temperatures and incubation periods vary with the kits and with the types of food

analysed. All ELISA kits are designed in a ‘‘sandwich’’ or capture format, i.e., antibody coated polystyrene wells are used to capture *Salmonella* antigen, and a second antibody to *Salmonella* conjugated with an enzyme is added to form an antibody-antigen-antibody (sandwich) complex. The sandwich complex is then determined by a colorimetric enzyme substrate, and the results are recorded either visually or with a spectrophotometer. Almost all *Salmonella* ELISA kits use alkaline phosphatase or horseradish peroxidase enzyme conjugates with a colorimetric substrate system. For example, in TekTM ELISA test system the detector antibodies are conjugated with horseradish peroxidase, which catalyzes the oxidation of tetramethylbenzidine and as a result, a blue color develops. The enzymatic reaction is stopped with sulphuric acid which at the same time changes the blue to a yellow color. The intensity of the resulting yellow color solution absorbance value is measured at 450 nm with a micro-ELISA reader, that allows the conclusion whether there are *Salmonella* antigens in the sample or not (Molla *et al*., 2003).

5.1.6 Rapid screening methods

Rapid screening methods would be performed directly on the food product without the need of enrichment of the sample, but none of the screening methods are available to detect the low number of *Salmonella* in foods.

Hydrophobic grid membrane filters (HGMF)/EF-18 agar method was evaluated against the conventional culture method (Association of Official Agricultural Chemists/ Bacteriological Analytical Manual, AOAC/BAM) using a total of 954 samples comprising 25 product categories. The HGMF/EF-18 method detected 653 *Salmonella* positive samples and AOAC/BAM method detected 654. The HGMF/EF-18 method with an overall false negative rate of 2% was determined to be equivalent in selectivity to AOAC/BAM procedure. The presumptive false positive rates were 0.3% for the HGMF/EF-18 and 7.95 for AOAC/BAM.

5.2 Genotypic methods

Genotyping which involves direct DNA-based analysis of chromosomal or extrachromosomal (plasmid) genetic material, has many advantages over traditional typing procedures. The major advantage lies in its increased discriminatory power to distinguish between two closely related strains. In examining the discriminatory ability of a particular method, it is useful to otherwise identical, when tested by other techniques. This genotyping techniques have many advantages for examples: DNA can always be extracted from bacteria so that all strains are typeable. The only exception here would be in the case of plasmid typing, which will be discussed later on, analytical strategies for the genotypic methods are similar and can be applied to DNA of any source, in general, genomic DNA is a stable characteristic and its composition is independent of cultural conditions or methods of preparation and it allows for statistical data analysis and is amenable to automation. Genotypic methods classified as restriction enzyme based methods and PCR based methods (Busch *et al*., 1999).

5.2.1 Restriction digestion based methods

5.2.1.1 Chromosomal DNA restriction profiles

For this type of analysis, chromosomal DNA is digested by restriction endonucleases and the generated fragments are separated by agarose gel electrophoresis. Restriction endonucleases are enzymes cutting DNA at a defined position within (or close to) a specific recognition sequence. Since restriction endonucleases are highly specific, complete digestion of a given DNA provides reproducible pattern of DNA fragments whereby the number and size of fragments depend on the DNA composition. Variation in the pattern of fragments is called restriction fragment length polymorphism (RFLP) and can result from a minor change of DNA such as a single base substitution within the restriction enzyme recognition, cleavage site, major changes like insertions, deletion and sequence rearrangements. A limitation of restriction enzyme digestion of whole chromosomes is the generation of a large number of DNA as different fragments. The separation by conventional gel electrophoresis leads to a smear-like appearance of the DNA on the gel and the

yielded band pattern are also too complex to be properly compared. To master this problem, the method was combined with nucleic acid probes (Busch *et al*., 1999).

5.2.1.2 Ribotyping

The use of rRNA-based probes for typing purposes is referred as 'ribotyping'. Many of the rRNA gene sequences found in the bacteria appears to have changed little during evolution, that means rRNA genes are very highly conserved. Therefore, probes specific for these sequences can detect a wide range of bacteria with similar rRNA sequences. rRNA genes are present in several copies and organised into operons, which contains individual genes coding for 16S, 23S and 5S rRNA. These individual genes are separated by non-coding spacer regions (Woese, 1987).

In practice, the steps of ribotyping can be arranged in that manner. Firstly, total DNA is isolated from cultured cells. Then usually by using 6bp recognising restriction enzyme DNA is cut into many small fragments which are separated by agarose gel electrophoresis. Subsequently, hybridization can be performed directly in gel or by transferring the fragments to blotting membrane with radioactively labelled probes which are targetted to either 16S, 23S or 5S rRNA genes (Towner, 1997).

The study was performed to ascertain the epidemic genetic types of *Salmonella* Ohio that have been circulating and causing human salmonellosis in Spain during the epidemiological alert of 1998 by Soto *et al*. (2000). 50 isolates of *Salmonella* serotype Ohio were used in ribotyping with *Hind*II which was selected to perform ribotyping because it detects a high numbers of restriction sites in the rRNA loci and yield a good discriminatory power within several salmonellae serotypes. *Hind*II ribotyping differentiated these Ohio isolates into eight (H1-H8) banding profiles. The h-ribotypes included 10 to 20 bands (of sizes 0.9-6.3 kbp). The two most frequent H-ribotypes (H2 and H4) showed only two mismatching fragments which could derive from a single insertion/deletion, whereas with regard to H1

showed 11 and 7 mismatching fragments, fact that implies genetic changes. Among 11 lineages (L1-L11), the two most frequent lineages which are L5 and L3 showed a closer relationship with one another. That means two Ohio lineages could be considered endemic in Spain.

5.2.1.3 Pulse field gel electrophoresis (PFGE)

The technique is capable of separating large DNA molecules in 50 kb-12 Mb range physically and by using electrical pulse system is called as pulsed field gel electrophoresis. Intact cells of bacteria are embedded in agarose gel in order to protect the DNA during the subsequent extraction. The agarose gel plugs are then treated with detergent and enzymes to isolate the DNA. The embedded chromosomal DNA is cut with rare-cutter restriction endonuclease to generate limited number (10 to 20) of high molecular weight restriction fragments which are ranging in size from 10 to 800 kbp. These fragments then are separated by agarose gel electrophoresis. The molecules subjected to the electrical fields applied alternatively in two directions. By switching the electrical field direction (pulses) frequently, the DNA can snake its way further into the gel and be separated on the basis of sizes. That is smaller fragments migrate faster than larger ones and the DNA fragments can be efficiently resolved (Busch *et al*., 1999).

The resulting electrophoretic patterns are highly specific for strains from a variety of organisms and also provide an opportunity to examine multiple variations throughout the genome of the organism to identify specific strains and accuracy links them with disease outbreaks. PFGE has great value in epidemiological analysis in the differentiation of pathogenic strains, and in the monitoring of their spread among communities. However, there are some disadvantages of current PFGE protocols which are involved time-consuming steps, tedious procedures for the purification of complete genomic DNA trapped in agarose, lengthy restriction enzyme digest, and extended electrophoresis times (Gautom, 1997).

5.2.1.4 Plasmid fingerprinting

Plasmid fingerprinting was the first molecular method to be used as a bacterial typing tool. Plasmids are double-stranded extrachromosomal DNA elements that are present in most clinical isolates (Tenover *et al*., 1985).

In the most basic application, plasmids would be isolated from each bacterial isolate and then separated electrophoretically in an agarose gel to determine their number and size. Plasmids of the same size, however, may actually be different. To circumvent this problem, one can treat the plasmid with a restriction enzyme.

If the two plasmids are different, they could be expected to be cut at different places and thus one would end up with various sizes of DNA fragments which would migrate differently on an agarose gel. Disadvantages of this method are; plasmids usually unstable, some organisms contain few or no plasmids and different plasmids can appear to be the same size even if it is relatively quick and easy method (Wachsmuth *et al*., 1991).

5.2.2 Polymerase chain reaction (PCR)-based methods

The polymerase chain reaction (PCR) is a technique widely used in molecular biology. It derives its name from one of its key components, a DNA polymerase used to amplify (replicate) a piece of DNA by in vitro enzymatic replication. As PCR progresses, the DNA thus generated is itself used as template for replication. This sets in motion a chain reaction in which the DNA template is exponentially amplified. With PCR, it is possible to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating millions or more copies of the DNA piece. PCR can be performed without restrictions on the form of DNA, and it can be extensively modified to perform a wide array of genetic manipulations (Kolmodin and Williams, 1997).

6. Polymerase chain reaction (PCR)

Almost all PCR applications employ a heat-stable DNA polymerase, such as *Taq* polymerase, an enzyme derived from the bacterium *Thermus aquaticus.* This DNA polymerase enzymatically assembles a new DNA strand from DNA building blocks, the nucleotides, using single-stranded DNA as template and DNA oligonucleotides (also called DNA primers or amplimers) required for initiation of DNA synthesis. The vast majority of PCR methods use thermal cycling which is alternately heating and cooling the PCR sample to a defined series of temperature steps. These different temperature steps are necessary to bring about physical separation of the strands in a DNA double helix (DNA melting), and permit DNA synthesis by the DNA polymerase to selectively amplify the target DNA or amplicon. The power and selectivity of PCR are primarily due to selecting primers that are highly complementary to the DNA region targeted for amplification, and to the thermal cycling conditions used.

Developed in 1983 by Kary Mullis, PCR is now a common and often indispensable technique used in biological research labs and microbiological analysis labs for applications to medical, food and environmental samples. These include DNA cloning for sequencing, DNA-based phylogeny, or functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints (used in forensics and paternity testing); and the detection and diagnosis of infectious diseases. Mullis won the Nobel Prize for his work on PCR. Mullis credits the psychedelic drug (Lysergic acid diethylamide, LSD) for helping his invention of the technique (Bartlett and Stirling, 2003).

6.1 PCR principle and procedure

PCR is used to amplify specific regions of a DNA strand (the DNA target). Deoxyribonucleic acid (DNA) is a molecule that encodes the genetic instructions used in the development and functioning of all known living organisms and many viruses. Along with RNA and proteins, DNA is one of the three major

macromolecules essential for all known forms of life. Genetic information is encoded as a sequence of nucleotides (guanine, adenine, thymine, and cytosine) recorded using the letters G, A, T, and C. Most DNA molecules are double-stranded helices, consisting of two long polymers of simple units called nucleotides, molecules with backbones made of alternating sugars (deoxyribose) and phosphate groups (related to phosphoric acid), with the nucleobases (G, A, T, C) attached to the sugars. DNA is well-suited for biological information storage, since the DNA backbone is resistant to cleavage and the double-stranded structure provides the molecule with a built-in duplicate of the encoded information.

These two strands run in opposite directions to each other and are therefore anti-parallel, one backbone being 3′ (three prime) and the other 5′ (five prime). This refers to the direction the 3rd and 5th carbon on the sugar molecule is facing. Attached to each sugar is one of four types of molecules called nucleobases (informally, bases). It is the sequence of these four nucleobases along the backbone that encodes information. This information is read using the genetic code, which specifies the sequence of the amino acids within proteins. The code is read by copying stretches of DNA into the related nucleic acid RNA in a process called transcription This can be a single gene, a part of a gene, or a non-coding sequence.

Most PCR methods typically amplify DNA fragments of up to 10 kbp, although some techniques allow for amplification of fragments up to 40 kbp in size (Cheng *et al*., 1994). A basic PCR set up requires several components and reagents (Sambrook and Russel, 2001). These components include the reagents as follows:

6.1.1 DNA template that contains the DNA region (target) to be amplified.

6.1.2 One or more primers, which are complementary to the DNA regions at the 5' (five prime) and 3' (three prime) ends of the DNA region.

6.1.3 DNA polymerase such as *Taq* polymerase or another DNA polymerase with a temperature optimum at around 70°C.

6.1.4 Deoxynucleoside triphosphates (dNTPs; also very commonly and erroneously called deoxynucleotide triphosphates), the building blocks from which the DNA polymerases synthesizes a new DNA strand.

6.1.5 Buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.

6.1.6 Divalent cations, magnesium or manganese ions; generally Mg^{2+} is used, but Mn^{2+} can be utilized for PCR-mediated DNA mutagenesis, as higher Mn^{2+} concentration increases the error rate during DNA synthesis (Pavlov *et al*., 2004)

6.1.7 Monovalent *cation* potassium ions.

The PCR is commonly carried out in a reaction volume of 15-100 µl in small reaction tubes (0.2-0.5 ml volumes) in a thermal cycler (Figure 9). The thermal cycler allows heating and cooling of the reaction tubes to control the temperature required at each reaction step. Many modern thermal cyclers make use of the Peltier effect which permits both heating and cooling of the block holding the PCR tubes simply by reversing the electric current. Thin-walled reaction tubes permit favorable thermal conductivity to allow for rapid thermal equilibration. Most thermal cyclers have heated lids to prevent condensation at the top of the reaction tube. Older thermocyclers lacking a heated lid require a layer of oil on top of the reaction mixture or a ball of wax inside the tube.

Figure 9 A thermal cycler for PCR.

The procedure of PCR usually consists of a series of 20 to 35 repeated temperature changes called cycles; each cycle typically consists of 2-3 discrete temperature steps. Most commonly PCR is carried out with cycles that have three temperature steps (Figure 10). The cycling is often preceded by a single temperature step (called hold) at a high temperature (>90°C), and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters. These include the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs in the reaction, and the melting temperature (Tm) of the primers (Rychlik *et al*., 1990).

Initialization step: This step consists of heating the reaction to a temperature of 94-96°C (or 98°C if extremely thermostable polymerases are used), which is held for 1-9 min. It is only required for DNA polymerases that require heat activation by hot-start PCR.

Denaturation step: This step is the first regular cycling event and consists of heating the reaction to 94-96°C for 20-30 s. It causes melting of DNA template and primers by disrupting the hydrogen bonds between complementary bases of the DNA strands, yielding single strands of DNA.

Annealing step: The reaction temperature is lowered to 50-65°C for 20-40 s. allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature (Ta) is about 3-5°C below the Tm of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis.

Extension/elongation step: The temperature at this step depends on the DNA polymerase used; *Taq* polymerase has its optimum activity temperature at 75-80°C (Lawyer *et al*., 1993) and commonly a temperature of 72°C is used with this enzyme. At this step, the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary

to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. As a rule-of-thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases in one minute.

Final elongation: This single step is occasionally performed at a temperature of 70-74°C for 5-15 min after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

Final hold: This step at 4-15°C for an indefinite time may be employed for short-term storage of the reaction.

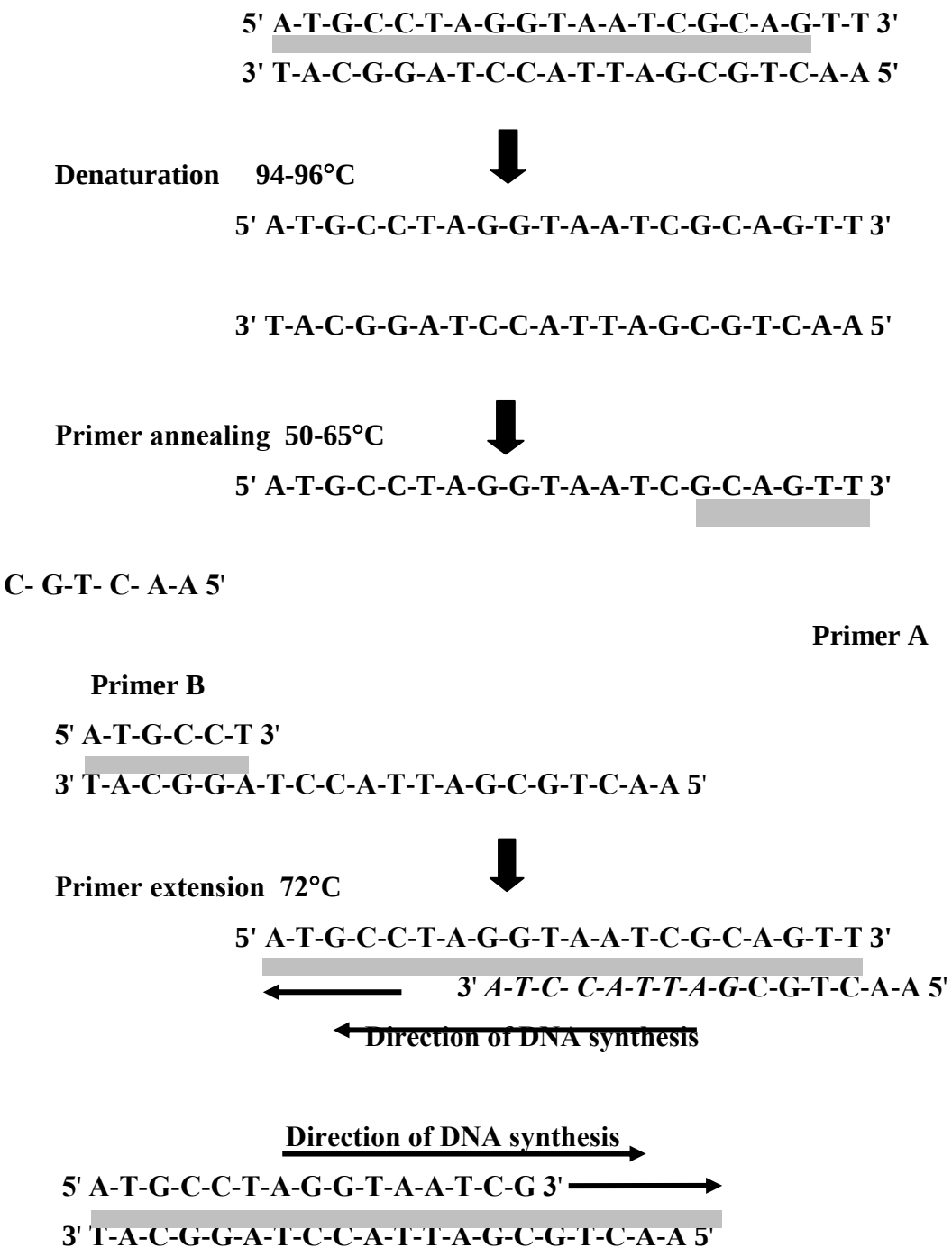

Figure 10 Schematic drawing of the PCR cycle: denaturation at 94-96°C, primer annealing at 50-65°C and primer extension at 72°C.

Source: Sambrook and Russel (2001)

6.2 PCR product analysis

Agarose gel electrophoresis is a method used in PCR product analysis to separate DNA, or RNA molecules by size. This is achieved by moving negatively charged nucleic acid molecules through an agarose matrix with an electric field (electrophoresis). Shorter molecules move faster and migrate farther than longer ones (Sambrook and Russel, 2001).

To check whether the PCR generated the anticipated DNA fragment (also sometimes referred to as the amplimer or amplicon), agarose gel electrophoresis is employed for size separation of the PCR products. The size(s) of PCR products is determined by comparison with a DNA ladder (a molecular weight marker), which contains DNA fragments of known size, run on the gel alongside the PCR products.

The objectives of PCR product analysis are shown as follows:

6.2.1 Estimation of the size of DNA molecules following restriction enzyme digestion in restriction mapping of cloned DNA.

6.2.2 Analysis of PCR products in molecular genetic diagnosis or genetic fingerprinting.

6.2.3 Separation of restricted genomic DNA prior to Southern transfer, or of RNA prior to Northern transfer.

For the factor of agarose gel electrophoresis, the most important factor is the length of the DNA molecule, smaller molecules travel farther. Conformation of the DNA molecule is also a factor. To avoid this problem linear molecules are usually separated, usually DNA fragments from a restriction digest, linear DNA PCR products, or RNA.

Increasing the agarose concentration of a gel reduces the migration speed and enables separation of smaller DNA molecules. The higher the voltage, the faster

the DNA migrates. Voltage is limited by the fact that it heats and ultimately causes the gel to melt. High voltages also decrease the resolution (above about 5 to 8 V/cm).

Conformations of a DNA plasmid that has not been cut with a restriction enzyme will move with different speeds (slowest to fastest): nicked or open circular, linearised, or supercoiled plasmid.

The most common dye used for agarose gel electrophoresis is ethidium bromide, usually abbreviated as EtBr. It fluoresces under UV light when intercalated into DNA (or RNA). By running DNA through an EtBr-treated gel and visualizing it with UV light, distinct bands of DNA become visible. Ethidium bromide is an intercalating agent commonly used as a nucleic acid stain in molecular biology laboratories for techniques such as agarose gel electrophoresis. When exposed to ultraviolet light, it will fluoresce with a red-orange color, intensifying almost 20-fold after binding to DNA. Ethidium bromide may be a very strong mutagen, and may be a carcinogen or teratogen, although this has never been definitively proven (Figure 11).

Figure 11 The molecular structure of ethidium bromide (3,8-Diamino-5-ethyl-6-phenylphenanthridinium bromide).

Source: Sambrook and Russel (2001)

SYBR Green I is another double stranded-DNA stain, produced by Invitrogen. It is more expensive, but 25 times more sensitive, and possibly safer than EtBr, though there is no data addressing its mutagenicity or toxicity in humans.

SYBR Safe is a variant of SYBR Green that has been shown to have low enough levels of mutagenicity and toxicity to be deemed nonhazardous waste under U.S. Federal regulations. It has similar sensitivity levels to EtBr but, like SYBR Green, is significantly more expensive (Invitrogen, 2010).

Loading buffers are added with the DNA in order to visualize it and sediment it in the gel well. Negatively charged indicators keep track of the position of the DNA. Xylene cyanol and Bromophenol blue are typically used. They run at about 5000 bp and 300 bp respectively, but the precise position varies with percentage of the gel. Other less frequently used progress markers are Cresol Red and Orange G which run at about 125 bp and 50 bp.

For the resolution limits of Gel electrophoresis**,** this technique can be used for the separation of DNA fragments ranging from 50 bp to several Mbp. However, it is normally used in a range of 100 bp to 20 kbp. Typical run times are about an hour.

Small nucleic acids are better separated by polyacrylamide gels, large DNA molecules are only able to move end-on in a process called "reptation" and are more difficult to separate. In general, higher concentrations of agarose are better for larger molecules; it will exaggerate the distances between bands. The disadvantage of higher concentrations is the long run times (sometimes days). Instead, these gels should be run with a pulsed field electrophoresis (PFE), or field inversion electrophoresis (Invitrogen, 2010).

6.3 Factors affecting the PCR

6.3.1 Denaturing temperature and time

The specific complementary association due to hydrogen bonding of single-stranded nucleic acids is referred to as "annealing": two complementary sequences will form hydrogen bonds between their complementary bases (G to C, and A to T or U) and form a stable double-stranded, anti-parallel "hybrid" molecule. One may make nucleic acid (NA) single-stranded for the purpose of annealing. If it is not single-stranded already as most RNA viruses, heating it to a point above the melting temperature (Tm) of the double- or partially-double-stranded form, and then flash-cooling it: this ensures the "denatured" or separated strands do not re-anneal. Additionally, if the NA is heated in buffers of ionic strength lower than 150 mM sodium chloride (NaCl), the melting temperature is generally less than 100^{o}C, which is why PCR works with denaturing temperatures of 91-97^{o}C.

A more detailed treatment of annealing / hybridization is given in an accompanying page, together with explanations of calculations of complexity, conditions for annealing / hybridization, etc.

Taq polymerase is given as having a half-life of 30 min at 95^{o}C, which is partly why one should not do more than about 30 amplification cycles: however, it is possible to reduce the denaturation temperature after about 10 rounds of amplification, as the mean length of target DNA is decreased: for templates of 300bp or less, denaturation temperature may be reduced to as low as 88^{o}C for 50% (G+C) templates, which means one may do as many as 40 cycles without much decrease in enzyme efficiency (Innis and Gelfand, 1990).

"Time at temperature" is the main reason for denaturation / loss of activity of *Taq*. Thus, if one reduces this, one will increase the number of cycles that are possible, whether the temperature is reduced or not. Normally the denaturation time is 1 min at 94^{o}C: it is possible, for short template sequences, to reduce this to 30 s or less. Increase in denaturation temperature and decrease in time may also work: Innis and Gelfand (1990) recommended 96^{o}C for 15 s.

6.3.2 Annealing temperature (Ta) and primer design

Primer length and sequence are of critical importance in designing the parameters of a successful amplification: the melting temperature of a NA duplex increases both with its length, and with increasing (G+C) content: a simple formula for calculation of the Tm is the equation as $Tm = 4(G + C) + 2(A + T)^{o}C$.

Thus, the annealing temperature chosen for a PCR depends directly on length and composition of the primer(s). One should aim at using a Ta about $5^{o}C$ below the lowest Tm of their pair of primers to be used (Innis and Gelfand, 1990). A more rigorous treatment of Ta is given by Rychlik *et al*. (1990): they maintain that if the Ta is increased by $1^{o}C$ every other cycle, specificity of amplification and yield of products <1kb in length are both increased. One consequence of having too low a Ta is that one or both primers will anneal to sequences other than the true target, as internal single-base mismatches or partial annealing may be tolerated: this is fine if one wishes to amplify similar or related targets; however, it can lead to "non-specific" amplification and consequent reduction in yield of the desired product, if the 3'-most base is paired with a target.

A consequence of too high a Ta is that too little product will be made, as the likelihood of primer annealing is reduced; another and important consideration is that a pair of primers with very different Tas may never give appreciable yields of a unique product, and may also result in inadvertent "asymmetric" or single-strand amplification of the most efficiently primed product strand.

Annealing does not take long: most primers will anneal efficiently in 30 s or less, unless the Ta is too close to the Tm, or unless they are unusually long.

6.3.3 Primer length

The optimum length of a primer depends upon its (A+T) content, and the Tm of its partner if one runs the risk of having problems such as previously described. Apart from the Tm, a prime consideration is that the primers should be complex enough so that the likelihood of annealing to sequences other than the chosen target is very low.

For example, there is a 1/4 chance (4^{-1}) of finding an A, G, C or T in any given DNA sequence; there is a 1/16 chance (4^{-2}) of finding any dinucleotide sequence (AG); a 1/256 chance of finding a given 4-base sequence. Thus, a sixteen base sequence will statistically be present only once in every 4^{16} bases (=4,294,967,296 or 4 billion). This is about the size of the human or maize genome, and 1000x greater than the genome size of *Escherichia coli* (*E. coli*). Thus, the association of a greater-than-17-base oligonucleotide with its target sequence is an extremely sequence-specific process, far more so than the specificity of monoclonal antibodies in binding to specific antigenic determinants. Consequently, 17-mer or longer primers are routinely used for amplification from genomic DNA of animals and plants. Too long a primer length may mean that even high annealing temperatures are not enough to prevent mismatch pairing and non-specific priming.

6.3.4 Degenerate primers

For amplification of cognate sequences from different organisms, or for "evolutionary PCR", one may increase the chances of getting product by designing "degenerate" primers: these would in fact be a set of primers which have a number of options at several positions in the sequence so as to allow annealing to and amplification of a variety of related sequences. For example, Compton (1990) describes using 14-mer primer sets with 4 and 5 degeneracies as forward and reverse primers, respectively, for the amplification of glycoprotein B (gB) from related herpesviruses. The reverse primer sequence was as follows:

TCGAATTCNCCYAAYTGNCCNT

Where Y = T + C, and N = A + G + C + T, and the 8-base 5'-terminal extension comprises an *Eco*RI site (underlined) and flanking spacer to ensure the restriction enzyme can cut the product (the New England Biolabs catalogue gives a good list of which enzymes require how long a flanking sequence in order to cut stub ends). Degeneracies obviously reduce the specificity of the primer(s), meaning mismatch opportunities are greater, and background noise increases; also, increased degeneracy means concentration of the individual primers decreases; thus, greater than 512-fold degeneracy should be avoided.

6.3.5 Elongation temperature and time

This is normally 70-72^{o}C, for 0.5-3 min. *Taq* actually has a specific activity at 37^{o}C which is very close to that of the *Klenow* fragment of *E.coli* DNA polymerase I, which accounts for the apparent paradox which results when the researcher tries to understand how primers which anneal at an optimum temperature can then be elongated at a considerably higher temperature. The elongation occurs from the moment of annealing, even if this is transient, which results in considerably greater stability. At around 70^{o}C the activity is optimal, and primer extension occurs at up to 100 bases/s. About 1 min is sufficient for reliable amplification of 2kb sequences (Innis and Gelfand, 1990). Longer products require longer times: 3 min is a good bet for 3kb and longer products. Longer times may also be helpful in later cycles when product concentration exceeds enzyme concentration (>1 nM), and when dNTP and / or primer depletion may become limiting.

6.3.6 Reaction buffer

Recommended buffers generally contain the reagents as follows:

6.3.6.1 10-50 mM Tris(hydroxymethyl)aminomethane Hydrochloric acid (Tris-HCl) pH 8.3

6.3.6.2 up to 50 mM Potassium chloride (KCl), 1.5 mM or higher Magnesium chloride ($MgCl_2$)

6.3.6.3 primers 0.2-1μM each primer

6.3.6.4 50-200 μM each dNTP

6.3.6.5 gelatin or BSA to 100 μg/ml

6.3.6.6 non-ionic detergents such as Tween-20, Nonidet P-40 and Triton X-100 (0.05-0.10% v/v)

Modern formulations may differ considerably; however, they are also generally proprietary (Innis and Gelfand, 1990).

PCR is supposed to work well in reverse transcriptase buffer**,** and *vice-versa*, meaning 1-tube protocols (with cDNA synthesis and subsequent PCR) are possible (Krawetz *et al*., 1989; Fuqua *et al*., 1990). Higher than 50 mM KCl or NaCl inhibits *Taq*, but some is necessary to facilitate primer annealing.

6.3.7 Magnesium ion ($Mg^{2+)}$ affects primer annealing; Tm of template, product and primer-template associations; product specificity; enzyme activity and fidelity. So allowances should be made for dNTPs, primers and template, all of which chelate and sequester the cation; of these, dNTPs are the most concentrated, so (Mg^{2+}) should be 0.5-2.5 mM greater than dNTP. A titration should be performed with varying (Mg^{2+}) with all new template-primer combinations, as these can differ markedly in their requirements, even under the same conditions of concentrations and cycling times/temperatures.

6.3.8 Primer concentrations should not go above 1 μM unless there is a high degree of degeneracy; 0.2 μM is sufficient for homologous primers.

6.3.9 Nucleotide concentration should not be above 50 μM.

6.3.10 Cycle number

The number of amplification cycles necessary to produce a band visible on a gel depends largely on the starting concentration of the target DNA: Innis

and Gelfand (1990) recommended from 40-45 cycles to amplify 50 target molecules, and 25-30 to amplify 3×10^5 molecules to the same concentration. This non-proportionality is due to a so-called plateau effect (Figure 12), which is the attenuation in the exponential rate of product accumulation in late stages of a PCR, when product reaches 0.3-1.0 nM. This may be caused by degradation of reactants (dNTPs, enzyme); reactant depletion (primers, dNTPs-former a problem with short products, latter for long products); end-product inhibition (pyrophosphate formation); competition for reactants by non-specific products; competition for primer binding by re-annealing of concentrated (10 nM) product (Innis and Gelfand, 1990).

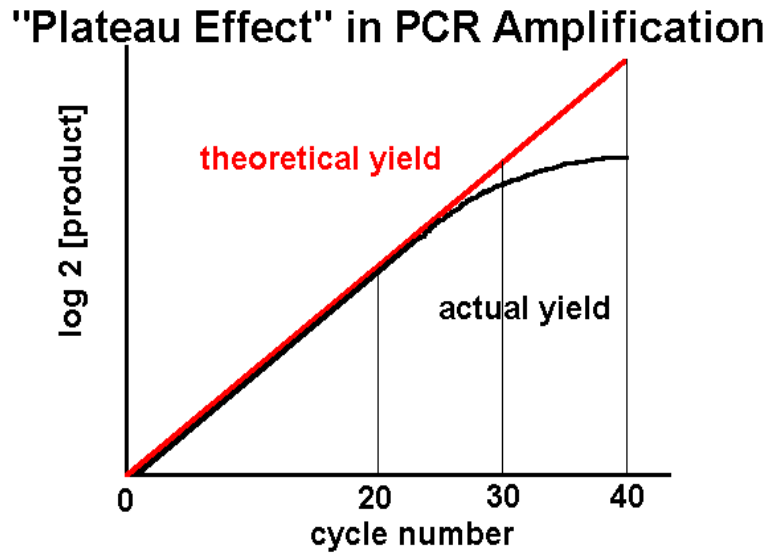

Figure 12 Plateau effect in PCR amplification.

Source: Innis and Gelfand (1990)

If desired product is not made in 30 cycles, take a small sample (1 µl) of the amplified mix and re-amplify 20-30x in a new reaction mix rather than extending the run to more cycles: in some cases where template concentration is limiting, this can give good product where extension of cycling to 40x or more does not.

6.3.11 PCR inhibitors in food and environmental samples

Many different PCR inhibitors have been identified in food. Fats, glycogen, polysaccharides, minerals as well as enzymes present in food may cause inhibition of the PCR. In milk samples, PCR inhibition is mainly dependent on the concentration of calcium, whereas the fat content seems to have only minor influence on the amplification efficiency. In seafood, mainly polysaccharides seem to be responsible for PCR inhibition. In addition, the glycogen content in the tissues of bivalve molluscs influences PCR efficiency. Generally, the ability of bivalve molluscs to filter the water may lead to concentration of different inhibitory substances. Plants carry many substances, such as polysaccharides, polyphenols, pectin and xylan, which may be co-extracted and thereafter hamper the PCR. Although for most of these polysaccharides (dextran, inulin, pectin or starch), no inhibitory effect was reported, some of them like dextran sulphate and gum affect the PCR efficiency. However, this effect was partly reversible by adding Tween 20, dimethyl sulphoxide or polyethylene glycol 400. In another study, acidic polysaccharides (ghatti gum, xylan, dextran sulphate), polyphenols as well as inulin and pectin found in tea extracts were proven to inhibit PCR amplification. Berries are generally rich in phenols (e.g. anthocyanin, flavonol, ellagitannin, proanthocyanidin and phenolic acids) and polysaccharides. Substances present in berries and tomatoes seem to especially inhibit real-time PCR assays using *Taq*Man probes, whereas conventional PCR assays are less affected. Environmental samples can be very diverse and derived from different compartments including soil, water or air. This large variety leads to the presence of many different PCR inhibitors including those already described above. In addition, dead biomass and soil may contain humic and fulminic acids, which inhibit PCR even at low concentrations. In sewage sludge, fats, proteins, polyphenols and heavy metals are found, and waste water contains polysaccharides, metal ions (e.g. iron and aluminium) and RNases all of them are common PCR inhibitors of environmental samples. Several different inhibitors have also been detected in animal. A large variety of water samples are frequently used for the detection of pathogens. To detect even low amounts of pathogens, large volumes of water are usually concentrated to very small volumes. However, this often results

in con-current concentration of the different inhibitors and increased interference with PCR. Similar problems may occur during the collection of air samples, which is usually carried out by passing large air volumes through a variety of filters or other binding material. By this, inhibitory airborne components are often enriched leading to failure of PCR (Schrader *et al*., 2012).

6.4 The advantages of PCR

The PCR method offers several advantages for rapid, reliable detection of microbial pathogens in food and promises to be a valuable addition and complement to the toolbox of food microbiologists. Several investigators have note certain advantages offered by PCR for diagnosis (Patel, 1994) as follows:

6.4.1 A short time requirement improves public health security and minimizes personal cost.

6.4.2 The method is able to identify microorganisms that are difficult to culture.

6.4.3 The culture and enrichment of pathogens are not necessary for quality control.

6.4.4 PCR reagents are more readily available and easier to store than to those required for serological procedures.

6.4.5 Animal models are not needed.

6.4.6 The choice of primer determines specificity, which contrasts with the frequent cross-reactivities of antisera utilized in immunoassays.

6.4.7 Elaborate diagnostic equipment and media are not required, thereby increasing the flexibility as to locations where PCR may be performed and eliminating many sample transport problems.

6.4.8 Automation of PCR in the future should result in excellent cost efficiency.

6.5 Variations on the basic PCR technique

6.5.1 Colony PCR

Bacterial colonies (*E. coli*) can be rapidly screened by PCR for correct DNA vector constructs. Selected bacterial colonies are picked with a sterile toothpick and dabbed into the PCR master mix or sterile water. The PCR is started with an extended time at 95°C when standard polymerase is used or with a shortened denaturation step at 100°C and special chimeric DNA polymerase (Pavlov, 2006).

6.5.2 Hot-start PCR

This is a technique that reduces non-specific amplification during the initial set up stages of the PCR. The technique may be performed manually by heating the reaction components to the melting temperature (95°C) before adding the polymerase. Specialized enzyme systems have been developed that inhibit the polymerase's activity at ambient temperature, either by the binding of an antibody or by the presence of covalently bound inhibitors that only dissociate after a high-temperature activation step. Hot-start/cold-finish PCR is achieved with new hybrid polymerases that are inactive at ambient temperature and are instantly activated at elongation temperature.

6.5.3 Multiplex-PCR

Multiplex-PCR is the method that uses multiple, unique primer sets within a single PCR reaction to produce amplicons of varying sizes specific to different DNA sequences. By targeting multiple genes at once, additional information may be gained from a single test run that otherwise would require several times the reagents and more time to perform. Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction, and amplicon sizes. Their base pair length should be different enough to form distinct bands when visualized by gel electrophoresis.

6.5.4 Nested PCR

Nested PCR increases the specificity of DNA amplification, by reducing background due to non-specific amplification of DNA. Two sets of primers are being used in two successive PCR reactions. In the first reaction, one pair of primers is used to generate DNA products, which besides the intended target, may still consist of non-specifically amplified DNA fragments. The product(s) are then used in a second PCR reaction with a set of primers whose binding sites are completely or partially different from and located 3' of each of the primers used in the first reaction. Nested PCR is often more successful in specifically amplifying long DNA fragments than conventional PCR, but it requires more detailed knowledge of the target sequences.

6.5.5 Overlap-extension PCR

This method is a genetic engineering technique allowing the construction of a DNA sequence with an alteration inserted beyond the limit of the longest practical primer length.

6.5.6 Quantitative PCR (Q-PCR)

Q-PCR is used to measure the quantity of a PCR product (preferably real-time). It is the method of choice to quantitatively measure starting amounts of DNA, cDNA or RNA. Q-PCR is commonly used to determine whether a DNA sequence is present in a sample and the number of its copies in the sample. The method with currently the highest level of accuracy is Quantitative real-time PCR. It is often confusingly known as RT-PCR (Real Time PCR) or RQ-PCR. QRT-PCR or RTQ-PCR is more appropriate contractions. RT-PCR commonly refers to reverse transcription PCR (see below), which is often used in conjunction with Q-PCR. QRT-PCR methods use fluorescent dyes, such as Sybr Green, or fluorophore-containing DNA probes (*Taq*Man) to measure the amount of amplified product in real time.

6.5.7 Reverse Transcription PCR (RT-PCR)

RT-PCR is a method used to amplify, isolate or identify a known sequence from a cellular or tissue RNA. The PCR is preceded by a reaction using reverse transcriptase to convert RNA to cDNA. RT-PCR is widely used in expression profiling, to determine the expression of a gene or to identify the sequence of an RNA transcript, including transcription start and termination sites and, if the genomic DNA sequence of a gene is known, to map the location of exons and introns in the gene. The 5' end of a gene (corresponding to the transcription start site) is typically identified by an RT-PCR method, named RACE-PCR, short for Rapid Amplification of cDNA Ends.

6.5.8 Thermal asymmetric interlaced PCR (TAIL-PCR)

TAIL-PCR is used to isolate unknown sequence flanking a known sequence. Within the known sequence TAIL-PCR uses a nested pair of primers with differing annealing temperatures; a degenerate primer is used to amplify in the other direction from the unknown sequence.

6.5.9 Touchdown PCR

This method is a variant of PCR that aims to reduce nonspecific background by gradually lowering the annealing temperature as PCR cycling progresses. The annealing temperature at the initial cycles is usually a few degrees (3-5°C) above the T_m of the primers used, while at the later cycles, it is a few degrees (3-5°C) below the primer T_m. The higher temperatures give greater specificity for primer binding, and the lower temperatures permit more efficient amplification from the specific products formed during the initial cycles (Patel, 1994).

6.5.10 Randomly amplified polymorphic DNA (RAPD)

The randomly amplified polymorphic DNA (RAPD) is PCR-based DNA fingerprinting technique using single, arbitrary designed, short (8-10 bp) nucleotide sequence as primer. Therefore, no prior sequence information of target

DNA is needed, and lower annealing temperature (30-40°C) is used in RAPD (Williams *et al*., 1990). At sufficiently low temperature, the arbitrary primer binds to many sequences with a variety of mismatches on the genomic DNA template (McClelland *et al*., 1995). The amplification process is initiated on those genomic regions in which two priming events occur on opposite DNA strands within a distance not exceeding a few thousand nucleotides. The outcome of the amplification reaction is determined by competition in which those products representing the most efficient pairs of priming sites separated by the most easily amplifiable sequences will prevail (McClelland *et al*., 1995). It should be emphasized that these results do not imply that all amplifications are the results of perfect pairing between the primer and the DNA template. The number of DNA segments amplified from bacterial samples with much smaller genomes can only be explained on the basis of mismatch between the primer and the DNA template (Williams *et al*., 1990). As shown in Figure 13, two primers must anneal to the template DNA on opposite strands, and in opposite orientations such that they face each other, in order to achieve synthesis of both strands of the DNA segment which lies between the two primers. Furthermore, primers must anneal within a reasonable distance of one another, in order for the successful PCR.

All classes of mutations (substitutions, insertions, deletions, invertions) can be potentially detected by means of random amplification fingerprinting even though most of the detected polymorphisms are supposed to be caused by substitutions. Single base substitutions within the primer binding site may prevent amplification by introducing a mismatch; this supported by the observation that most single base changes in a primer sequence result in complete change in the amplification pattern (Williams *et al*., 1990).

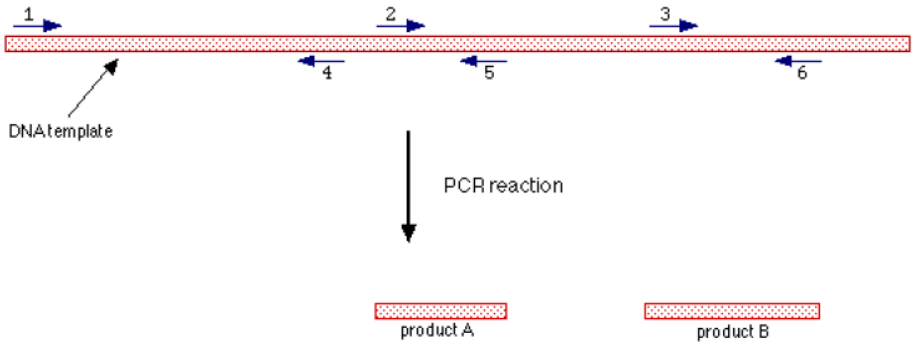

Figure 13 Schematic representation of RAPD-PCR.

Source: Williams *et al*. (1990)

Reaction products are resolved by gel electrophoresis, and patterns generated from different genomic DNAs (DNAs from different individual or strains) using the same primer are easily compared to identify possible differences. DNA fragments that are amplified from genomic DNA but not from another. Any difference between the patterns of amplified fragments reveals a polymorphism in that it arises from a difference in template sequence which inhibits primer binding or otherwise interferes with amplification of the corresponding fragment.

The RAPD technique has further advantages over other systems of genetic documentation because in RAPD, no previous genetic and molecular knowledge is required, a universal set of primers of arbitrary nucleotide sequence can be applied, a large number of primers are available, and only small amount of DNA is required. No southern blotting or radioactive probes are required, and the protocol is relatively quick and simple. RAPDs allow quick detection of usually 50-60 polymorphic DNA markers in 4-5 amplification reactions and speed in the assay of the high number of samples. The ease and simplicity of RAPD technique makes it ideal for genetic mapping, plant and animal breeding applications, DNA fingerprinting, with particular utility for studies of population genetics (Williams *et al*., 1990).

RAPD fingerprinting was carried out by using four arbitrary primers, OPA-10, OPR-03, OPI-06 and OPJ-09, to assess the genetic diversity of twenty-six *S. enterica* isolates which were obtained from hospital laboratories and commercial poultry producers in Mauritius. All clinical isolates were subjected to biochemical and serological testing for confirmation. Primer OPA-10 produced a single clear band of 1 kbp with all of the *Salmonella* isolates. Samples O and P did not produce the same bands. A larger number of bands were observed with primer OPR-03. A clear band of approximately 2.2 kbp was obtained with all isolates except O, P, Q and R.

Similarly, each produced a band of 1 kbp. Primer OPJ-09 produced two clear bands of approximately 2250 bp and 1800 bp from all the isolates of *Salmonella* except Q, X, Z, and A. Primer OPI-06 produced the greatest number of bands, ranging between 220 kbp to 2200 kbp. RAPD analysis of *Salmonella* isolates from Mauritius showed that they were genetically diverse. Four primers were selected for testing the samples by amplification. From all four banding patterns, it is clear that isolates O and P, which were mistakenly identified as *Salmonella* by biochemical and serological analyses, were later confirmed to be *Proteus* species and not *Salmonella*. In this respect, primer OPA-10 is potentially useful for the identification and differentiation of the *Salmonella* species from other Gram-negative bacteria (Khoodoo *et al*., 2002).

RAPD analysis was performed for the molecular genetic typing of 30 *S. enterica* subsp. *enterica* strains isolated from chickens and duck in Thailand. Six different primers were tested for their discriminatory ability. They found that most of the different serovars produced different RAPD types using only one primer. For example, all eight *S.* Enteritidis showed identical patterns using primer 4 and this pattern was different from the pattern using primer 4 for all other tested strains. Of these same eight strains, five showed an identical pattern with primer 1, while remaining three strains showed individual patterns with primer 1. Using all six primers the RAPD could differentiate between all of the *S.* Enteritidis isolates (Chansiripornchai *et al*., 2000).

Another study was prepared by Soto *et al*. (1999) for typing of *S. enterica* strains assigned to 12 serotypes. The series of organisms used included 235 strains (326 isolates) collected mainly from clinical samples.

RAPD reaction were carried out with the following three primers; primer S, primer B, primer C, respectively. With primer S, 21 amplified DNA band profiles were differentiated; each of these profiles included 2 to 5 fragments which were 250 to 2000 bp long. With primer B, 14 amplified DAN band profiles were differentiated; each of these profiles included 4 to 7 fragments which were 400 to 2500 bp long and with primer C, 40 amplified DNA band profiles were obtained. Most of these profiles were defined by considering only bands that were 1700 bp long or smaller. Different amplified fragments appeared to be characteristic of different serotypes. In another study, RAPD described to analyse 23 *Salmonella* spp. and 16 non-*Salmonella* spp. By using single oligonucleotide primer (du-primer) synthesized on the basis of N-terminal amino acid sequence of *Salmonella* dulcitol 1-phosphate dehydrogenase (D1PDH). Among three to five prominent bands produced the two bands at about 460 and 700 bp were detected all the strains of *Salmonella*, but some of the non-*Salmonella* bacteria showed no bands and the others showed different band patterns with some primer and same conditions (Miyamoto *et al*., 1998).

Previous study was performed to evaluate the efficiency of RAPD-PCR method for identification of *Salmonella* serotypes. For this purpose 10 different random oligonucleotide primers, 8-10 bp in size, were used with 14 different *Salmonella* serotypes and 16 non-*Salmonella* strains. Three of these primers which were primer 7, primer 6, and primer 3 gave distinguishable band patterns. Primer 7 gave 550 bp strong amplification band with 11 of the 14 *Salmonella* serotype, but some of the non-*Salmonella* strains gave same band. Primer 3 and primer 6 produce different fragments with *Salmonella* serotypes. Primer 3 gave a distinguishable amplification pattern and strong band with *S.* Typhimurium. According to this result, it was decided that further analysis should be performed with different *S.* Typhimurium isolates and also other *Salmonella* serotypes, especially closely related

serotype *S.* Enteritidis to decide whether this band can be used as a specific marker for detection and identification of *S.* Typhimurium.

7. Viable cell detection

Molecular methods targeting nucleic acids have revolutionized microbial detection. DNA-based methods, named polymerase chain reaction (PCR), are rapid, versatile, sensitive, precise, and allow specific detection and/or quantification of microorganisms of interest in food, environmental, and clinical samples. Despite these advantages, broad application is still hampered by some challenges. Apart from inhibition of amplification by substances naturally found in many environmental samples, the inability to differentiate between viable and nonviable cells and the resulting overestimation of microbial targets is considered a major disadvantage of PCR (Wang and Levin, 2006). Whereas the first limitation is greatly remedied by the incorporation of internal amplification controls, the second one was addressed by amplification of RNA instead of DNA. In contrast to the highly persistent DNA, RNA degrades more rapidly after cell death. Detection of RNA and especially of the highly unstable mRNA thus tends to indicate the presence of live cells far better than the detection of DNA. In addition, messenger RNA (mRNA) is only produced by metabolically active cells, making mRNA is suitable to specifically detect living microorganisms (Bleve *et al*., 2003; Morin *et al*., 2004). Nevertheless the same labile nature qualifying mRNA as a suitable target for detecting live cells at the same time makes working with it more challenging. Degradation can occur by inadequate sample processing and storage or as a result of sample contamination with RNA-degrading enzymes. Quantification of live cell numbers is further complicated by the fact that the expression levels of many mRNA species greatly depend on the physiological status of the cells, which is typically an unknown factor. It is likely that when slow-growing or dormant cells are present in a sample, the RNA content of such cells is beneath the detection limit of the RNA-PCR, while the cells remain essentially viable and even active. In addition, it is to be noted that, despite the labile nature of mRNA, false-positive signals from residual transcripts can occur in the case of high

levels of dead bacteria (>10^4 CFU/ml) (Sheridan *et al*., 1998; Vaitilingom *et al*., 1998).

An alternative approach to detect viable cells by PCR (herein referred to as v-PCR) was presented in 2003 by Nogva *et al*. (2003) by introducing the concept of EMA-PCR (ethidium bromide monoazide PCR). The invention of the alternative molecule propidium monoazide (PMA) in 2006 (Nocker *et al*., 2007) similarly resulted in the term PMA-PCR. The distinction between viable and non-viable cells for both membrane impermeant dyes is based on membrane integrity. Microbiological samples are treated with a nucleic acid intercalating dye that selectively enters cells with compromised cell membranes, whereas an intact cell membrane presents a barrier for this molecule. Once inside a (dead) cell, the dye intercalates into the cell's DNA to which it is believed to covalently crosslink after exposure to strong visible light due to the presence of an azide group. Photolysis converts the azide group into a highly reactive nitrene radical (DeTraglia *et al*., 1978), which can react with any organic molecule in its proximity. Reaction with DNA can be assumed to occur with a high probability considering the spatial proximity of the intercalated dye. The modification was empirically found to strongly inhibit its amplification (Rudi *et al*., 2005a; Nocker and Camper, 2006). At the same time when the cross-linking with DNA occurs, any unbound excess dye reacts with water molecules. The resulting hydroxylamine (Graves *et al*., 1981) is no longer reactive, preventing reaction of the dye with DNA extracted from intact cells (Nocker and Camper, 2009).

Viable PCR is a promising technique because it makes use of the speed and sensitivity of the molecular detection while at the same time providing viability information. EMA and PMA have since their invention been applied to a wide variety of microorganisms including bacterial vegetative cells (Rudi *et al*., 2005; Pan and Breidt, 2007; Cawthorn and Witthuhn, 2008; Bae and Wuertz, 2009; Delgado *et al*., 2009; Nocker *et al*., 2009; Viscogliosi *et al*., 2009; Agusti *et al*., 2010; Soejima *et al*., 2011), bacterial spores (Rawsthorne *et al*., 2009), fungi (Vesper *et al*., 2008), viruses (Graiver *et al*., 2010; Fittipaldi *et al*., 2012; Sanchez *et al*., 2012), yeast and

protozoa (Brescia *et al*., 2009; Fittipaldi *et al*., 2010). The addition of a pre-treatment step to the sample analysis to inhibit the amplification of DNA from membrane-damaged cells has been used in combination with end point PCR (Brescia *et al*., 2009), real-time or quantitative PCR (qPCR) (Rudi *et al*., 2005), reverse transcription PCR (Graiver *et al*., 2010), isothermal amplification (Lu *et al*., 2009; Chen *et al*., 2011), denaturing gradient gel electrophoresis (DGGE) (Nocker *et al*., 2007), terminal restriction fragment length polymorphism (TRFLP) (Rogers *et al*., 2008), microarray technology (Nocker *et al*., 2009), and next generation sequencing (Nocker *et al*., 2010). Apart from providing evidence of feasibility, the research has illustrated the urgent need for adding viability information to DNA-based detection methods in diverse fields ranging from testing of food and water safety to clinical microbiology.

Despite its advantages, there is evidence demonstrating that v-PCR using DNA-intercalating dyes has practical and theoretical limitations especially when applied to environmental samples (Wagner *et al*., 2008). Apart from sample-specific challenges, the two dyes both seem to have specific advantages and disadvantages. Whereas the greatest concern with EMA lies in its lack of specificity for intact bacterial cells, the greatest concern with PMA is the generation of false-positive signals due to incomplete signal suppression. For a successful application of v-PCR, the factors, which can influence the outcome of the resulting data, including the choice of the dye, its concentration, the incubation conditions, the light source, the presence of a high number of dead cells, the presence of high levels of suspended solids or biomass in the analyzed samples, the salt concentration in the reaction mix, the pH of the reaction mix, the length of the target gene; and the sequence of the target gene among others, have to be considered. These parameters and their potential implication to increase the chances of generating specific, reproducible, sensitive and accurate data. The consideration of the critical factors in the experimental design ultimately increase the chances of the integrating viable dyes in routine diagnostics.

Although molecular-based assays significantly reduce assay time, simplify detection procedure, and lower the detection limit, there remains one major drawback to circumvent–the inability to differentiate live bacteria from dead ones since both

dead and live cells could be amplified by DNA-based assays. Live bacteria are the primary target for food microbiological analysis rather than dead cells since they are the ones capable of causing foodborne infections in human. Therefore, these assays give potential false positive results. To achieve more reliable and accurate results, research on various live detection techniques (mRNA-based PCR), EMA and PMA techniques, have been undertaken and yielded promising results.

7.1 mRNA-based

One of these techniques is called reverse-transcriptase PCR (RT-PCR), utilizing mRNA as a cell viability marker. mRNA has a short half life (0.5 to 50 min) and degrades rapidly upon cell death, hence can be a good candidate for live bacteria detection (Takayama and Kjelleberg, 2000). Similarly to PCR, RT-PCR can also become real-time (qRT-PCR) by employing fluorescent dyes or probes. So far, there are RT-PCR and qRT-PCR assays developed for various foodborne pathogens, including *E. coli* (de Wet *et al.*, 2008), *Listeria. monocytogenes* (*L. monocytogenes*) (Klein and Juneja, 1997), *Salmonella* (Jacobsen and Holben, 2007; Gonzalez-Escalona *et al.*, 2009) and *Vibrio cholerae* (*V. cholerae*) (Bej *et al.*, 1996). However, live detection relied on mRNA is unreliable as mRNA stability and quantity is heterogeneous, depending on environmental conditions and intrinsic factors of the target gene. Besides, RT-PCR and qRT-PCR are generally of poor efficiency, specificity, and sensitivity when compared with DNA-based PCR. For instance, an investigation of RT-PCR for the detection of viable *E. coli* O157:H7 from environmental or food samples revealed that among several genes studied, only mRNA from the *rfbE* gene was reliable for live detection, however, a cell level up to 10^7 CFU was necessary (Jacobsen and Holben, 2007).

7.2 Nucleic acid dyes

Besides the utilization of mRNA, other live detection techniques involve the use of chemicals such as nucleic acid dyes: Ethidium bromide monoazide (EMA) and propidium monoazide (PMA) (Figure 14) as promising agents to discriminate live

cells from dead ones. These techniques are based on the membrane integrity of cells. EMA and PMA can enter only the membrane-compromised dead cells, upon photolysis by strong visible light, the azide group of EMA or PMA convert into a highly active nitrene which then covalently binds with DNA in dead cells (Figure 15), while the remaining free EMA is simultaneously degraded by reacting with water molecules. Cross-linking of EMA or PMA with DNA is reported to strongly inhibit PCR amplification of modified DNA, thus PCR or qPCR analysis coupled with EMA treatment could successfully eliminate false positive results by selectively excluding DNA from dead cells. In 2003, EMA was initially incorporated into PCR as an effective live detection assay by Nogva (Nogva *et al.*, 2003). Since then, EMA-PCR and EMA-qPCR have been developed for detecting a variety of viable bacteria, including *Campylobacter jejuni* (*C. jejuni*), *L. monocytogenes*, *E. coli* O157: H7, *S.* Typhimurium, *Vibrio. vulnificus* (*V. vulnificus*) and *Vibrio. parahaemolyticus* (*V. parahaemolyticus*) (Rudi *et al.*, 2005; Nocker and Camper, 2006; Wang and Levin, 2006; Wang *et al*., 2012). However, further studies revealed one major drawback of EMA-potential penetration into viable cells, depending on the bacterial species (Nocker and Camper, 2006; Flekna *et al.*, 2007; Cawthorn and Witthuhn, 2008). For example, EMA was reported to enter viable *E. coli* and cause 60% genomic DNA loss of log-phase viable cell (Nocker and Camper, 2006).

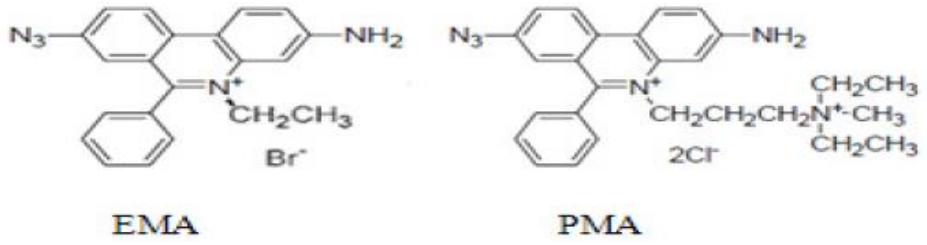

Figure 14 Chemical structures of EMA and PMA.

Source: Biotium (2009)

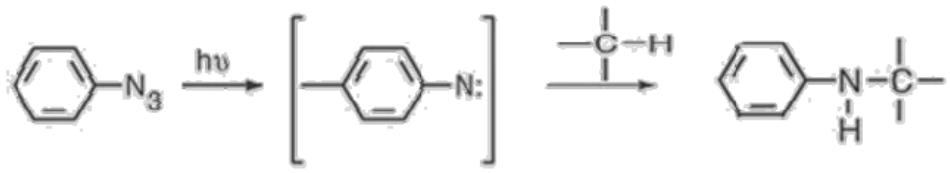

Figure 15 Photoactive cross-linking reaction of a simple azide.

Source: Invitrogen (2010)

8. Microorganism stress response

Stress refers to the imposition of detrimental nutritional conditions, toxic chemicals and suboptimal physical conditions or any deleterious factor or condition that adversely affects microbial growth or survival. According to this practical definition, many food processing treatments are considered stresses (Yousef and Juneja, 2002).

Stresses encountered by microorganisms vary in magnitude and outcome including mild stress, moderate stress and severe stress. The word “mild” describes sublethal stress levels that does not result in viability loss, but reduce or arrest growth rate. “Moderate” stress not only arrests microbial growth but also causes some loss in cell viability. “Extreme” or “severe” describes a stress level that is normally lethal to the cells, resulting in death of the majority of the population.

Stresses that food microbiota encounter include uncontrollable pre-harvest environmental factors (e.g., radiation and dry air) and the deliberate post-harvest application of preservation factors. Basicly, there are various deleterious factors provoking stress response in foodborne microorganisms at the links of the food chain, including production, processing, storage, distribution and consumption (Table 4).

Table 4 Deleterious factors likely to provoke stress response in foodborne microorganisms at various links of the food chain, including production, processing, storage, distribution and consumption.

Factor	Stage in the food chain pre-harvest (Environmental)	Processing	Storage and distribution	Consumption site
Heat shock	Weather-related Composting	Mild Processing	Temperature control failure	Cooking Reheating
Cold shock	Weather-related	Refrigeration	Refrigeration	Refrigeration fluctuation
Acidity	Acid rain Irrigation water Fermentation (e.g., silage production) Spoilage and decay (vegetation or product)	Food fermentations Additives (e.g., acidulents, organic acids, acidic salts)	Spoilage by acid producers	Acidic additives during food preparation (e.g. vinegar and lemon juice)
Osmotic shock	Soil salinity Irrigation water	Additives (e.g., salt) Concentration Dehydration		Additives in food preparation
Starvation	Non-nutritious environment	-	-	-
Oxidation	Air exposure of anareobic microbiota	Exposure to air Oxidative sanitizers	Exposure to air Oxidative sanitizers	Exposure to air

For stress response of microorganism, once cells sense a stress, the cells respond in various ways. Bacteria sense stresses that change membrane fluidity (e.g., cold shock), alter cell protein structure or disrupt ribosomes (e.g., heat), or affect nucleic acids (e.g., γ radiation). At the molecular level, stress response includes transcription leading to the synthesis of regulatory proteins. The resulting regulation may lead to the synthesis of other proteins that cope with the imposed stress. Microbial response to stress may produce these outcomes: production of proteins that repair damage, maintain the cell, or eliminate the stress agent; transient increase in resistance or tolerance to deleterious factors; cell transformation to a dormant state, i.e., spore formation or passage to the viable-but-not-cultarable state; evasion of host organism defenses and adaptive mutations (Yousef and Juneja, 2002).

For *Salmonella* stress response**,** cells must manage stresses ranging from acidic to basic pH, high to low osmolarity, high to low temperature, various types of oxidative stress and a variety of anti-microbial compounds encountered through its journey from the natural environment to an infected host. The defenses used to survive these encounters can be specific or can provide cross protection to a variety of hostile or stress conditions. Inside the host, *Salmonella* species escape the humoral immunity by invading professional and non professional phagocytes in which a new set of defense factors acts (Foster and Spector, 1995).

The ability of bacteria to sense and respond to unexpected changes in the environment is important for their survival. This is true for pathogens that not only encounter potentially lethal environmental extremes in the natural environment but must also resist a battery of host defense factors (Foster and Spector, 1995). Under adverse cultural or environmental conditions such as nutrient limitation, changes in temperatures, pH, etc., bacteria activate “stress responses” that substantially improve their chances of survival in unfavorable environments (CJ, 1994). These responses are particularly manifested to host defense systems during infection, lethal or sub lethal inhibitory effects of chemical/physical treatments with disinfectant and heat treatment and conditions in preserved and minimally processed food.

Thus, even though the foods receive heat treatment or treatment with acids, additives or preservatives to suppress bacterial metabolism, it may be insufficient and would enable bacteria to survive and cause spoilage or food-borne illness in consumers. Simply regulating the temperature or increasing the concentrations of the acid or preservatives is not an easy solution as consumers demand minimally processed and additive free products (Abee and Wouters, 1999).

Bacterial response to one stress can increase resistance to other stresses (cross protection) and can change a number of other aspects of bacterial metabolism. One such effect can be increased potential to interact with hosts including the ability to invade and colonize hosts during food-borne disease. Thus, stress can produce more resistant, persistent and dangerous pathogens. All pathogenic bacteria possess the ability to evade or surmount body defenses, long enough to cause a sufficient reaction, which is then manifested clinically as the disease or illness, while opportunistic pathogens will cause illness in the event of the pre-disposal weakness in these defenses. This is particularly true of gastrointestinal pathogens such as *L. monocytogenes* and *Salmonella* species which must circumvent many different stresses in order to arrive at the site of infection. This includes the acid barrier of the stomach, the physical barrier of the epithelial cells lining the gastrointestinal tract and various immune defenses including the initial onslaught of macrophages. These organisms have developed elaborate systems for sensing stress and for responding to those stresses in a protective fashion (Gahan and Hill, 1999).

8.1 Acid stress response

One well characterized adaptive response is to acid stress. The acid stress response (ASR) is the complex phenomenon, involving a number of changes in the proteins expression and many associated events at the level of gene regulation. A number of molecular approaches have identified numerous interesting genes involved both in sensing and responding to stress and in virulence. Acidic pH is one of the most frequent stress condition encountered by microbial systems. Acid mine drainage, acid rain, and weak acids produced by microorganisms themselves all

contribute to acid stress. The ability to survive and even to flourish during these conditions is crucial to survival. Some microorganisms (acidophiles) have evolved to the point where the preferred ecological niche is an extremely acidic environment (Foster and Spector, 1995).

S. Typhimurium is a neutrophilic bacterium that can grow over a wide range of pH conditions (pH 5-9) because of physiologically triggered pH homeostasis mechanisms (Foster, 1991). Mechanisms of pH homeostasis include the use of H^+ antiport systems to maintain the internal pH at a relatively constant level (~7.6) over a wide range of external pH conditions (Foster and Hall, 1990). *S.* Typhimurium is capable of growing in minimal media with a pH as low as 5.0 but below pH 4.0 the cells undergo a rapid acid death apparently due to inability to maintain internal pH suitable for viability. However, recently it has been demonstrated that *S.* Typhimurium can be adapted to survive this harsh environment (Foster, 1991).

This organism is shown to have acid stress management including an inducible pH homeostasis system that functions beyond normal constitutive pH homeostasis. The acidification tolerance response has been reported to occur if cells are pre-incubated (pre-shock-adapted) at pH levels near 6 (optimum, pH 5.8) for one hour prior to exposure at pH 3.3 (acid shock). Under these conditions, survival of adapted cells is 100 to 1,000 fold better than that of unadapted cells. In one of the studies, analysis of polypeptide profiles with polyacrylamide gel electrophoresis (PAGE) has revealed that 18 polypeptides change during pH 5.8 pre-shock exposure. Of these, 12 are induced and 6 are reported to be repressed during adaptation. This is controlled by the ferric uptake regulator (Fur). Thus, it was found that, exposure to stress required protein synthesis and as such represented a newly described genetic response to acidic stress. It has been shown that mutations in the *fur* locus eliminate induction of several acid inducible genes. They also prevent synthesis of inducible pH homeostasis system and thus confer an extremely acid sensitive phenotype on the cell. Fur has been reported to sense internal pH directly (Foster, 1991).

8.2 Peroxide stress response

The inducible systems in bacteria, that protect against oxidative stresses are either OxyR or SoxRS regulated and are switched on by hydrogen peroxide and superoxide, respectively (Carmel-Harel and Storz, 2000). The detoxification of superoxide is a two step process in *Salmonella*; the first step involves the conversion of superoxide to hydrogen peroxide by superoxide dismutases, and the second step is a catalase mediated destruction of hydrogen peroxide. The consequent accumulation of hydrogen peroxide is known to activate the regulator OxyR which then leads to the transcriptional activation of the genes involved in the thioredoxin and glutaredoxin systems. These systems restore protein functions by reducing oxidized residues. After treatment with H_2O_2, OxyR activates the expression of many genes which have clear antioxidant roles. The protection against the toxic effects of peroxides is provided by hydroperoxidase I (katG) and alkyl hydroperoxide reductase (ahpCF), which directly eliminate the oxidants In addition, several of the genes of the oxyR regulon in *S.* Typhimurium have been found to be induced by the macrophage environment, including *ahpCF* and *dps* (Francis *et al*., 1997). *S.* Typhimurium strains carrying mutations in slyA (a transcriptional regulator), *rpoS*, and *recA* (a positive regulator of the SOS DNA repair pathway) showed increased sensitivity to reactive oxygen species as well as attenuated virulence in mice (Atlung and Brondsted, 1994).

8.3 Temperature stress response.

Temperature is one of the major stresses faced by all living organisms. As refrigeration is commonly used method for extending the shelf life of food, it is necessary to understand the cold stress response of the food-borne pathogens. *S.* Typhimurium is a major food-borne microbial pathogen which primarily contaminates poultry products causing salmonellosis in humans. Following processing poultry products are typically stored under refrigerated conditions until consumed. Therefore, the ability of this pathogen to survive at cold temperatures is of concern. Also, this pathogen has been reported to survive in food products held at

cold storage at 5°C for up to 8 months and can cause diarrhea in humans if contaminated food is consumed raw or undercooked.

S. Typhimurium cultures, when transferred from 37ºC to 5ºC or 10ºC, showed an initial lag period in growth with an approximate generation time of 10 to 25 h. The changes in cellular physiology and changes in protein expression profiles in response to cold temperatures have been demonstrated in a number of microorganisms including *E. coli, S.* Enteritidis and *V. vulnificus* (Bryan *et al*., 1999). The ability of these microorganisms to survive in cold temperatures requires proteins termed cold shock proteins (CSPs), which are synthesized at the onset of cold temperature shift (Thieringer *et al*., 1998). The *cspA* gene that codes for major cold shock protein, CSP, has been cloned, sequenced and characterized in *E. coli* and *S.* Enteritidis (Newkirk *et al.*, 1994).

MATERIALS AND METHODS

Materials

1. Bacterial strains

A total of 80 strains of *Salmonella* spp. and 20 strains of non-*Salmonella* spp. used in this study were donated from the following culture collection sources:

1.1 Department of Medical Science, Ministry of Public Health, Bangkok, Thailand: *S*. Paratyphi A DMST 15673 (A), *S*. Virchow DMST 17239 (B), *S.* Enteritidis DMST 15676 (D1), *S.* Enteritidis (10 strains), *S*. Fresno DMST 19197 (D2), *S.* Aberdeen DMST 19198 (F), *S*. Poona DMST 19199 (G1), *S*. Cerro DMST 19200 (K), *S.* Minnesota DMST 19201 (L), *S*. Chicago DMST 19202 (M), *S*. Alachua DMST 19203 (O), *S*. Bangkok DMST 7121 (P), *S*. Wandsworth DMST 19204 (Q), *S*. Waycross DMST 19205 (S), *S. enterica* subsp. *salamae* ser. 17:gt:- (U), *S*. Bergen DMST 19206 (X), *S*. Enteritidis (10 strains), *E. coli* O124 DMST 672 (Enteroinvasive *E. coli*, EIEC), *E. coli* O18 DMST 652 (Enteropathogenic *E. coli*, EPEC), *E. coli* O126 DMST 674 (EPEC), *E*. coli O6 DMST 644 (Enterotoxigenic *E. coli*, ETEC), *E. coli* O168 DMST 693 (ETEC), *E. coli* O111 DMST 667 (Veroxytotoxin-producing *E. coli*, VTEC), *E. coli* O157:H7 DMST 12743 (VTEC), *E.coli*, *Citrobacter freundii* (*C. freundii*) DMST 16368, *Enterobacter aerogenes* (*E. aerogenes*) DMST 8841 (ATCC 13048), *Klebsiella pneumoniae* (*K. pneumoniae*) DMST 7592 (ATCC 700603), *Pseudomonas fluorescens* (*P. fluorescens*) DMST 16077, *Shigella sonnei* (*S. sonnei*) DMST 17564 and *Yersinia enterocolitica* (*Y. enterocolitica*) DMST 8012

1.2 Department of Food Science and Technology, Faculty of Agro-Industry, Kasetsart University: *S.* Agona (B), *S*. Derby (B), *S*. Schwarzengrund (B), *S*. Stanley (B), *S. enterica* subsp. *enterica* ser. 1,4,5,12:i:- (B), *S. enterica* subsp. *enterica* ser. 4, 12:i:- (B), *S. enterica* subsp. *enterica* ser. 4,5,12:i:- (B), *S.* Montevideo (C1), *S.* Tennessee (C1), *S.* Newport (C2-3), *S.* Amsterdam (E1), *S.* Anatum (E1), *S*. Orion

(E1), *S.* Orion var15^{+} (E1), *S.* Weltevreden (E1), *S.* Senftenberg (E4), *S.* Kedougou (G2), *S.* Worthington (G2), *S.* Rissen (C1) (10 strains), *S.* Braenderup (7 strains), *S.* Panama (10 strains), *S.* Corvallis (6 strains), *Proteus mirabilis* (*P. mirabilis*), *Lactobacillus plantarum* (*L. plantarum*) PD110 and *L. monocytogenes* V7

1.3 Microbiological Resources Centre, Institute of Scientific and Technological Research, Patumthanee, Thailand: *Bacillus cereus* (*B. cereus*) ATCC 11778, *E. coli* ATCC 8739, *S.* Typhimurium ATCC 13311 (B), *S.* Typhimurium DT104 2486 (B), *S.* Typhimurium DT104 2572 (B), *S.* Typhimurium DT104 2582 (B) and *S. aureus* ATCC 13565

1.4 Department of Microbiology, Faculty of Public Health, Mahidol University, Bangkok, Thailand: *V. parahaemolyticus* VP293

2. Culture media

2.1 Buffered Peptone Water (Oxoid, UK)
2.2 Lysine Iron Agar (Oxoid, UK)
2.3 Nutrient Agar (Merck Laboratories, Darmstadt, Germany)
2.4 Nutrient Broth (Merck Laboratories, Darmstadt, Germany)
2.5 Stock cell culture media (Merck Laboratories, Darmstadt, Germany)
2.6 Tetrathionate Broth (Oxoid, UK)
2.7 Triple Sugar Iron Agar (Oxoid, UK)
2.8 Trypticase Soy Broth (Merck Laboratories, Darmstadt, Germany)
2.9 Xylose Lysine Deoxycholate (Merck Laboratories, Darmstadt, Germany)
2.10 API Test Kits (Merck Laboratories, Darmstadt, Germany)

3. Chemicals

3.1 Chemicals for DNA extraction

3.1.1 Ethanol (Merck Laboratories, Darmstadt, Germany)

3.1.2 Ethylenediaminetetraacetic acid (UNIVAR, Australia)

3.1.3 Isoamyl alcohol (Merck Laboratories, Darmstadt, Germany)

3.1.4 Sodium chloride (UNIVAR, Australia)

3.1.5 Sodium dodecyl sulphate (SDS, Agax Finechem, Australia)

3.1.6 Tris-(hydroxymethyl)-aminomethane (Riedel-deHaen, Germany)

3.1.7 10 mM Tris-Hydrochloric acid buffer containing 1 mM EDTA, pH 8.0 (TE Buffer, Sigma, USA)

3.2 Chemicals for PCR reaction

3.2.1 Deionized water

3.2.2 Deoxynucleotide triphosphate (dNTP, New England Biolab, USA)

3.2.3 Magnesium chloride (QIAGEN, Germany)

3.2.4 PCR buffer (QIAGEN, Germany)

3.2.5 Primer (National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand)

3.2.6 *Taq* polymerase (QIAGEN, Germany)

3.3 Chemicals for PCR products detection

3.3.1 Agarose (low melting temperature, BioWhittaker Molecular Applications, USA)

3.3.2 Ethidium bromide monoazide (Sigma, USA)

3.3.3 Ethidium bromide (Fluka, Switzerland)

3.3.4 Lambda/*Hind*III/*Eco*RI DNA marker (Fermentus, Germany)

3.3.5 Loading dye (bromophenol blue, Bio-Rad, USA)

3.3.6 Tris-acetate buffer containing 1 mM EDTA (Sigma, USA)

3.3.7 40 mM Tris-acetate buffer containing 1 mM EDTA, pH 8.0 (TAE buffer, Sigma, USA)

3.4 Chemicals for stress treatment

3.4.1 Sodium hydroxide (Merck Laboratories Germany)

3.4.2 Hydrochloric acid (Merck Laboratories, Germany)

3.4.3 Hydrogen peroxide (Merck Laboratories, Germany)

3.4.4 Sodium hypochlorite (Merck Laboratories, Germany)

3.4.5 Phosphate buffer saline pH 7 (PBS buffer, Sigma, USA)

4. Equipments

4.1 Coarse balance (Sartorius BA-1602, Japan)

4.2 Laboratory blender stomacher (Seward model BA 7021, UK)

4.3 Vortex mixer (model Genie II, USA)

4.4 Thermal cycler PCT 200 (MJ Research, USA)

4.5 pH meter (Microcomputer pH-vision 6071, China)

4.6 Microcentrifuge (Spectrafuge, USA)

4.7 Gel documentation system (Vilber Lourmat, France)

4.8 UV illuminator (Spectorline, USA)

4.9 Ultraviolet lamp (Sylvania, USA)

4.10 Gel electrophoresis system (Bio 101, USA)

4.11 Laminar flow cabinet (Gelman Science model no. CF 43S, UK)

4.12 Incubator (Memmert model 700 D 06063, Germany)

4.13 Refrigerator (Sharp model SJ-51G, Thailand)

4.14 Microwave (Whirlpool model VIP 27, Sweden)

4.15 Water bath (Memmert, Germany)

4.16 Tungsten halogen lamp (Philips, USA)

Methods

The methods are divided into 3 parts according to the results and discussion.

1. The consistency of primer 3-based RAPD-PCR for various *Salmonella* serotypes detection

Total 80 strains of *Salmonella* spp. and 20 strains from non-*Salmonella* spp. (as previously described in the materials) were used in this study. All strains were detected by random amplified polymorphic DNA (RAPD) PCR analysis. The DNA patterns from RAPD-PCR were analysed comparing between 38 strains of *Salmonella* spp. and 20 strains from non-*Salmonella* spp. Moreover, the reproducibility of RAPD-PCR was studied by the analysis of RAPD-PCR product patterns from 5 serotypes of *Salmonella* spp.

1.1 Bacterial Cultivation

The cultures were maintained in stock cell culture media and stored at 4°C. Stock cell culture medium was prepared by using 10 g proteose peptone, 5 g meat extract, 3 g yeast extract, 5 g sodium chloride, 0.8 g di-sodium hydrogenphosphate anhydrous, 10 g agar per liter of distilled water (WHO National *Salmonella* and *Shigella* Center). The cultures were grown overnight in nutrient broth (NB) (Merck, Germany) at 37°C. For cultivation**,** a single colony of each bacterial pure culture grown on nutrient agar (NA) was inoculated into 10 ml of freshly prepared NB. After aerobically overnight incubation at 37°C, a loopful of each culture suspension was transferred to 10 ml NB and incubated further at 37°C for 18 h. Each bacterial culture used was serially ten-fold diluted with 0.1% Buffered Peptone Water (BPW) to obtain a final concentration of approximately 10^6 CFU/ml. The bacterial cell enumeration as colony forming unit CFU/ml was determined by spread plating with appropriate dilutions onto duplicate plates of NA and the inverted plates were then incubated aerobically at 37°C for 24 h. The experiment was done in triplicate.

1.2 Random Amplified Polymorphic DNA (RAPD) PCR analysis

1.2.1 Primer

The 20-mer oligonucleotides (named primer 3: 3'-GGAGAGTAACGGGTAGCGCC-5') used to screen for RAPD-PCR was randomly designed from the nucleotide sequence of *S.* Typhimurium IFO 12529 *gat*D gene encoding a galactitol-1-phosphate dehydrogenase (Trevanich *et al.*, 2010). This single primer was synthesized by the National Center for Genetic Engineering and Biotechnology (BIOTEC, Thailand).

1.2.2 DNA extraction

One millilitre of bacterial culture was taken to 1.5 ml microtube. The cells were centrifuged at 14,000 rpm, 4°C for 3 min. The pellets were washed twice with 100 µl of TE buffer and resuspended in 100 µl of TE buffer, after the supernatants were discarded. For DNA extraction, the simple boiling was done according to Miyamoto *et al*. (1998) with some modifications. The cells were lysed by boiling in a water bath for 5 min. After centrifugation, the supernatant of the extracted DNA was kept at -20°C until analysis.

1.2.3 RAPD-PCR amplifications

The PCR mixture consisted of 500 ng of template DNA, 50 ng of primer 3, 2 mM of Magnesium chloride, 0.2 mM of each deoxynucleotide triphosphate and 1 unit of *Taq* DNA polymerase (QIAGEN, Germany) and deionized water for a final volume of 25 µl. The RAPD-PCR reaction was performed using a PCT 200 thermocycler (MJ Research, USA). The amplification conditions were as follows: 30 cycles at 95°C for 1 min (DNA denaturation), 50°C for 30 s (primer annealing), 72°C for 30 s (primer extension). The positive control was a PCR mixture containing template DNA and the negative control was PCR mixer without DNA template.

1.2.4 RAPD-PCR products analysis

The amplified RAPD-PCR products were analyzed by agarose gel electrophoresis. Ten microliters of each amplification sample were mixed with DNA-loading solution (Bio-Rad, USA) and loaded onto 1.0% agarose gel (BioWhittaker Molecular Applications, USA). Electrophoresis was run in 1 × TAE buffer at a constant voltage of 50 V for 1.5 h. The agarose gel was stained in 10 µg/ml ethidium bromide solution and visualized with UV illuminator using a Gel Documentation System (Vilber Lourmat, France). Lambda/*Hind*III/*Eco*RI DNA (Fermentas, USA) was used as a DNA molecular weight marker to determine the molecular weight of amplified DNA.

2. The efficiency of primer 3-based RAPD-PCR for detection *Salmonella* spp. in various *in vitro* stress treatments and primer 3-based RAPD-PCR involving culturing step for detection in food process models

2.1 Bacterial strain

The multi-drug resistant (Streptomycin, Sulphonamides, Vancomycin, Gentamicin and Ampicillin) *S.* Enteritidis type strain DMST 15676 obtained from Department of Medical Sciences, Ministry of Public Health, Thailand was selected as representative to test in this study. The culture was grown at 37°C for 18 h in NB (Merck, Germany) to stationary phase. Viable counts were obtained by plating an appropriate dilution made in 0.1% BPW onto NA and incubated at 37°C for 24 h. The population of the culture was estimated by calculating the average number of the colony-forming units from five agar plates.

2.2 *In vitro* stress treatments

One millilitre of approximately 1.2×10^8 CFU/ml of *S.* Enteritidis culture was treated by eight *in vitro* stress treatments. After that, one millilitre of stressed culture with percentage survival of ≥90%was taken to 1.5 ml microtube. The cell

suspension was centrifuged at 14,000 rpm 4°C for 3 min and the cell pellet was washed twice with TE buffer. After centrifugation and discarding the supernatant, one hundred microlitres of TE buffer were added to the cell pellet. The suspension was boiled for 5 min for DNA extraction. Then, DNA was used directly as a template for RAPD-PCR analysis as described in 1.2.3. The RAPD-PCR products were loaded onto gel electrophoresis and detected under UV illumination as described previously. All treatments were performed in triplicate. The *in vitro* stress treatments performed in this study were as follows.

2.2.1 UV light stress treatment

S. Enteritidis culture was placed in a sterile petri dish with the lid removed. The petri dish was exposed to the UV irradiation 254 nm light source (1,200 mW/cm^2) with 5 cm distance for 20 min at 25°C (McKillip *et al*., 1998).

2.2.2 Ethanol stress treatment

S. Enteritidis culture was inoculated to 10 ml of 50% ethanol for 5 min (Ngwai *et al*., 2007).

2.2.3 Acidic stress treatment

S. Enteritidis culture was inoculated to 10 ml hydrochloric acid (HCl) pH 4.0 for 5 min (Ngwai *et al*., 2007).

2.2.4 Alkaline stress treatment

S. Enteritidis culture was inoculated to 10 ml sodium hydroxide (NaOH) pH 10 for 5 min (Sampathkumar *et al*., 2003).

2.2.5 Hydrogen peroxide stress treatment

S. Enteritidis culture was inoculated to 10 ml hydrogen peroxide solution (0.38 mM) for 5 min (Wong *et al*., 1994).

2.2.6 Sodium hypochlorite stress treatment

S. Enteritidis culture was inoculated to 10 ml sodium hypochlorite solution (200 ppm) for 5 min (Ngwai *et al*., 2007).

2.2.7 Heat stress treatment (52°C)

S. Enteritidis culture was inoculated to 10 ml NB and incubated at 52°C for 15 min (Kobayashi *et al*., 2005).

2.2.8 Cold stress treatment

S. Enteritidis culture was inoculated to 10 ml NB and stored at 0°C and 4°C for 24h.

2.2.9 The survival test after stress treatments

After *S.* Enteritidis cultures were treated with each stress treatment as described previously, the cell survival was checked by plating technique on NA. One hundred microlitres of treated samples were washed twice by PBS and counted aerobically onto NA at 37°C for 24 h. Then, all stressed samples were also detected for *S.* Enteritidis by RAPD-PCR. The colony on NA was counted and the percentage survival was calculated as following:

$$\frac{\text{No. of viable cells counted on NA after stress treatment}}{\text{No. of viable cells counted on NA before stress treatment}} \times 100$$

2.3 Stress treatments in various food process models

2.3.1 Cold stress treatments of fresh ground chicken meat

S. Enteritidis type strain DMST 15676 was grown in NB to stationary phase at 37°C for 18 h. The culture was centrifuged at 14,000 rpm for 3 min and resuspended in 5 ml of PBS. After washing with PBS twice, the cells were diluted to ten-fold serial dilutions in sterile 0.1% (w/v) peptone solution and plated in duplicate on NA for enumeration. Before bacterial inoculation, the perchased fresh ground chicken meat was tested for *Salmonella* spp. by RAPD-PCR (as described in 1.2.3) involving 20-h culturing in NB and conventional method as described in 2.3.2.2 to confirm that the meat sample used was not contaminated with *Salmonella* spp. After that, one millilitre of the cell culture at a final concentration of approximately 1.2×10^8 CFU/g was inoculated to each fresh ground chicken meat sample (10 g) in each sterile flask. After mixed thoroughly, the samples were chilled at 4°C and frozen at 0°C and -20°C for 1,5 and 10 days. The experiments were triple repeated. To detect for *S.* Enteritidis gene by RAPD-PCR, 10 g samples were taken to 90 ml of sterile NB, then incubated at 37°C for 20 h. Afterthat, one millilitre of the sample was taken to 1.5 ml microtube. The cell suspension was centrifuged at 14,000 rpm, 4°C for 3 min and the cell pellet was washed twice with TE buffer. One hundred microlitres of TE buffer were added to the cell pellet. The suspension was boiled for 5 min for DNA extraction. Then, DNA was analysed for RAPD-PCR. All treatments were performed in triplicate.

2.3.2 Acid stress treatments in Thai traditional fermented pork and chicken meat (Nham)

2.3.2.1 Nham preparation

Ground pork and chicken meat were brought from local fresh market near Kasetsart University at Jatujak district, Bangkok. To confirm that the meat samples used for Nham fermentation were not contaminated with *Salmonella* spp., the meat samples were presumptively determined for *Salmonella* spp. by RAPD-PCR involving 20-h culturing in NB and conventional method. Afterthat, the non-

Salmonella spp. contaminated meat samples were used for making Nham. Thai traditional Nham recipe (unpublished data) consisted of ground pork or chicken meat (91.4%), minced garlic (5%), cooked rice (1.6%) and salt (2.0%). Three batches of each Nham were prepared as following. The ground pork or chicken meat was mixed thoroughly with minced garlic, cooked rice and salt. The samples were inoculated with the different inoculum levels in order to achieve the final concentrations (1.1×10^2, 1.1×10^4 and 1.1×10^6 CFU/g) of *S.* Enteritidis DMST 15676. The samples were fermented at 30±1ºC for 5 days. The samples of each Nham after 0, 1, 2, 3, 4 and 5 days of fermentation were taken for *Salmolnella* detection by RADP-PCR involving 20 h pre-enrichment compared to the conventional method. and pH measurement. For *Salmonella* spp. detection from Nham sample, the 10 g of sample was placed in 90 ml of NB and mixed thoroughly by stomacher. All treatments were performed in triplicate.

2.3.2.2 *Salmonella* analysis (ISO 6579 (2002) with some modification)

To detect *Salmonella* spp.before and after fermentation, meat sample or Nham sample (10 g) was placed in 90 ml of NB. The sample was incubated at 37ºC for 20 h. A 1 ml of aliquot of NB enrichment broth was transferred to 10 ml each of the tetrathionate (TT) broth and 1 ml of NB broth was taken to detect the presence of *S.* Enteritidis gene. The TT broth was incubated at 37ºC. After 24 h incubation, one loopful of each broth was streaked onto double plates of XLD. The plates were incubated at 37ºC for 24 h and examined for the presence of typical *Salmonella* colonies. Three suspected colonies from each plate were inoculated onto triple sugar iron agar (TSI) and lysine iron agar (LIA) slant. After 24 h of incubation at 37ºC, the isolates with typical *Salmonella* were confirmed by API test kits (ISO, 2002).

2.3.2.3 pH measurement

In order to prepare the samples for analysis, after removed the polyethylene case, 10 g Nham were thoroughly ground and mixed with 5 ml sterile distilled water until the homogeneous samples were obtained. The pH values of samples were determined by using a standard pH meter (Microcomputer pH-vision 6071, China) according to AOAC (2002).

3. The development of primer 3-based EMA-RAPD-PCR involving culturing step to discriminate viable *Salmonella* cells from dead cells

3.1 Bacterial Cultivation

S. Typhimurium ATCC 13311, *S.* Enteritidis DMST 15676, *S. sonnei* DMST 17564, *E. coli* O124 DMST 672, *E. aerogenes* DMST 8841, *K. pneumoniae* DMST 7592, and *C. freundii* DMST 16368 were used throughout this study. For cultivation**,** a single colony of each bacterial pure culture grown on NA was inoculated into 10 ml of freshly prepared NB. After aerobically overnight incubation at 37°C, a loopful of each culture suspension was transferred to 10 ml NB and incubated further at 37°C for 18 h. Each bacterial culture used was serially ten-fold diluted with 0.1% BPW to obtain a final concentration of approximately 10^6 CFU/ml. The bacterial cell enumeration as colony forming unit CFU/ml was determined by spread plating with appropriate dilutions onto duplicate plates of NA and the inverted plates were then incubated aerobically at 37°C for 24 h. The experiment was done in triplicate.

3.2 Heat treatment of bacterial cells

Microcentrifuge tubes (1.5 ml capacity) containing 1.0 ml of 1.2×10^6 cells suspended in sterile 0.9% NaCl solution were heated at 72°C in a water bath for 15 min as previously described by Nocker and Camper (2006). The heat-treated tubes were immediately cooled to room temperature (RT). The absence of viable cells was presumptively determined by streaking onto NA and then, the cell culture was aerobically incubated at 37°C for 18 h.

3.3 Determination of the minimum amount of EMA to inhibit DNA amplification of dead *Salmonella* cells

Two *Salmonella* serovars (*S.* Typhimurium ATCC 13311 and *S.* Enteritidis DMST 15676) were used as model microorganisms in this study. Ethidium bromide monoazide (EMA) (cat. no: E2028, Sigma, U.S.A.) was dissolved in sterile deionized water for making a stock EMA solution and stored at -20°C in the dark until use. EMA from the stock solution was added to each microcentrifuge tube containing 1.0 ml of 1.2×10^6 cells at the final concentrations of 1.0, 2.0, 3.0, 4.0 and 5.0 μg/ml, respectively. The tubes were agitated and then placed in the dark at RT for 5 min, set into crushed ice with their lids off, and exposed to light from a Tungsten halogen lamp (650 W) for 5 min at a fixed distance of 20 cm to activate and photolyse EMA as described by Nocker and Camper (2006). Each sample after EMA-treatment was used to perform RAPD-PCR as described elsewhere below. Positive control was viable *Salmonella* cells without EMA treatment and negative control was PCR reaction mixture without target DNA template. The experiment was done in triplicate. Comparison to no EMA treatment, the toxicity to viable cells of *S.* Typhimurium and *S.* Enteritidis after EMA treatment at various concentrations tested was determined by colony counting on NA.

3.4 Optimization of light exposure time and distance to activate and photolyse EMA

To optimize the light exposure time for cross linking of dead *Salmonella* DNA to EMA, the viable and heat-killed cells of *S.* Typhimurium ATCC 13311 and *S.* Enteritidis DMST 15676, which were treated with 3.0 μg/ml. EMA, were exposed to the Tungsten halogen lamp (650 W) for 5, 10 and 15 min, respectively at a fixed distance of 20 cm. To optimize the light exposure distance in EMA photolysis, the viable and heat-killed cells treated with 3.0 μg/ml EMA were exposed to the Tungsten halogen lamp for 5 min at different distances of 10, 20 and 30 cm, respectively. The experiment was carried out in triplicate.

3.5 Effect of EMA on the ratios of viable and dead cells

To evaluate the effect of the proportion of viable cells and dead cells on the EMA-RAPD-PCR technique for detection of *Salmonella* spp., heat-killed *S.* Enteritidis DMST 15676 cells were mixed with the viable *S.* Enteritidis cells in the ratios of the viable cells representing 100, 1.0, 0.1 and 0.01% of the total concentration of 1.3×10^6 cells, respectively. After EMA treatment (3.0 µg/ml EMA, 20 cm and 5 min light exposure), the resulting cells were centrifuged at 14,000 rpm at 4°C for 3 min prior to DNA extraction for RAPD-PCR amplification.

3.6 Detection of *Salmonella* cells in artificially contaminated chicken meat by EMA-RAPD-PCR

Chicken meat was decontaminated by autoclave at 121°C for 15 min. A sample of 25 g of autoclaved chicken was aseptically transferred into 225 ml of NB, processed in a stomacher homogenizer (Seward model BA7021) at medium speed for 2 min, and tested for the absence of *Salmonella* spp. by the conventional method (ISO, 2002) with some modifications. The homogenized sample used as positive control was artificially contaminated with viable *S.* Enteritidis DMST 15676 at a final concentration of approximate 2.2×10^6 CFU/ml, while negative control was prepared by using dead *S.* Enteritidis DMST 15676 at the same concentration instead of viable one. Similarly, the other homogenized samples were spiked separately with the viable and dead cells of each range of mixed bacterial cultures in the ratio of 1:1 as follows: *S.* Enteritidis DMST 15676 and *S. sonnei* DMST 17564 or *E. coli* O124 DMST 672 or *E. aerogenes* DMST 8841 or *K. pneumoniae* DMST 7592 or *C. freundii* DMST 16368. Each sample was then incubated at 37°C for 20 h. After pre-enrichment step, a 1.0 ml portion of each artificially contaminated chicken sample was centrifuged at 14,000 rpm, 4°C for 3 min to harvest the cells. The obtained pellets were subsequently washed twice with sterile 0.9% NaCl solution and centrifuged at 14,000 rpm, 4°C for 3 min. A 0.5 ml of sterile 0.9% NaCl solution was added to resuspend. The suspensions were treated with EMA at a final concentration of 3.0 µg/ml for 5 min and then placed in the dark at RT for 5 min. The samples were placed on ice with

the caps open to avoid excessive heating and exposed to Tungsten halogen lamp (650 W) for 5 min at a distance of 20 cm. The EMA-treated samples were centrifuged at 14,000 rpm, 4°C for 3 min. The resulting precipitate was washed twice with 100 µl of TE buffer and centrifuged at 14,000 rpm, 4°C for 3 min. The precipitate was resuspended in 100 µl of TE buffer and boiled in water bath for 5 min. The suspension was centrifuged at 14,000 rpm, 4°C for 3 min and the supernatant (5 µl) was directly used as the template DNA solution for the RAPD-PCR analysis.

3.7 Detection of *Salmonella* spp. in naturally contaminated chicken products by EMA-RAPD-PCR

The total of 240 chicken products including 60 of chicken breasts, 60 of leg muscles, 60 of gizzard, and 60 of eggs, were purchased from three local fresh markets and three local supermarkets nearby Bangkhen District, Bangkok Province. Briefly, 25 g of each sample was homogenized in 225 ml of NB with a stomacher homogenizer at a medium speed for 2 min. The homogenates were incubated at 37°C for 20 h. A 1 ml aliquot of the homogenate was removed for detecting the presence of *Salmonella* spp. by the conventional method. For the EMA-RAPD-PCR analysis, 1.0 ml of the homogenate was taken out and pelleted by centrifugation at 14,000 rpm at 4°C for 3 min, subsequently washed twice in sterile 0.9% NaCl solution and resuspended in 0.5 ml of sterile 0.9% NaCl solution. The resulting suspension was then treated with EMA at optimized conditions as previously described above. DNA extraction of the EMA-treated sample was also prepared as previously mentioned. The supernatant (5 µl) obtained was directly used as a template DNA solution for the EMA-RAPD-PCR analysis.

3.8 Detection of *Salmonella* spp. by conventional method (ISO, 2002) with some modifications

A 25 g of each sample was homogenized with 225 ml NB and incubated at 37°C for 24 h. A 1.0 ml portion of the pre-enrichment was transferred into 10 ml TT broth and incubated at 37°C for 24 h. Samples from the TT selective enrichment

broth were streaked on XLD agar and incubated at 37°C for 24 h. Suspected *Salmonella* spp. colonies with good isolation were randomly selected and examined further in TSI and LIA slants.

RESULTS AND DISCUSSION

1. The consistency study of RAPD-PCR for various serotype *Salmonella* spp. detection

The major disadvantages of conventional methods used for *Salmonella* detection are time-consuming and labor-intensive, and are not proper for monitoring of the pathogenic contamination in the process line food products. Therefore, polymerase chain reaction (PCR), a molecular technique, is widely used in the identification and the rapid detection of *Salmonella* spp. in foods. In this study, 20-mer oligonucleotide primer 3-based random amplified polymorphic DNA (RAPD) PCR was used for the rapid detection of *Salmonella* spp. in foods. This part aimed to study the consistency of the single primer-based RAPD-PCR for the detection of various *Salmonella* serotypes.

1.1 The detection of various *Salmonella* spp. by RAPD-PCR

The primer used in this study was the single primer on the sequence of *S.* Typhimurium IFO 12529 *gatD* gene, which was single-stranded DNA containing 20-mer oligonucleotides; named primer 3: 3'-GGAGAGTAACGGGTAGCGCC-5' (Trevanich *et al*., 2010). The consistency of this single primer in RAPD-PCR method for detection of *Salmonella* spp. was studied. Total 38 *Salmonella* serotypes and 13 non-*Salmonella* spp. (as previously described in the materials) were used in this study. Figure 16, 17 and 18 showed the RAPD-PCR band patterns from 38 strains of *Salmonella* spp. Between two to six prominent DNA bands were produced, RAPD-PCR products showed the similar DNA band (approximated 770 bp) obtained from various strains of *Salmonella* spp. with primer 3. The results indicated that primer 3 was highly specific for all *Salmonella* strains used.

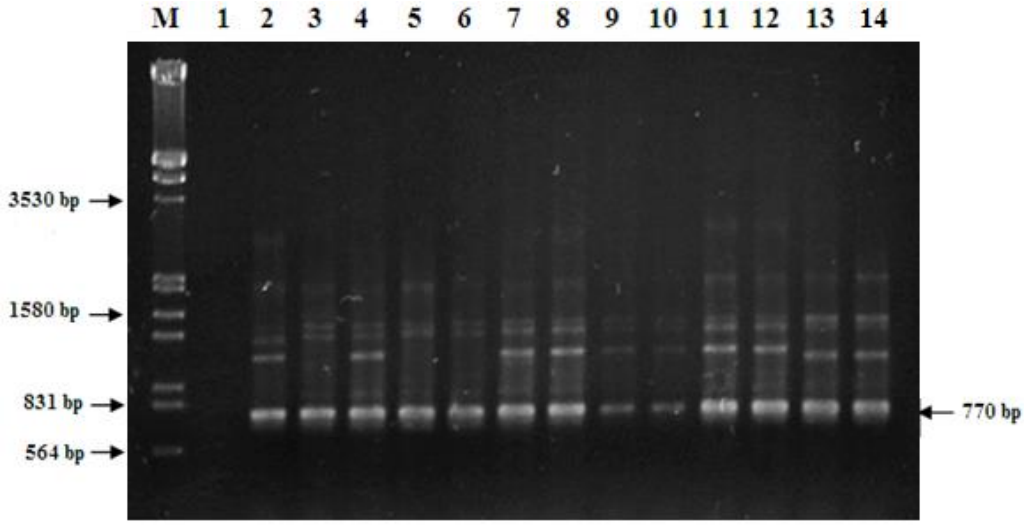

Figure 16 RAPD-PCR band patterns generated with primer 3 and DNAs from various *Salmonella* spp.: lane M, Lambda/*Hind* III/*Eco*RI marker; lane 1, no DNA template (control sample); lane 2, *S.* Paratyphi A; lane 3, *S.* Agona; lane 4, *S.* Derby; lane 5, *S.* Schwarzengrund; lane 6, *S.* Stanley; lane 7, *S.* Typhimurium; lane 8, *S.* Typhimurium DT104 2486; lane 9, *S.* Typhimurium DT104 2487; lane 10, *S.* Typhimurium DT104 2582; lane 11, *S. enterica* subsp. *enterica* ser. 4,5,12:I; lane 12, *S. enterica* subsp. *enterica* ser. 1,4,5,12:I; lane 13, *S. enterica* subsp. *enterica* ser. 4,12:I; lane 14, *S.* Montevideo.

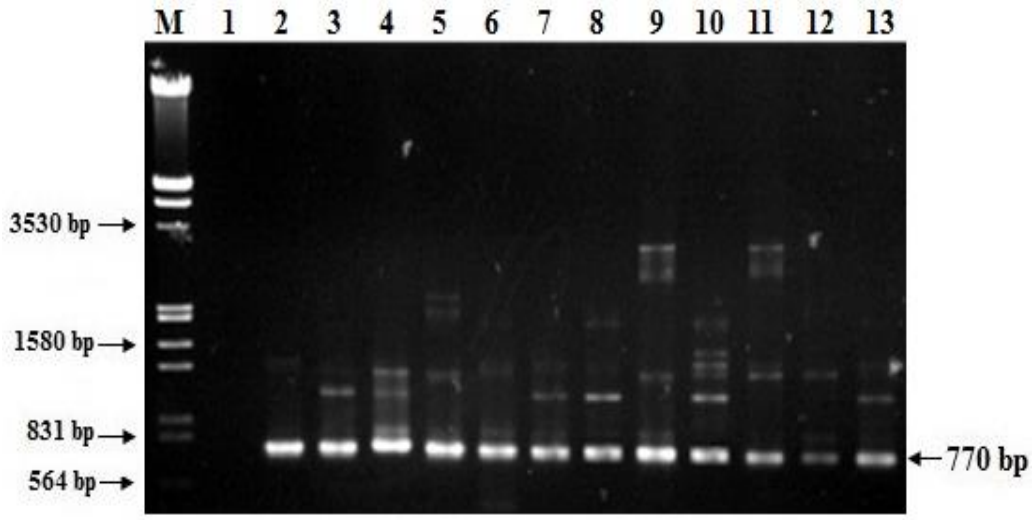

Figure 17 RAPD-PCR band patterns generated with primer 3 and DNAs from various *Salmonella* spp.: lane M, Lambda/*Hind* III/*Eco*RI marker; lane 1, no DNA template (control sample); lane 2, *S.* Poona DMST 19199; lane 3, *S.* Kedougou; lane 4, *S.* Worthington, lane 5, *S.* Cerro DMST 19200; lane 6, *S.* Minnesota DMST 19201; lane 7, *S.* Chicago DMST 19202; lane 8, *S.* Alachua DMST 19203; lane 9, *S.* Bangkok DMST 7121; lane 10, *S.* Wandsworth DMST 19204; lane 11, *S.* Waycross DMST 19205; lane 12, *S.* Salamaeser, 17:gt; lane 13, *S.* Bergen DMST 19206.

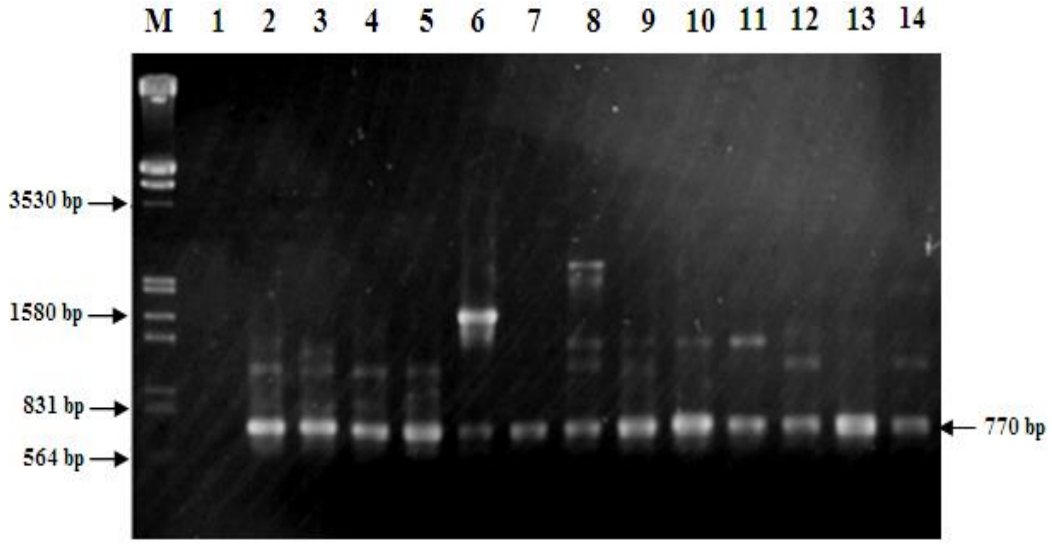

Figure 18 RAPD-PCR band patterns generated with primer 3 and DNAs from various *Salmonella* spp.: lane M, Lambda/*Hind* III/*Eco*RI marker; lane 1, no DNA template (control sample); lane 2, *S.* Rissen; lane 3, *S.* Tennessee; lane 4, *S.* Virchow; lane 5, *S.* Newport; lane 6, *S.* Enteritidis; lane 7, *S.* Fresno; lane 8, *S.* Amsterdam; lane 9, *S.* Anatum; lane 10, *S.* Orion; lane 11, *S.* Orion var15^{+}; lane 12, *S.* Weltevreden; lane 13, *S.* Senftenberg; lane 14, *S.* Aberdeen DMST 19198.

1.2 The detection of various non-*Salmonella* spp. by RAPD-PCR

The specificity of primer 3 was also confirmed with 20 bacteria strains other than *Salmonella* spp. (Figures 19-20), including gram-negative bacteria of the family Enterobacteriaceae, which are closely related to *Salmonella* spp., such as *E. coli*, *C. freundii*, *E. aerogenes*, *K. pneumonia*, *P. mirabilis*, *Y. enterocolitica* and *S. sonnei*; other gram-negative bacteria: *P. fluorescens* and *V. parahaemolyticus* and some strains of gram-positive bacteria: *S. aureus*, *L.plantarum*, *B. cereus* and *L. monocytogenes*.

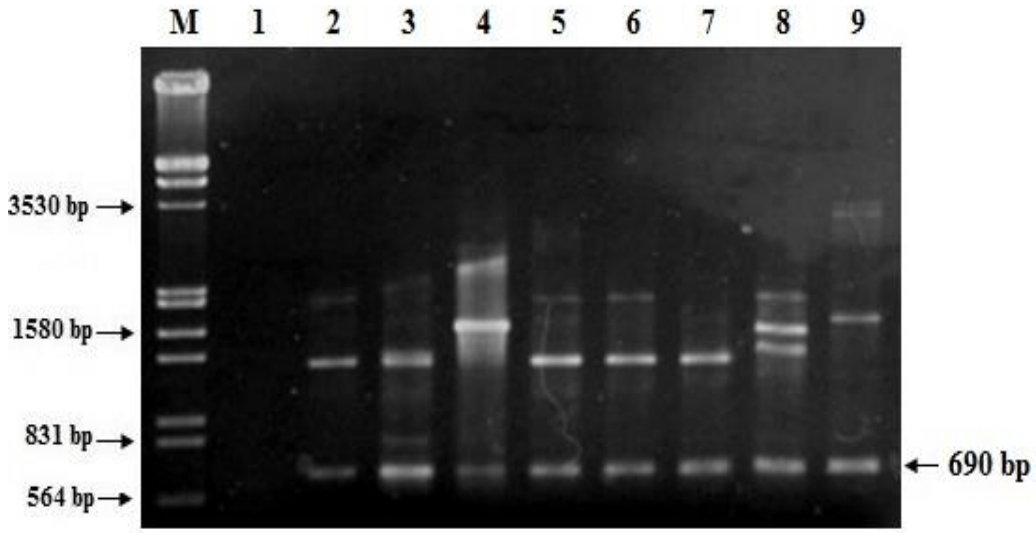

Figure 19 RAPD-PCR band patterns generated with primer 3 and DNAs from various non-*Salmonella* spp.: lane M, Lambda/*Hind* III/*Eco*RI marker; lane 1, no DNA template (controlsample); lane 2, *C. freundii* DMST 16368; lane 3, *E. aerogenes* DMST 8841; lane 4, *K. pneumonia* DMST 7592; lane 5, *P. mirabilis*; lane 6, *P. fluorescens* DMST 16077; lane 7, *S. sonnei* DMST 17564; lane 8, *Y. enterocolitica* DMST 8012; lane 9, *B. cereus* ATCC 11778; lane 10, *L. plantarum* PD110; lane 11, *L. monocytogenes* V7; lane 12, *S. aureus* ATCC 13565; lane 13, *V. parahaemolyticus* VP293.

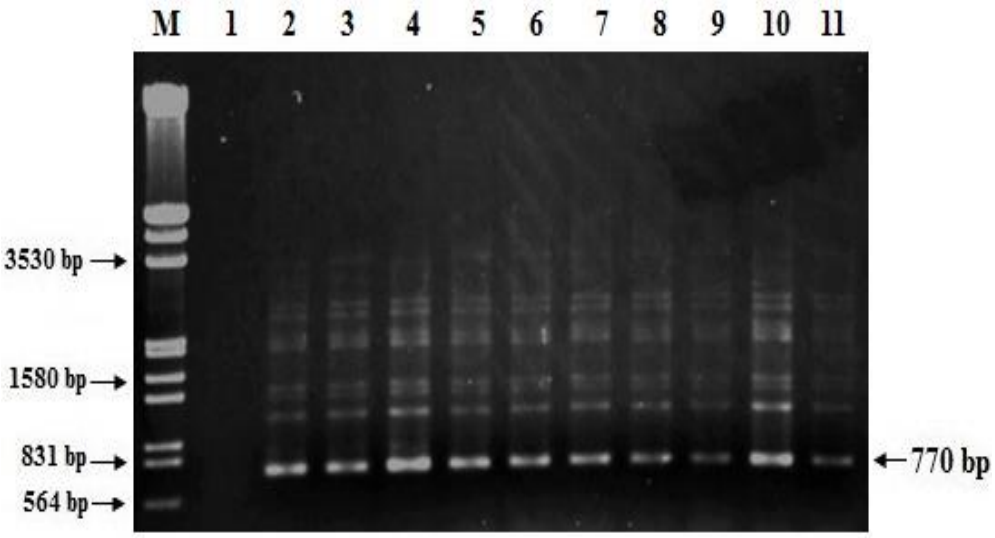

Figure 20 RAPD-PCR band patterns generated with primer 3 and DNAs from various *E. coli*.: lane M, Lambda/*Hind* III/*Eco*RI marker; lane 1, no DNA template (control sample); lane 2, *E. coli* O157:H7 DMST 12743 (VTEC); lane 3, *E. coli* O111 DMST 667 (VTEC); lane 4, *E. coli* O124 DMST 672 (EIEC); lane 5, *E. coli* O168 DMST 693 (ETEC); lane 6, *E. coli* O6 DMST 644 (ETEC); lane 7, *E. coli* O126 DMST 674 (EPEC); lane 8, *E. coli* O18 DMST 652 (EPEC); lane 9, *E. coli*.

Results showed that non-*Salmonella* spp. did not give 770 bp product which was specific for *Salmonella* spp. Some bacteria showed no RAPD-PCR bands and others showed different band patterns from one another. These band patterns were obviously different from those of the *Salmonella* strains tested. However, all of the pathogenic *E. coli* strains used (Figure 20) showed very similar RAPD band patterns, in which one RAPD-PCR band at about 690 bp was detected. Several PCR-based detection methods for *Salmonella* spp. have been reported, but each of these methods has some limitations. The PCR based on the IS200 insertion sequence failed to detect *S.* Agona and *S.* Arizona. Rahn *et al*. (1992) have developed a PCR method which amplifies an internal sequence within the *invA* gene of *S.* Typhimurium for detection of *Salmonella* spp., but a low sensitivity and a few false-positive results from non-*Salmonella* strains were found. Similarly, Miyamoto *et al*. (1998) could

developed RAPD-PCR by the determination of the 980 bp nucleotide sequence of *S.* Typhimurium IFO 12529 *gatD* gene as a target gene.

1.3 The detection of various *Salmonella* serotypes by RAPD-PCR

To prove the reproducibility of *Salmonella* detection by RAPD-PCR in various serotypes (Figures 21-25), 43 strains of five *Salmonella* serotypes including *S.* Rissen (10 strains), *S.* Braenderup (7 stains), *S.* Panama (10 stains), *S.* Enteritidis (10 stains) and *S.* Corvallis (6 strains) were tested. These *Salmonella* strains were isolated from the chicken, pork meat and beef samples from Thai fresh markets in 2005. The results showed that some strains of different *Salmonella* serotypes could gave different band patterns, but all strains produced the main 770 bp product by amplifying with primer 3. The non-specific band was observed with the low intensity of DNA band, and some non-specific band was noted only in some PCR runs. For the *S.* Enteritidis (Figure 21), interestingly, *S.* Enteritidis DMST 15676 showed the true specific band of 770 bp and 1584 bp after RAPD-PCR amplification; whereas, other *S.* Enteritidis strains presented only the specific band of 770 bp. This result confirmed that 770 bp has been shown to be the only one specific band of *S.* Enteritidis detection, similar to other serotypes.

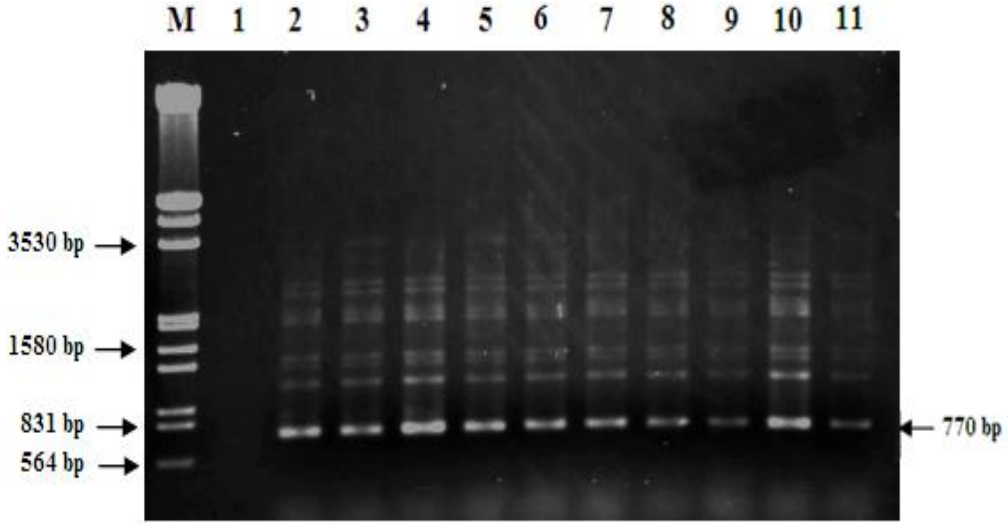

Figure 21 RAPD-PCR band patterns generated with primer 3 and DNAs from *S.* Rissen: lane M, Lambda/*Hind* III/*Eco*RI marker; lane 1, no DNA template (control sample); lanes 2-11, *S.* Rissen.

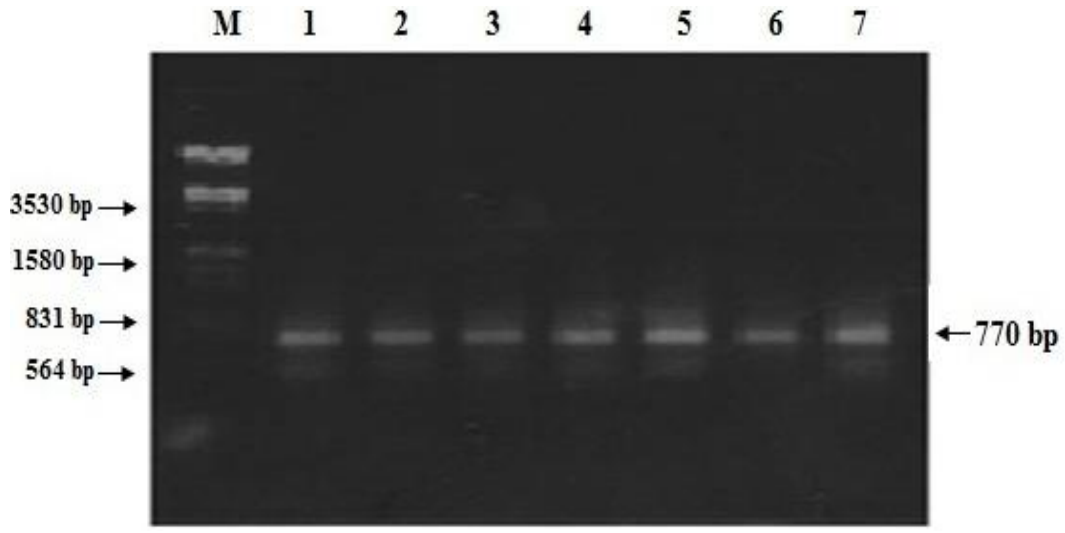

Figure 22 RAPD-PCR band patterns generated with primer 3 and DNAs from *S.* Braenderup: lane M, Lambda/*Hind* III/*Eco*RI marker; lanes 1-7, *S.* Braenderup.

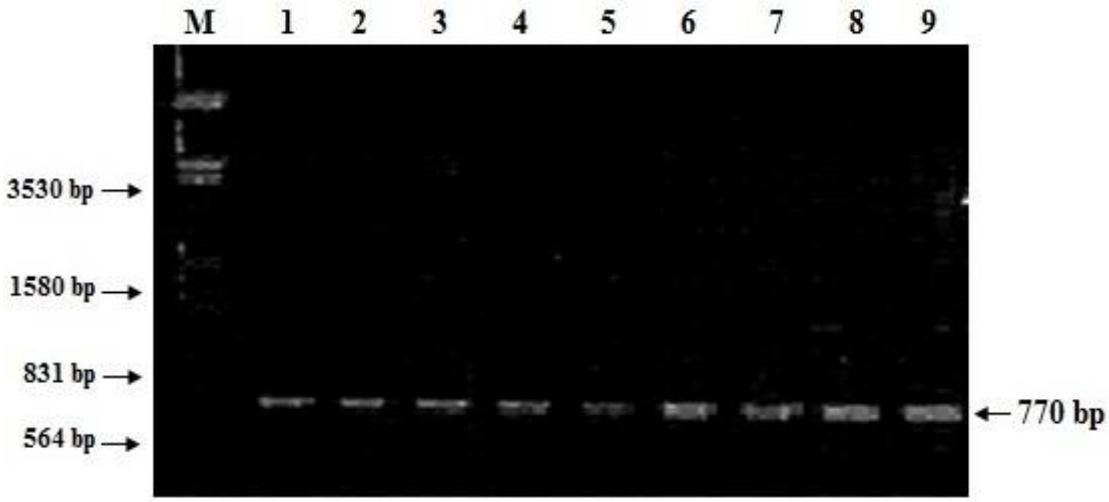

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Figure 23 RAPD-PCR band patterns generated with primer 3 and DNAs from *S.* Panama: lane M, Lambda/*Hind* III/*Eco*RI marker; lanes 1-9, *S.* Panama.

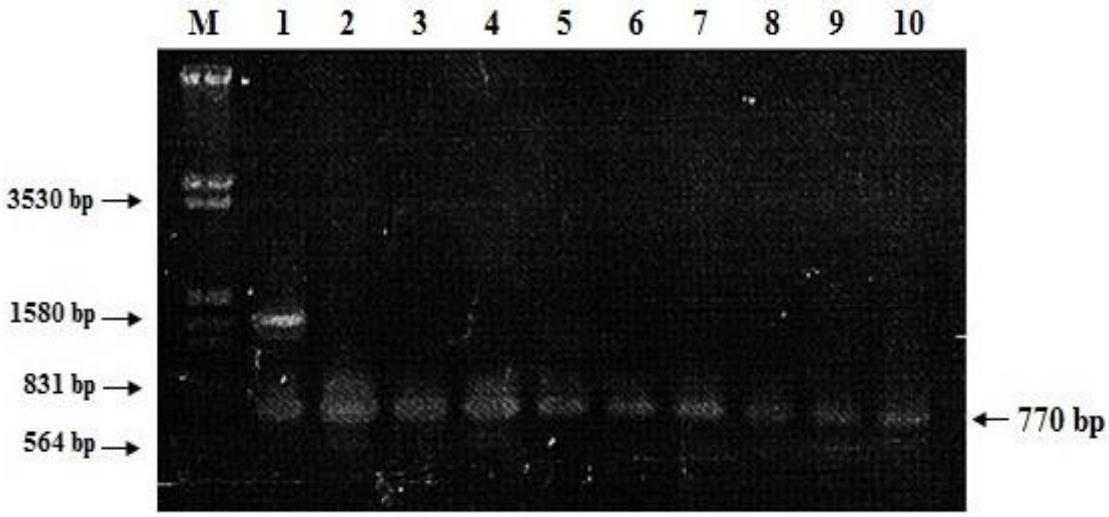

Figure 24 RAPD-PCR band patterns generated with primer 3 and DNAs from *S.* Enteritidis: lane M, Lambda/*Hind* III/*Eco*RI marker; lane 1, *S.* Enteritidis DMST 15676; lanes 2-10, *S.* Enteritidis.

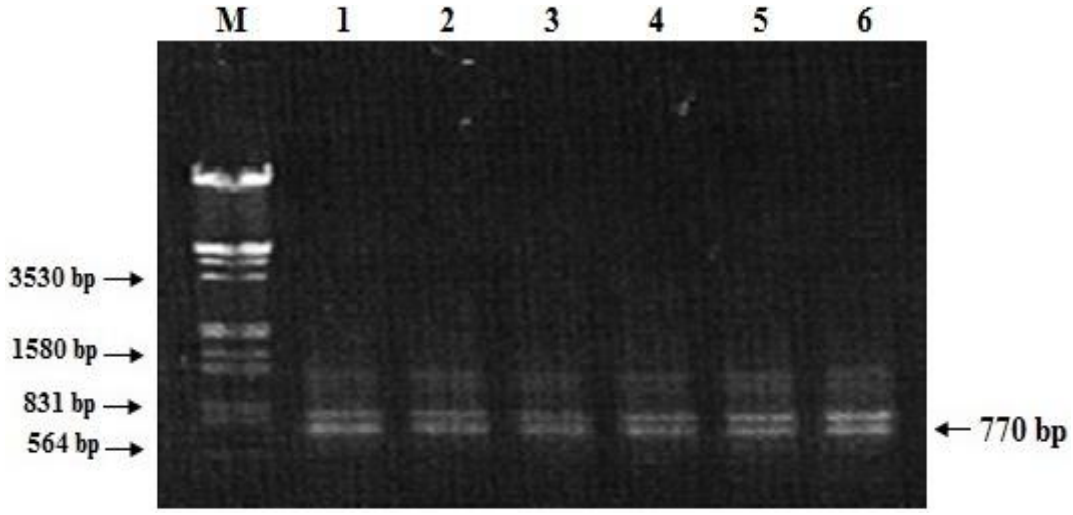

Figure 25 RAPD-PCR band patterns generated with primer 3 and DNAs from *S.* Corvallis: lane M, Lambda/*Hind* III/*Eco*RI marker; lanes 1-6, *S.* Corvallis.

Among the bands produced, only the 770 bp band was considered for analysis of the RAPD-PCR amplification pattern because all *Salmonella* spp. tested produced a completely reproducible pattern with the 770 bp band, whereas bands outside this size had variations between different runs. The 770 bp band was considered present or absent regardless of its intensity under UV, and the 770 bp band was absent, when the non-*Salmonella* DNA was detected. Interestingly, the present single primer-based PCR is promising detection of *Salmonella* spp. since the method is not based on antigenicity, motility, or antibiotic susceptibility of *Salmonella* spp. Besides the specificity, the sensitivity of the method is also influent on the success of the RAPD-PCR method for rapid detection. The previous study reported that the lowest amount of *Salmonella* DNA template that could be detected was 0.1 ng, when DNA was prepared by CTAB method. For determining sensitivity in terms of whole cell lysis, 2.3×10^3 cells per ml of culture are required to give a positive result with this RAPD-PCR using primer 3 (Trevanich *et al*., 2010). The detection limit of *S.* Typhimurium was higher than that reported by Rahn *et al*. (1992) who used the *invA* gene-based PCR assay and detected 3.0×10^2 cells of *S.* Typhimurium. Miyamoto *et al*. (1998) reported that *Salmonella*-specific RAPD bands were detected by ethidium bromide staining method even when pure genomic DNA prepared by CTAB from as

few as 2.8×10^2 cells was used. However, in this study, the preparation of DNA from pure *Salmonella* culture suspension was simple and rapid by boiling the cells in TE buffer for 5 min. The method could reduce many cumbersome steps, the possibility of cross contamination, and the cost. Similar results were obtained by the works of Piknova *et al*. (2002) and Malorny *et al*. (2003) used boiling for DNA extraction before PCR. This simple boiling procedure was found optimal for the DNA extraction although this procedure gave higher detection limit than that of lysis in SDS/NaOH at 85-95°C for 15 min or microwave heating for 10 min (Trkov *et al*., 1999). De Medici *et al*. (2003) reported that the *Salmonella* cells could be lysed easily by boiling at 100°C for 15 min because *Salmonella* lack a thick cell wall. Thus, the *Salmonella* DNA was released into suspension. They also found that boiling, alkaline lysis and nucleospin tissue showed no significant difference in effectiveness of DNA extraction, and they selected boiling as the preferred extraction method. Overall, the PCR product of 770 bp was the specific band for *Salmonella* spp. detection of single primer based RAPD-PCR. This method was highly reproducible for the detection of *Salmonella* spp.

The results of this part confirmed the hypothesis that the single primer-based RAPD-PCR used in this study could detect various serotypes of *Salmonella* spp. with the specific DNA product of approximate 770 basepairs. The RAPD-PCR containing 20-mer oligonucleotide single-stranded DNA primer, named primer 3 was designed on the basis of *S.* Typhimurium *gat*D gene encoding galactitol-1-phosphate dehydrogenase. The primer 3 could produce the specific DNA product of approximate 770 basepairs from all 80 *Salmonella* strains. No 770-bp DNA band was amplified from any DNA samples of 20 non-*Salmonella* bacteria. The specific 770 bp DNA band was high specificity and consistency for the various serotype *Salmonella* detection.

2. The efficiency study of primer 3-based RAPD-PCR for detection of *Salmonella* spp. in various *in vitro* stress treatments and primer 3-based RAPD-PCR for detection in food process models

The previous part indicated primer 3-based RAPD-PCR used in this study was high sensitivity, specificity and consistency for the detection of *Salmonella* spp. However, according to the reduction of *Salmonella* contamination in food processing, disinfectants, sanitizers, heat treatment, acid and alkali treatment are used in food plant. Those conditions may damage *Salmonella* cell or DNA and change the normal cell to stressed cell. These injured or stressed cells may be not detected by RAPD-PCR. Therefore, the effect of various stress conditions *in vitro* and food processing conditions on the efficiency of RAPD-PCR for *Salmonella* detection was investigated in this part.

2.1 The efficiency of RAPD-PCR for detection of stressed *S.* Enteritidis *in vitro*

The RAPD-PCR is used commonly to investigate pathogen contaminated in food (Way *et al*., 1993; Hilton *et al*., 1996). In this study, we used this method to investigate the multi-drug resistant (Streptomycin, Sulphonamides, Vancomycin, Gentamicin and Ampicillin) *S.* Enteritidis type strain DMST 15676 at a final concentration of approximately 1.2×10^7 CFU/ml. *S.* Enteritidis type strain DMST 15676 was treated by various conditions *in vitro* including UV irradiation 20 min, ethanol (50%) 5 min, hydrochloric acid (pH 4) 5 min, sodium hydroxide (pH 10) 5 min, hydrogen peroxide (0.38 mM) 5 min, sodium hypochlorite (200 ppm) 5 min, heat treatment (52°C) 15 min and cold treatment (0 and 4°C) 24 h. After stress treatments, the percentage survival under each stress treatment was determined by plating technique on NA and XLD agars. The percentages of stressed cells from all treatments were not less than 90%. Then, the stressed cells were determined directly by RAPD-PCR to confirm that this technique did not give false negative with stressed *S.* Enteritidis contaminated in food sample or environment. Under the conditions *in vitro* used in this study, all stressed *S.* Enteritidis gave the same positive signal of 770-

bp DNA band as the control sample (non-stressed *S.* Enteritidis) by RAPD-PCR with primer 3 (Figure 26). The results indicated that the stress treatments used in this study did not destroy the *S.* Enteritidis DNA at the annealing site of primer 3 or the 770 bp target DNA. Therefore, primer 3 could anneal the target DNA site of 770 bp of the stressed cells. With the same reason, when *S.* Enteritidis DNA was extracted by boiling for 5 min, 770 bp PCR products were still successfully amplified by primer 3. For the high numbers of *S.* Enteritidis cells tested (1.2×10^7 cells), most cells at approximately 10^6 CFU/ml were injured by each stress treatment used as the percentage survival of stresses cells was not less than 90%. RAPD-PCR could detect those numbers of the stressed cells because the detection limit of this method was at least 10^3 viable cells. In case of the low numbers of *S.* Enteritidis cells (less than 10^3 cells), RAPD-PCR could not detect the presence of stressed *S.* Enteritidis cells. Therefore, the technique should be used in combination with pre-enrichment culturing step to increase the number of bacterial cells over detection limit. The stressed conditions used in this study did not affect the efficiency of RAPD-PCR for specific detection of *S.* Enteritidis at high concentration of cells.

The obtained results similarly agreed with the study of Tamanai-Shacoori *et al*. (2005). They compared the profiles of RAPD product from peracetic acid (PAA) treated and untreated *E. coli* H10407 by testing twenty different 10-mer primers. They found that no changes in the RAPD pattern of PAA-treated *E. coli* with 50% of the primers used. However, the other primers could discriminate the variations of the RAPD profiles after the PAA treatment, including the loss of some amplification fragments and the appearance of new amplicons. Moreover, the clear differences observed between the RAPD profiles of stressed and non-stressed cell were also reported by Warner and Oliver (1998) and Jolivet-Gougeon *et al*. (2000). Jolivet-Gougeon *et al*. (2000) published the genetic rearrangements of the stressed *E. coli* H10407 cell in the osmotic condition. Our results strongly indicated that the stress treatments used in the present study did not affect the annealing site of primer 3 to amplify the target DNA gene of 770 bp product.

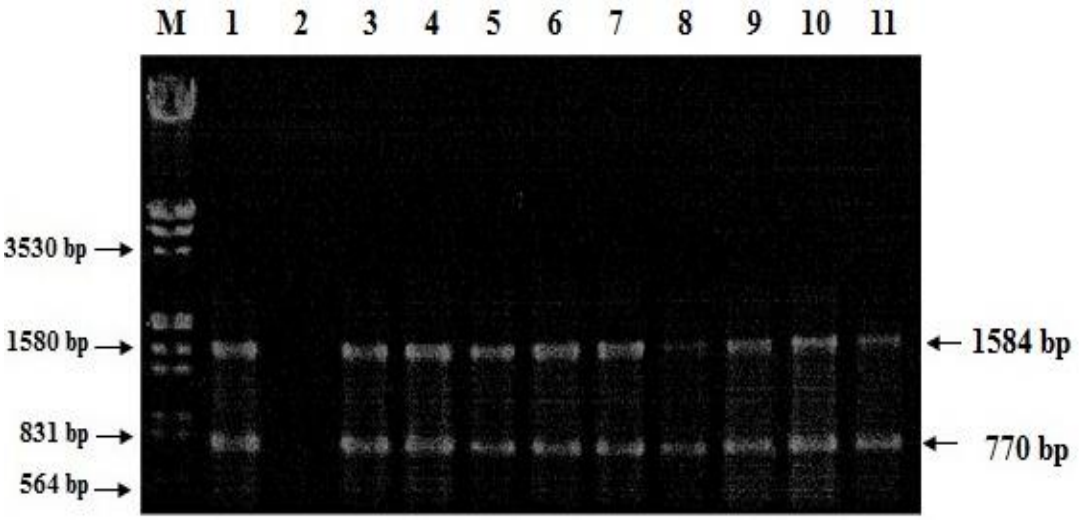

Figure 26 RAPD-PCR product patterns obtained from genomic DNAs of *S.* Enteritidis cells treated by various stress conditions: lane M, Lambda/*Hin*d III/*Eco*RI marker; lane 1, positive control; lane 2, negative control; lane 3, UV Irradiation 20 min; lane 4, ethanol (50%) 5 min; lane 5, hydrochloric acid (pH 4) 5 min; lane 6, sodium hydroxide (pH 10) 5 min; lane 7, hydrogen peroxide (0.38 mM) 5 min; lane 8, sodium hypochlorite (200 ppm) 5 min; lane 9, 52°C for 15 min; lane 10, 4°C for 24 h; lane 11, 0°C for 24 h.

2.2 The efficiency of RAPD-PCR for detection of stressed *S.* Enteritidis in various food process models

2.2.1 The detection of stressed *S.* Enteritidis in fresh ground chicken meat subjected to the chilling and freezing storage

The fresh ground chicken meat samples were artificially spiked with the final concentration of 1.2×10^7 CFU/g, then the samples were chilled at 4°C, frozen at 0°C and -20°C for 10 days. After 1, 5 and 10 days storage, *S.* Enteritidis was detected directly by RAPD-PCR (Figure 27). The results of RAPD-PCR for detection of stressed *S.* Enteritidis in fresh ground chicken meat after the storage at 4°C, 0°C and -20°C showed that the specific bands of 770 bp were generated in all

samples. Therefore, primer 3-based RAPD-PCR involving 20-h culturing step in NB could detect the *S.* Enteritidis cells in all fresh ground chicken meat models after the chilling and freezing temperatures at different storage times. Basically, food matrixs could affect the efficiency of RAPD-PCR analysis as they could prevent the annealing site of primer and react with PCR components. Therefore, the false negative result was obtained. The pre-enrichment step in NB for 20 h could prevent such inhibitory effects of food matrixs by various aspects including dilution of inhibitory substances in food. This step also highly enhanced the detection of the viable and healthy bacterial cells even if the stressed cells originally contaminated food at low number. Nutrient borth could help in recovery of the injured cells and then increase of the viable and healthy cells as well as reduction of DNA from the dead cells.

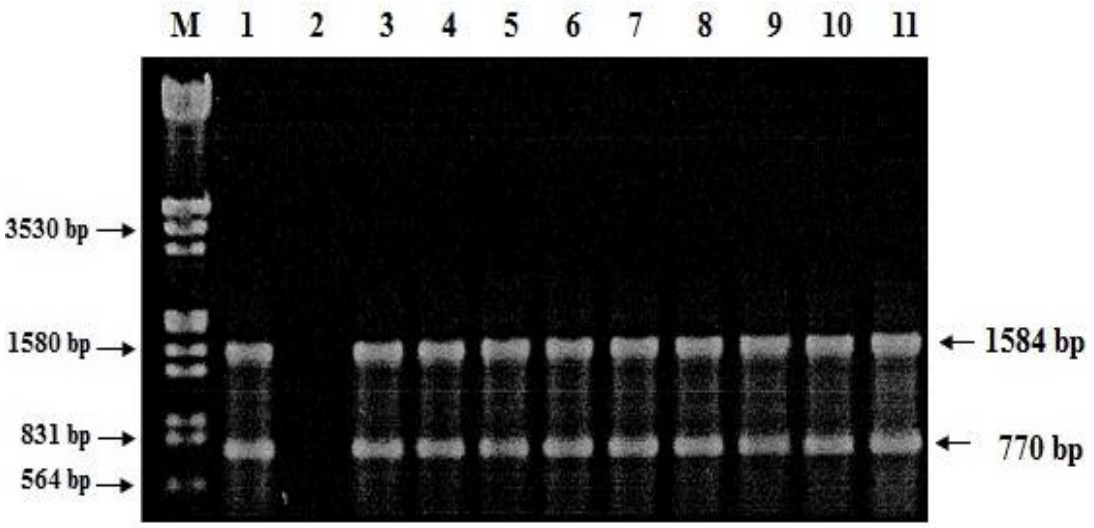

Figure 27 RAPD-PCR product patterns obtained from genomic DNAs of *S.* Enteritidis cells in fresh poultry meat: lane M, Lambda/*Hind* III/*Eco*RI marker; lane 1, positive control; lane 2, negative control; lane 3, 4°C for 1 day; lane 4, 4°C for 5 days; lane 5, 4°C for 10 days; lane 6, 0°C for 1 day; lane 7, 0°C for 5 days; lane 8, 0°C for 10 days; lane 9, -20°C for 1 day; lane 10, -20°C for 5 days; lane 11, -20°C for 10 days.

Warner and Oliver (1998) reported the different RAPD profiles of *V. vulnificus* cells in the starvation period of artificial seawater. This result

conformed that some stress condition such as the high concentration of salt could destroy DNA template of the stressed cells. Although many researchers reported that RAPD-PCR could detect DNA alterations from stressed cell induced by chemical or physical agents following direct and/or indirect interaction with the genomic DNA, the RAPD-PCR technique with primer 3 did not show the difference of PCR products between stressed and non-stressed cell. The stress conditions used in this study did not destroy *Salmonella* DNA, especially the DNA target site for primer 3 binding. Alternatively, there were more than one site that primer 3 could anneal to *Salmonella* DNA template. The investigation of DNA alteration in PCR profile depended on the strength of stress condition, content of amplified altered DNA, primer, template and PCR conditions.

2.2.2 The detection of stressed *S.* Enteritidis in Thai fermented food (Nham)

The fermented pork and chicken meat were produced and inoculated with *S.* Enteritidis at 10^2, 10^4 and 10^6 CFU/g, respectively. Then, the Nham samples were fermented within 5 days for the study of acid stress model of Thai fermented food (Table 5). For the pH values determination, Nham pH values were gradually decreased throughout the fermentation process. The pH values of the pork Nham and chicken Nham were decreased from 5.97 to 4.39 and 6.11 to 4.38, respectively after the fermentation for 5 days. The pH values of the samples inoculated with different inoculum levels of *S.* Enteritidis were not much different. For the detection of spiked *S.* Enteritidis in 5 day-fermented Nham, *S.* Enteritidis was detected by RAPD-PCR involving 20-h culturing in NB and the result was compared to the conventional method. All samples showed positive results of *S.* Enteritidis by both detection methods. *S.* Enteritidis could be isolated from Nham throughout the 5 days fermentation and could be found in all samples even in the samples initially inoculated with the lowest inoculum levels in this study (10^2 CFU/g**)**. During the fermentation period of Nham, the lactic acid bacteria could produce organic acid such as lactic acid and acetic acid which played an important role in the reduction of acidity in Nham. It has been generally known that the contaminated pathogens in

Nham could be destroyed with pH lower than 4.6 with the reason that high acidity causes harm to bacterial cells. The bacterial cells were stressed, injury or even death. Paukatong and Kunawasen (2001) reported the agreement that the pathogens including *Salmonella* spp., *S. aureus*, and *L. monocytogenes* were survived in Nham with pH higher than 4.6. This report was in agreement with the study of stressed *S.* Typhimurium cells in liquid medium (*in vitro*) that the stressed *S.* Typhimurium cells were destroyed by the acidity in liquid medium of the pH lower than 4.5 (Alvarez-Ordonez *et al*., 2009). However, the present study showed that *S.* Enteritidis could survive in Nham for 5 days of fermentation while the pH values of Nham decreased to 4.38 and 4.39 in chicken and pork Nham, respectively, even in the initially low inoculum levels (10^2 CFU/g) spiked samples. This result was correlated to the research of Osiriphun *et al*. (2004), who studied quantitative risk assessment of *Salmonella* spp. in fermented pork sausage (Nham) in Thailand and found 10^2 CFU/g of *Salmonella* spp. in Nham on 7 days of fermentation at the pH level of 4.1. *S.* Enteritidis could be tolerant to high acidity in Nham because the food matrix such as lipid, protein, etc. worked as a barrier for protection of the bacterial cells against acidity. The food composition could prevent absorbance of acid passing through the cell membrane. The stressed cells could be resuscitated and grown after pre-enrichment step and then detected by both RAPD-PCR and the conventional method. The pre-enrichment step could also prevent the DNA amplification from the dead cells due to the cell dilution by the enrichment broth. This study indicated that RAPD-PCR is a powerful and reliable for the detection of the stressed *S.* Enteritidis contaminated in the high acid food samples and can be potentially used for monitoring of *S.* Enteritidis along the food production chain in HACCP and risk assessment.

Table 5 pH change and *Salmonella* detection of pork Nham and chicken Nham during fermentation.

Incubation time (days)	Inoculum concentration (CFU/g)	Pork Nham			Chicken Nham		
		pH[1]	Conventional method	RAPD-PCR	pH[1]	Conventional method	RAPD-PCR
0	1.1×10^2	5.96±0.01	+	+	6.11±0.01	+	+
	1.1×10^4	5.97±0.01	+	+	6.10±0.01	+	+
	1.1×10^6	5.95±0.01	+	+	6.10±0.00	+	+
1	1.1×10^2	5.29±0.05	+	+	5.27±0.02	+	+
	1.1×10^4	5.13±0.03	+	+	5.15±0.02	+	+
	1.1×10^6	5.24±0.02	+	+	5.23±0.02	+	+
2	1.1×10^2	4.92±0.02	+	+	4.80±0.01	+	+
	1.1×10^4	4.93±0.01	+	+	5.11±0.01	+	+
	1.1×10^6	4.91±0.02	+	+	4.91±0.01	+	+
3	1.1×10^2	4.85±0.01	+	+	4.75±0.02	+	+
	1.1×10^4	4.70±0.01	+	+	4.82±0.03	+	+
	1.1×10^6	4.80±0.03	+	+	4.77±0.01	+	+

[1]mean ± standard deviation values

Table 5 (continued)

Incubation time (days)	Inoculum concentration (CFU/g)	Pork Nham			Chicken Nham		
		pH[1]	Conventional method	RAPD-PCR	pH[1]	Conventional method	RAPD-PCR
4	1.1×10^2	4.68±0.01	+	+	4.60±0.01	+	+
	1.1×10^4	4.67±0.02	+	+	4.68±0.01	+	+
	1.1×10^6	4.62±0.01	+	+	4.65±0.02	+	+
5	1.1×10^2	4.39±0.02	+	+	4.38±0.01	+	+
	1.1×10^4	4.47±0.01	+	+	4.46±0.02	+	+
	1.1×10^6	4.39±0.02	+	+	4.54±0.04	+	+

[1]mean ± standard deviation values

The results in this part indicated that the primer 3-based RAPD-PCR could detect the stressed cells treated by various *in vitro* conditions including UV irradiation, ethanol, sanitizer, acidity, alkaline, heat and cold. The stressed cells from the chilled and frozen foods also gave the same positive signal of 770-bp DNA band as the non-stressed cells. Apart from this result, the primer 3-based RAPD-PCR involving culturing in NB could detect the stressed *Salmonella* cells in pork and chicken meat Nham represented as high acid food (pH < 4.6). These obtained results confirmed the hypothesis that those various stress conditions did not affect the amplification of 770-bp DNA band by primer3-based RAPD-PCR. Primer 3-based RAPD-PCR has potential to detect both healthy and stressed *S.* Enteritidis.

3. The development of primer 3-based EMA-RAPD-PCR involving culturing step for reliable detection of only viable *Salmonella* spp. in foods

The previous part revealed that primer 3-based RAPD-PCR could detect both normal and stressed *Salmonella* cells with the positive signal of 770-bp DNA band. This technique could improve the sensitivity, specificity and reduce the time for *Salmonella* detection in foods. However, many researches indicated that PCR technique still had one crucial disadvantage in that it could not distinguish between DNA originated from viable and dead cells. This could be the cause of an overestimation of targeted viable cells and dead cells in PCR. Therefore, in this part, a photoreactive dye or nucleic acid dye (ethidium bromide monoazide, EMA) was used as a DNA-binding dye to inhibit the amplification of the dead cell DNA in the RAPD-PCR reaction.

For more than a decade, many types of PCR methods have been designed to detect *Salmonella* spp. from foods, clinical specimens and the environment including random amplified polymorphic DNA (RAPD) PCR, nested PCR, multiplex PCR, and real time PCR (Oliveira *et al*., 2002; Wang and Yeh, 2002; Yeh *et al*., 2002; Taitt *et al*., 2004; Wolffs *et al*., 2007; Omiccioli *et al*., 2009; Munoz *et al*., 2010; Trevanich *et al*., 2010; Alves *et al*., 2012). RAPD-PCR is one of the rapid, simple and economical PCR methods. It requires a single arbitrary oligonucleotide primer with no nucleotide

sequence information on the target gene. Generally, RAPD-PCR has low stringency in amplifying the DNA because the primer can anneal to several locations on the DNA strand. The polymorphic DNA product patterns can be used as genetic markers in the epidemic investigation (Maripandi *et al*., 2007). However, the standard PCR can confirm the presence of bacteria, but it is unable to differentiate between viable and dead bacteria. Trevanich *et al.* (2010) developed an optimized 18 h method involving one step culturing in NB and single primer-based PCR assay for detection of *Salmonella* spp. in foods. A single primer was used containing 20-mer oligonucleotide (named primer 3), which could amplify the specific DNA product of approximate 770 base pairs in all 38 *Salmonella* strains tested. This procedure was relatively high sensitivity and specificity even if the high population of non-*Salmonella* spp. was also present in the food sample after 14-h pre-enrichment in NB. However, the remaining DNA from dead *Salmonella* spp. cells may affect the reliability of the *Salmonella* spp. detection. Although the pre-enrichment in NB was performed in order to increase the population of viable cells, such DNA was still presented due to its relatively long persistence in the range of several days to a month (Masters *et al.*, 1994). Therefore, this study aimed to improve the reliability of PCR-based method for specific detection of only viable *Salmonella* spp. in food samples by applying EMA with the RAPD-PCR procedure due to only viable *Salmonella* cells have the ability to cause disease to consumer. Based on the previous knowledge, the developed EMA-RAPD-PCR in this research was the first study that EMA was used to combined with RAPD-PCR to amplify DNA from only viable *Salmonella* cells.

3.1 Optimization of EMA concentration and the light exposure for EMA-RAPD-PCR

The factors affecting the ability of EMA treatment for removing interference from DNA of dead cells were investigated. Results showed that EMA at optimized conditions before RAPD-PCR amplification can be effectively used as a method for detection of viable cells of *S.* Typhimurium and *S.* Enteritidis. The nucleic acid dye, EMA was used to combine with RAPD-PCR for *Salmonella* detection. The minimum amount of EMA to prevent amplification of DNA derived

from heat-killed cells was found to be in a range of 3.0–5.0 µg/ml. A concentration of 5.0 µg/ml EMA or less did not inhibit the amplification of target DNA from viable cells of *S.* Typhimurium and *S.* Enteritidis (Figure 28A and 28B). Additionally, there was no bacteriocidal effect on both of tested *S.* Typhimurium and *S.* Enteritidis and RAPD-PCR results of viable cells and viable cells treated with 3.0 µg/ml EMA were identical (data not shown). However, EMA at the concentration of 2.0 µg/ml or less could not completely inhibit the amplification of DNA from heat-killed cells of both serovars (Figure 28A and 28B). Therefore, the concentration of 3.0 µg/ml EMA was used to treat cells throughout this study. EMA at a concentration of 2.0 µg/ml or less could not completely inhibit the amplification of DNA from heat-killed cells of both serovars (Figure 29A and 29B). Therefore, the concentration of 3.0 µg/ml EMA was used to treat cells throughout this study.

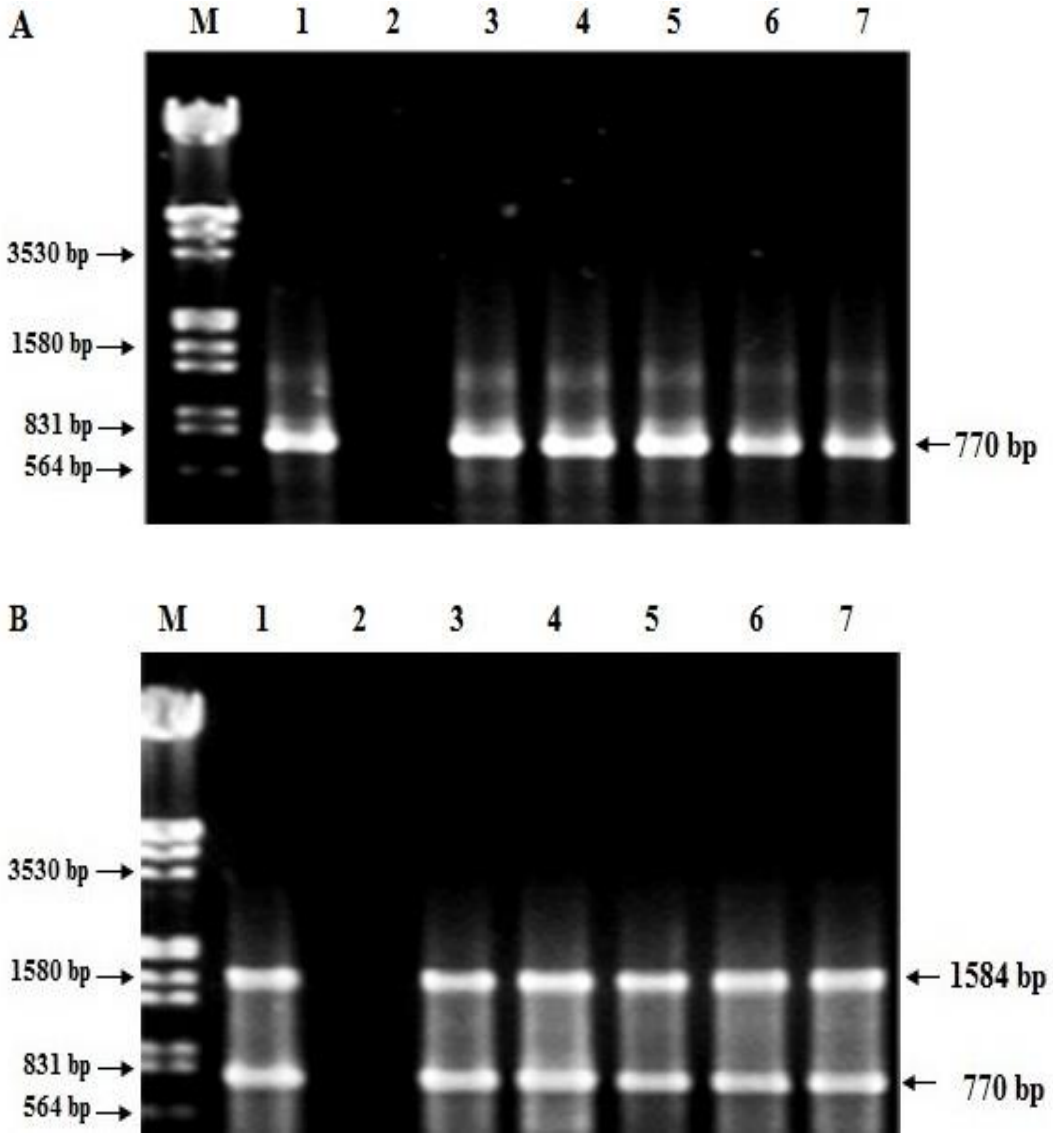

Figure 28 RAPD-PCR product patterns obtained from genomic DNAs of viable *S.* Typhimurium cells (A) and viable *S.* Enteritidis cells (B) treated with different amounts of EMA: lane M, DNA marker; lane 1, positive control; lane 2, negative control; lane 3, 1.0 μg/ml EMA; lane 4, 2.0 μg/ml EMA; lane 5, 3.0 μg/ml EMA; lane 6, 4.0 μg/ml EMA; lane 7, 5.0 μg/ml EMA.

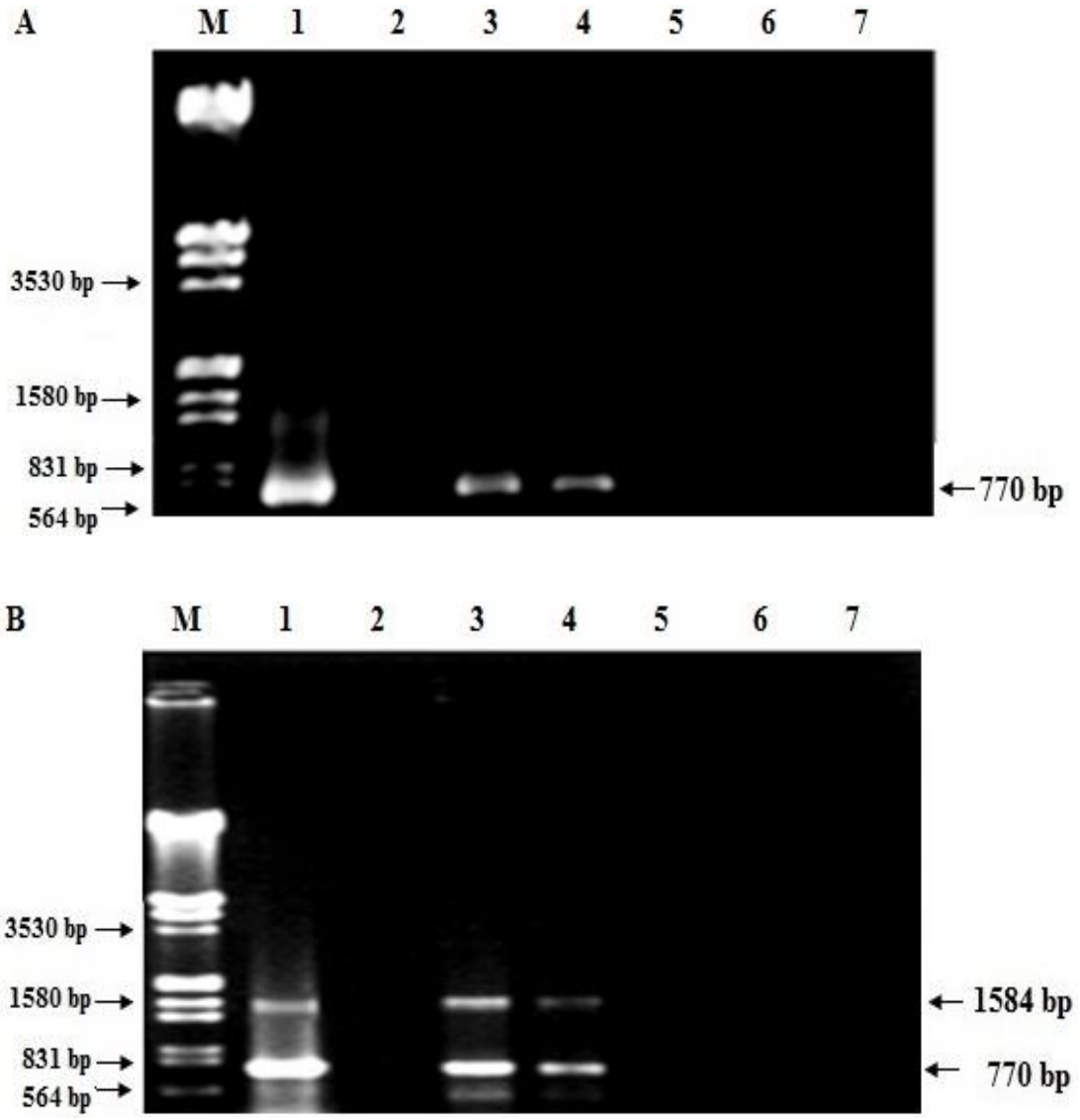

Figure 29 RAPD-PCR product patterns obtained from genomic DNAs of dead *S.* Typhimurium cells (A) and dead *S.* Enteritidis cells (B) treated with different amounts of EMA: lane M, DNA marker; lane 1, positive control; lane 2, negative control; lane 3, 1.0 µg/ml EMA; lane 4, 2.0 µg/ml EMA; lane 5, 3.0 µg/ml EMA; lane 6, 4.0 µg/ml EMA; lane 7, 5.0 µg/ml EMA.

Regarding EMA-RAPD-PCR, EMA, a DNA-intercalating dye can enter only the dead cells with a damaged cell membrane and cell wall and form irreversible covalent-linkage with DNA during photoactivation. Although EMA could enter viable cells, it was pumped out of the cells by the active transport system. Therefore,

only the DNA amplification of heat-killed cells treated with EMA was inhibited (Hixon *et al.*, 1975; Nogva *et al*., 2003). Up-to-date, the EMA-PCR technique has been successfully used to differentiate viable cells from dead cells with *E. coli* 0157:H7, *S.* Typhimurium, *L. monocytogenes*, *C. jejuni* and *V. vulnificus* as model organisms (Nogva *et al.*, 2003; Rudi *et al.*, 2005; Wang and Levin, 2006; Pan and Breidt, 2007; Wang and Mustapha, 2010). Our study demonstrated that the minimum amount of EMA to completely inhibit the RAPD-PCR-amplification of DNA derived from *S.* Typhimurium and *S.* Enteritidis heat-killed cells was ≥ 3.0 µg/ml. A concentration of 2.0 µg/ml EMA or less could not completely inhibit the amplification of DNA from heat-killed cells because the amount of EMA was not sufficient to bind to the DNA strands thoroughly. The inhibition of viable cell amplification was not notable in all concentrations of EMA used in our study. However, Lee and Levin (2006) reported the DNA amplification of viable cells could be inhibited at a concentration of EMA which was higher than 50 µg/ml. The optimum conditions of the EMA treatment depend on the bacterial strains and the PCR techniques. The study by Wang and Levin (2006) demonstrated that the amplification of *V. vulnificus* heat-killed cells by real-time PCR was inhibited by 2.5 µg/ml EMA and a concentration higher than 3.0 µg/ml EMA could also inhibit PCR amplification from viable cells. For our RAPD-PCR procedure to detect *Salmonella* spp., a concentration of 3.0 µg/ml EMA was suitable to be used to prevent the DNA amplification from heat-killed cells.

In addition, for The optimization of the light exposure period to activate and photolyse EMA, the 1.2×10^6 *Salmonella* cells were treated with 3.0 µg/ml EMA and exposed to the T halogen bulb (650 W) for 5, 10, and 15 min, respectively at a distance of 20 cm. After the RAPD-PCR amplification, increasing the duration time of photo-exposure did not affect the DNA amplification (Figure 30A and 30B). All studied exposure periods (5, 10 and 15 min) prevented the DNA amplification of heat-killed cells (Figure 31A and 31B*).*

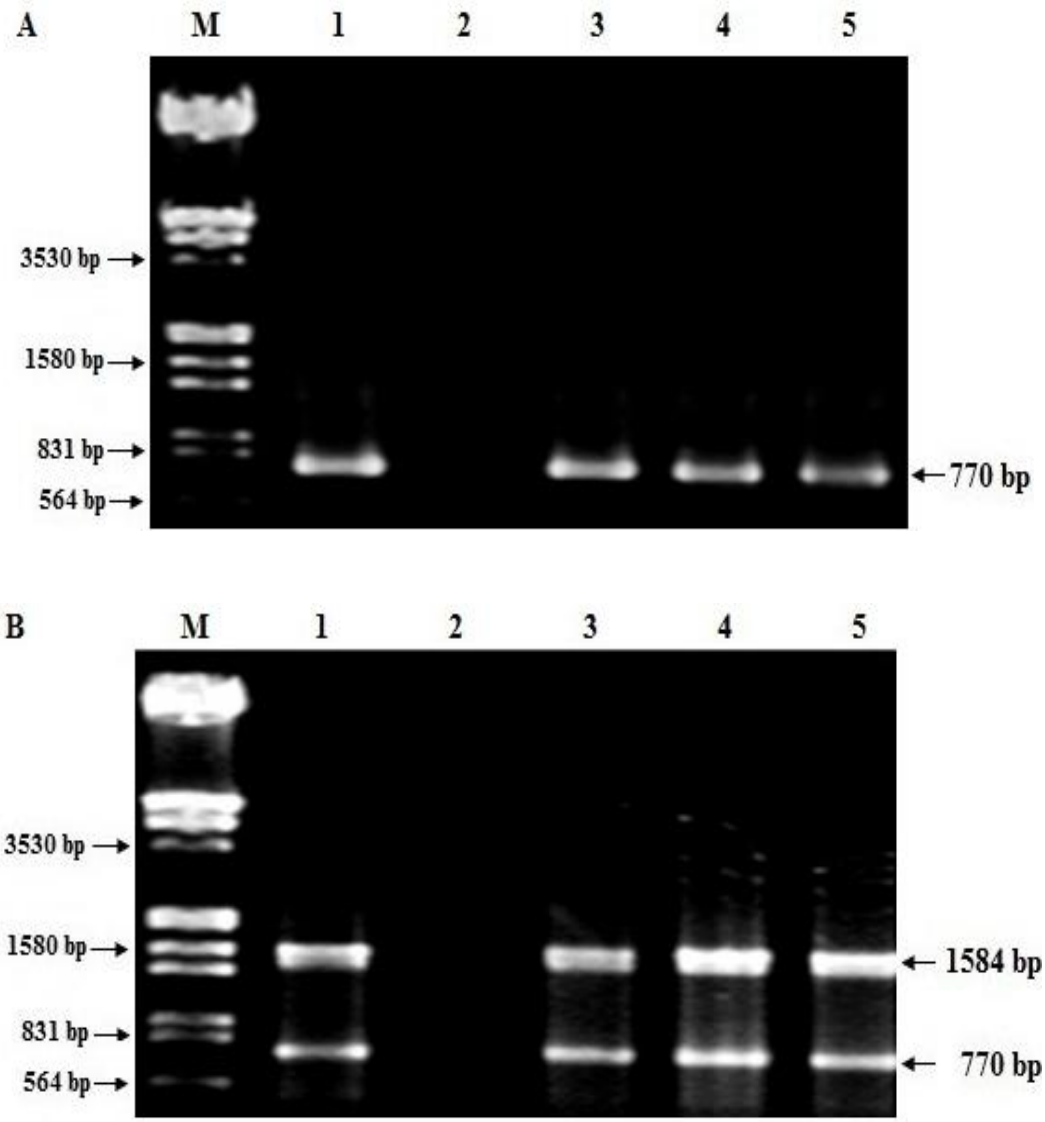

Figure 30 RAPD-PCR product patterns obtained from genomic DNAs of viable *S.* Typhimurium cells (A) and viable *S.* Enteritidis cells (B) treated with 3.0 µg/ml EMA and exposed with different durations of light: lane M, DNA marker; lane 1, positive control; lane 2, negative control; lane 3, 5 min; lane 4, 10 min; lane 5, 15 min.

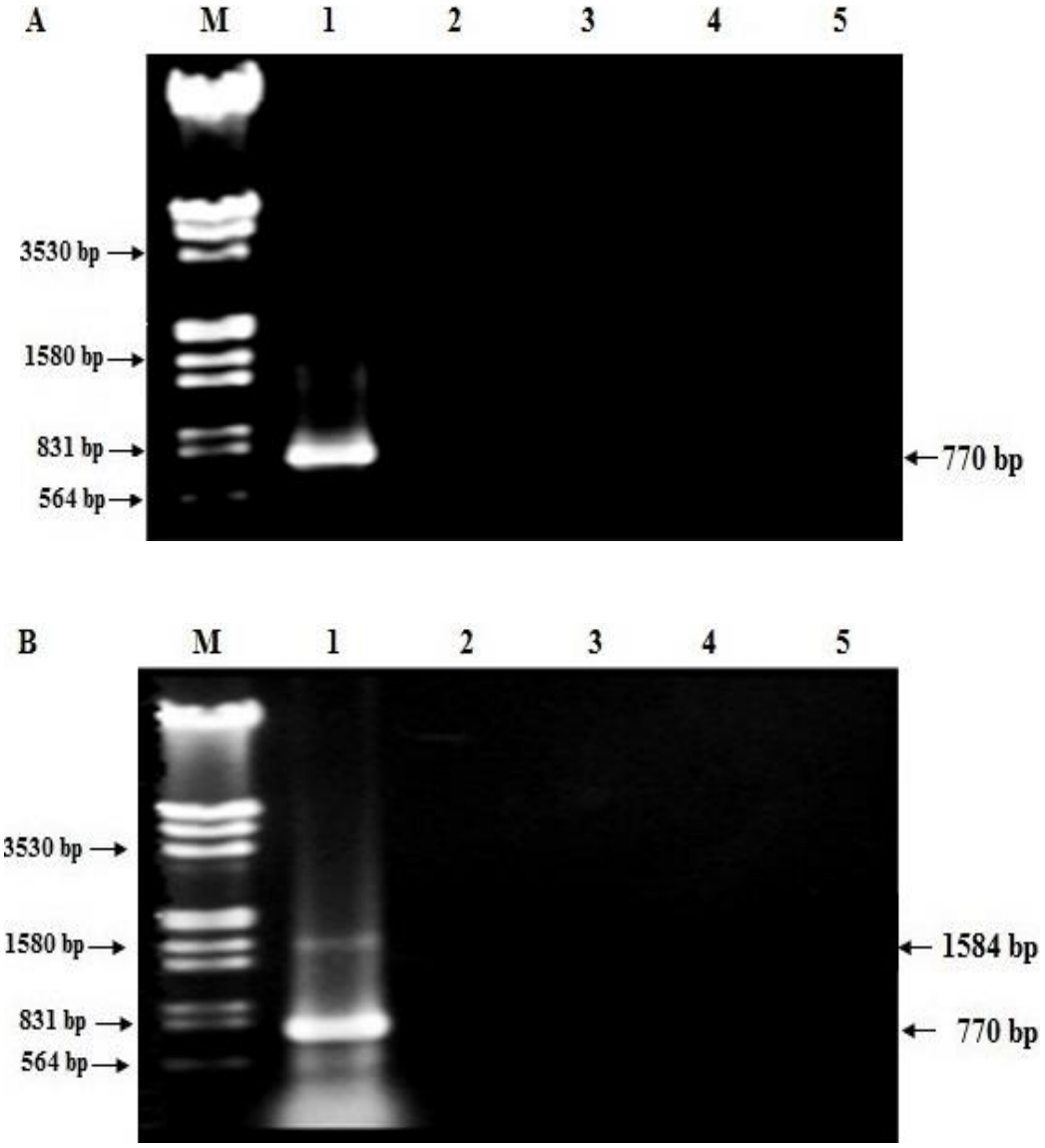

Figure 31 RAPD-PCR product patterns obtained from genomic DNAs of dead *S.* Typhimurium cells (A) and dead *S.* Enteritidis cells (B) treated with 3.0 µg/ml EMA and exposed with different durations of light: lane M, DNA marker; lane 1, positive control; lane 2, negative control; lane 3, 5 min; lane 4, 10 min; lane 5, 15 min.

The optimum light exposure distance was also tested. A distance for the halogen light exposure of 10, 20, and 30 cm did not affect the DNA amplification of viable cells (Figure 32A and 32B). However, EMA in heat-killed cells inhibited the amplification at an exposure distance of 10 and 20 cm. The DNA amplification of

heat-killed cells was not completely inhibited at 30 cm (Figure 33A and 33B). Thus, the suitable exposure distance for both *Salmonella* serovars was 20 cm. The light intensity at the exposure distance of 30 cm was not enough to activate the EMA for forming irreversible cross-linkage with DNA of the dead cell. The suitable light exposure time for cross linking of dead bacterial DNA to EMA was at least 5 min. Based on the results, the increase of the photolysis duration did not affect the DNA product yield from RAPD-PCR amplification. Apart from the above factors, the presence of high levels of suspended solids or biomass in the analyzed samples, the salt concentration in the reaction mixture, the pH of the reaction mix and the length of the target gene are also important factors for the success of the EMA-PCR technique (Fittipaldi *et al*., 2012).

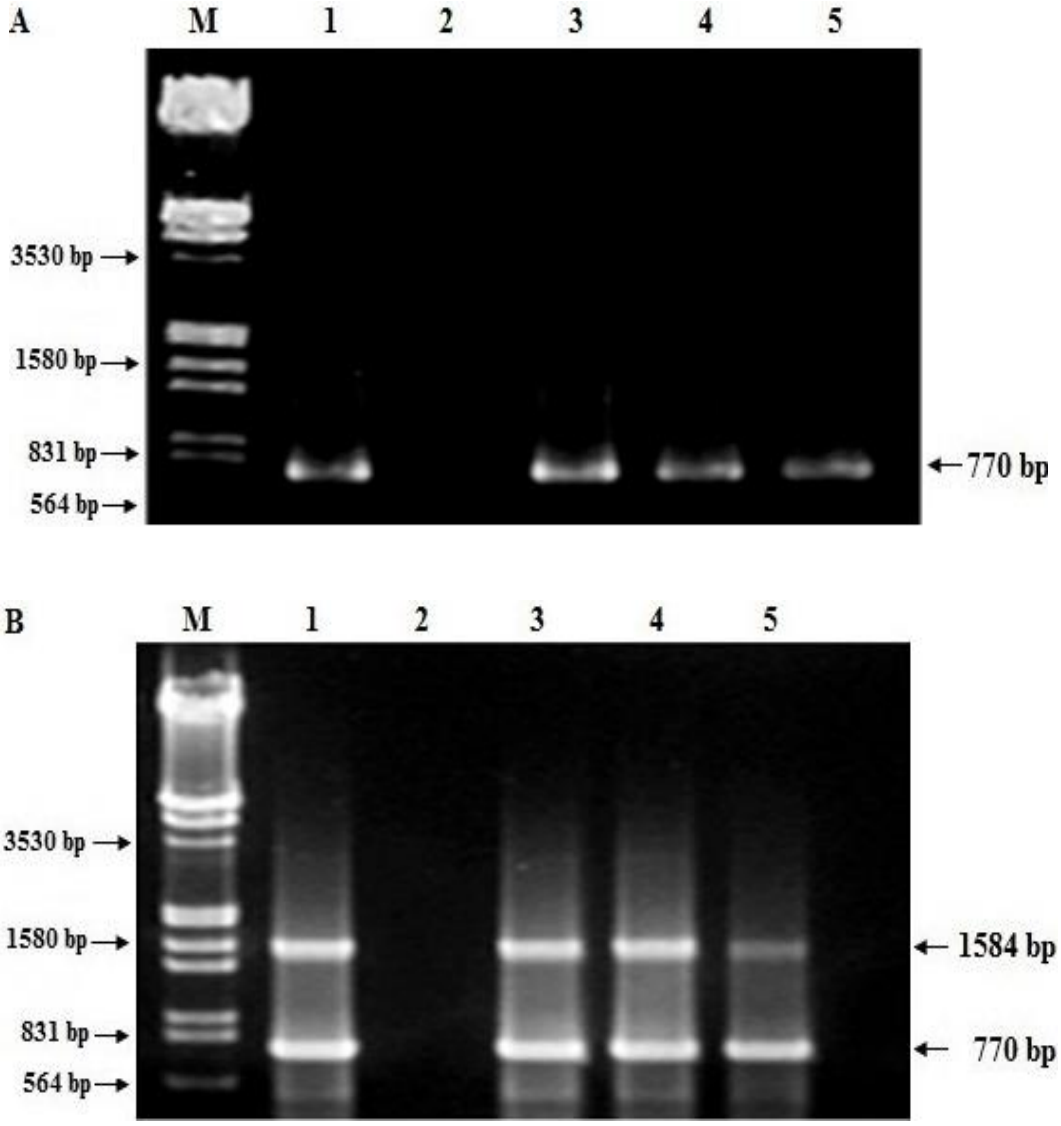

Figure 32 RAPD-PCR product patterns obtained from genomic DNAs of viable *S.* Typhimurium cells (A) and viable *S.* Enteritidis cells (B) treated with 3.0 µg/ml EMA and exposed with different light exposure distances: lane M, DNA marker; lane 1, positive control; lane 2, negative control; lane 3, 10 cm; lane 4, 20 cm; lane 5, 30 cm.

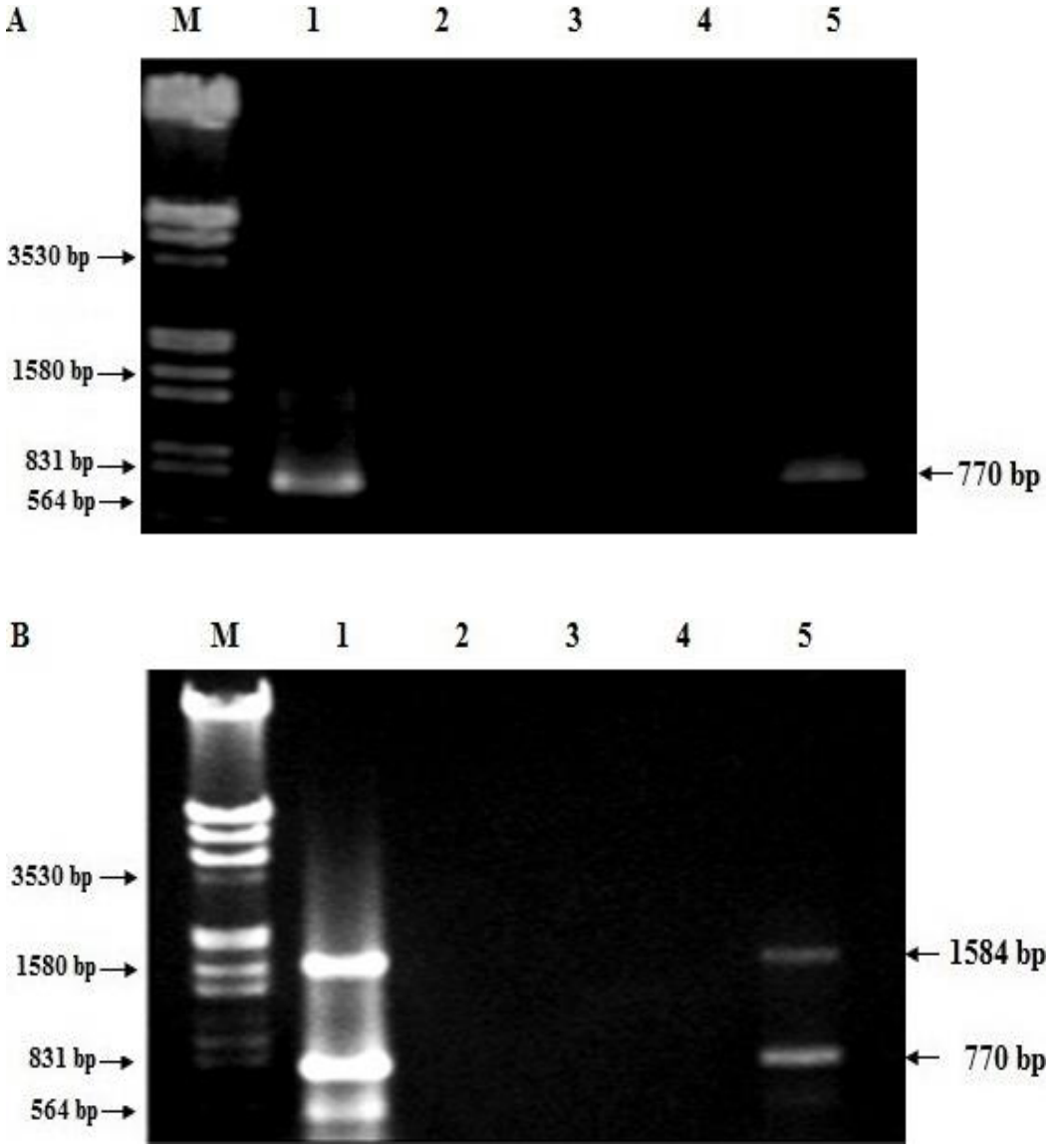

Figure 33 RAPD-PCR product patterns obtained from genomic DNAs of dead *S.* Typhimurium cells (A) and dead *S.* Enteritidis cells (B) treated with 3.0 µg/ml EMA and exposed with different light exposure distances: lane M, DNA marker; lane 1, positive control; lane 2, negative control; lane 3, 10 cm; lane 4, 20 cm; lane 5, 30 cm.

3.2 The effect of EMA on the ratios of viable and dead *Salmonella* cells

To study the proportion of viable cells and dead cells affected the inhibition of the DNA amplification from dead cells by EMA-RAPD-PCR method

(Figure 31), the heat-killed *S.* Enteritidis DMST 15676 cells were mixed with their viable cells in the ratios of viable cells representing 100, 1.0, 0.1 and 0.01% of the total 1.3×10^6 cells, respectively. The results showed that the lowest ratio of the viable cells that could be detected by EMA-RAPD-PCR method was 0.1% of the total 1.3×10^6 cells. The obtained results suggested that the total number of viable cells, which could be detected by this EMA-RAPD-PCR, was not lower than 1.3×10^3 cells. This result was correlated with the study of Trevanich *et al*. (2010), who reported that the detection limit of RAPD-PCR using primer 3 for detection of pure *Salmonella* was 3.2×10^3 cells. Detection limit in our present study was also the same as reported by Wang and Mustapha (2010), who found that the detection limit of pure *Salmonella* culture was at least 10^3 CFU/ml when an optimized EMA-Real-Time-PCR was used. However, our detection limit was higher than that reported by Rahn *et al.* (1992), who detected as low as 3.0×10^2 cells of *S*. Typhimurium by using the *invA* gene-based PCR assay. Miyamoto *et al*. (1998) reported that *Salmonella*-specific RAPD bands were detected by ethidium bromide staining method even when pure genomic DNA prepared by CTAB from as few as 2.8×10^2 cells was used. For the application of RAPD-PCR method to detect a low number of *Salmonella* cells in food or environment samples, the pre-enrichment step should be used prior to RAPD-PCR amplification to increase the number of viable cells (Wang and Mustapha, 2010). Interestingly, Banihashemi *et al.* (2012) reported that amplicon length was one of critically important factors in signal reduction of target DNA amplification from heat-killed cells when using PMA-PCR. Their results showed that PMA treatment prior to PCR amplification of short DNA amplicon (< 200 bp) did not fully inhibit the effects of heat-killed *S. enterica* and *C. jejuni* as such weak short target band product was still observed. Our target DNA amplicons generated from *Salmonella* spp. by primer 3-based RAPD-PCR were relatively longer in size than that of 200 bp. Therefore, use of longer amplicons as the target in this study can highly improve the effectiveness of EMA-RAPD-PCR in terms of specific detection of viable *Salmonella* cells.

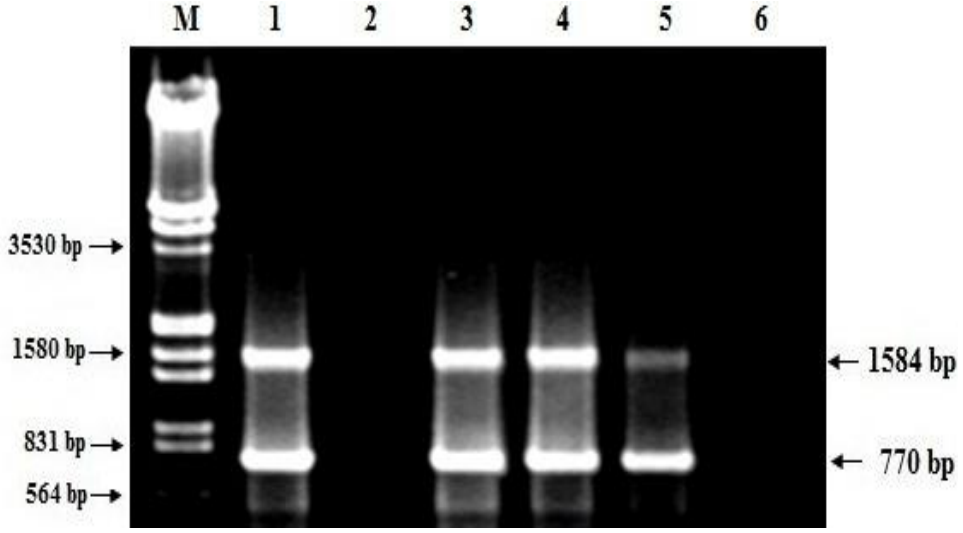

Figure 34 RAPD-PCR product patterns obtained from genomic DNAs of EMA-treated viable *S.* Enteritidis cells mixed with dead cells in various ratios: lane M, DNA marker; lane 1, positive control; lane 2, negative control; lane 3, 100% EMA-treated viable cells; lane 4, 1.0% EMA-treated viable cells; lane 5, 0.1% EMA-treated viable cells; lane 6, 0.01% EMA-treated viable cells.

3.3 The detection of *Salmonella* cells in artificially contaminated chicken meat by EMA-RAPD-PCR

The effect of the viable and dead bacterial mixtures of the same high concentrations on the effectiveness of EMA-RAPD-PCR was also tested. This technique was applied to detect DNA mixtures obtained from the same high concentrations of viable or dead *S.* Enteritidis cells at the ratio of 1:1 with other viable or dead bacteria including *S. sonnei* DMST 171564, *E. coli* O124 DMST 672, *E. aerogenes* DMST 8841, *K. pneumoniae* DMST 7592, or *C. freundii* DMST 16368. These mixed bacterial cultures were artificially contaminated into the autoclaved chicken meat samples. The results shown in Figure 35 and 36 indicated that only DNA of the viable *S.* Enteritidis cells could be amplified by our developed EMA-RAPD-PCR technique while no amplification of any products was generated from dead *S.* Enteritidis cells mixed with other dead bacterial cells tested. Additionally,

several faint band products were observed when dead *S.* Enteritidis cells mixed with other viable bacterial cells tested (data not shown). However, no *S.* Enteritidis specific bands of about 770 and 1584 bp among other bands produced was detected by the EMA-RAPD-PCR analysis. These indicated that the designed primer 3 for RAPD-PCR was highly specific to *S.* Enteritidis and other *Salmonella* spp. EMA could effectively inhibit DNA amplification of the dead cells from *S.* Enteritidis mixed with other dead bacteria used and also the naturally contaminated bacteria in food samples. The results also indicated that EMA treatment was not affected by both soluble and insoluble materials originated from chicken meats and bacterial cells. Therefore, other viable or dead bacterial cultures even dead *Salmonella* cells did not affect the specific amplification of viable *Salmonella* cells by the EMA-RAPD-PCR technique used. From the result obtained, it implied that the EMA-RAPD-PCR method was significantly important for discrimination of viable and dead cells when higher numbers of bacteria were present. In order to improve the reliability of the EMA-RAPD-PCR when low numbers of viable *Salmonella* cells were present, 20-h pre-enrichment step in NB was performed prior to EMA-RAPD-PCR to increase the number of viable cells.

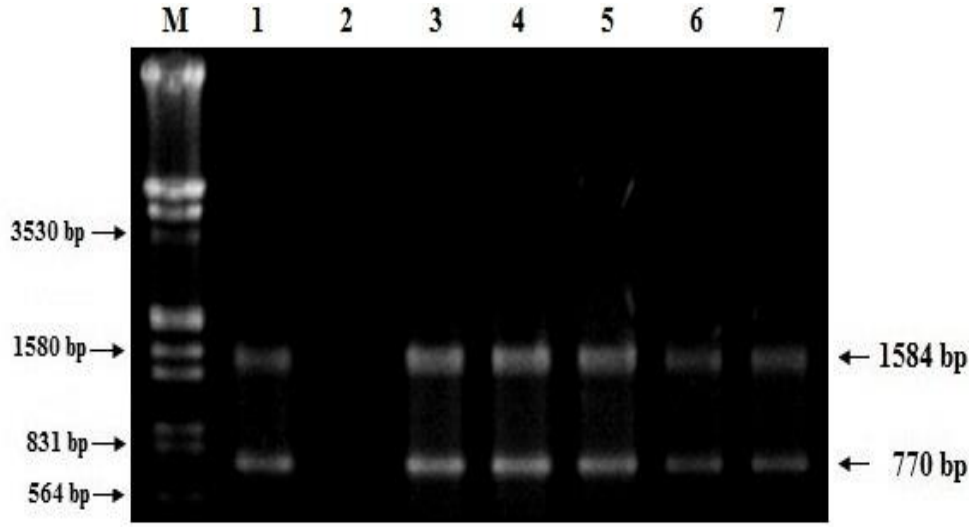

Figure 35 RAPD-PCR product patterns obtained from genomic DNAs of viable *S.* Enteritidis cells mixed with other dead organisms artificially contaminated in chicken meats as determined by EMA-RAPD-PCR: lane M, DNA marker; lane 1, positive control; lane 2, negative control; lane 3, *S.* Enteritidis DMST 15676 and *S. sonnei* DMST 17564; lane 4, *S.* Enteritidis DMST 15676 and *E. coli* O124 DMST 672; lane 5, *S.* Enteritidis DMST 15676 and *E. aerogenes* DMST 8841; lane 6, *S.* Enteritidis DMST 15676 and *K. pneumoniae* DMST 7592; lane 7, *S.* Enteritidis DMST 15676 and *C. freundii* DMST 16368.

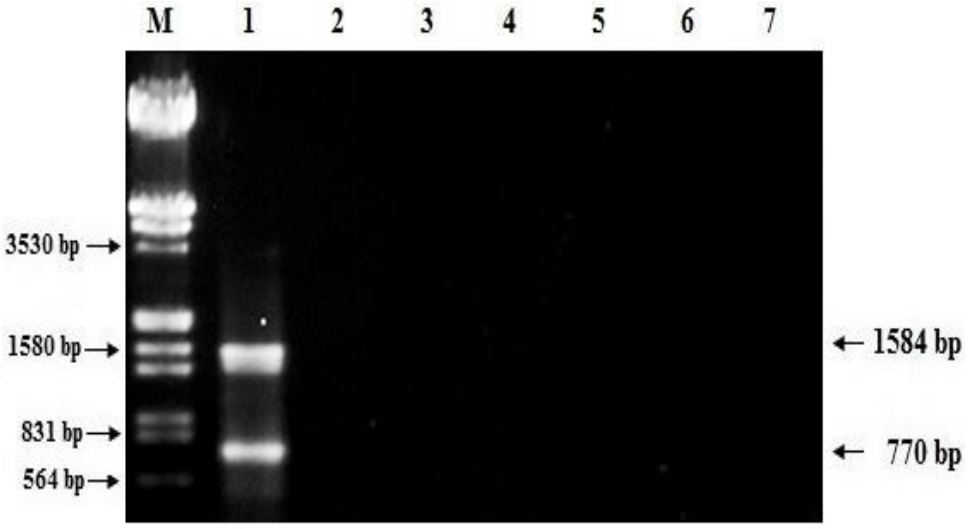

Figure 36 RAPD-PCR product patterns obtained from genomic DNAs of dead *S.* Enteritidis cells mixed with other dead organisms artificially contaminated in chicken meats as determined by EMA-RAPD-PCR: lane M, DNA marker; lane 1, positive control; lane 2, negative control; lane 3, *S.* Enteritidis DMST 15676 and *S. sonnei* DMST 17564; lane 4, *S.* Enteritidis DMST 15676 and *E. coli* O124 DMST 672; lane 5, *S.* Enteritidis DMST 15676 and *E. aerogenes* DMST 8841; lane 6, *S.* Enteritidis DMST 15676 and *K. pneumoniae* DMST 7592; lane 7, *S.* Enteritidis DMST 15676 and *C. freundii* DMST 16368.

3.4 Detection of *Salmonella* spp. in naturally contaminated chicken products by EMA-RAPD-PCR

In this present study, the detection results obtained from naturally-contaminated food samples were observed. Two hundred and forty of chicken products purchased from three local fresh markets and three supermarkets were analyzed (Table 6). The results revealed that of the 120 samples from fresh markets, 34 (28%) were positive: 9 samples of leg muscle, 7 samples of breast, 18 samples from gizzards and no positive samples detected from eggs. For the supermarket samples, of the 120 samples, 5 (4.1%) gizzard samples were positive; other samples were not contaminated with *Salmonella* spp. All true positive samples, in which the

presence of *Salmonella* spp. was confirmed by the conventional method, obviously showed *Salmonella* specific band of 770 bp generated by the EMA-RAPD-PCR analysis.

Table 6 Comparison between the EMA-RAPD-PCR method and the conventional method for detection of *Salmonella* spp. in chicken products from fresh markets and supermarkets.

Sample sources	Sample types	Number of samples tested	Positive number of samples tested	
			EMA-RAPD-PCR Method	Conventional method
Fresh markets	Leg muscle	30	9	9
	Breast	30	7	7
	Gizzard	30	18	18
	Egg	30	0	0
	Total	120	34 (28%)	34 (28%)
Supermarkets	Leg muscle	30	0	0
	Breast	30	0	0
	Gizzard	30	5	5
	Egg	30	0	0
	Total	120	5 (4.1%)	5 (4.1%)
Total		240	49 (16.25%)	49 (16.25%)

From the results, *S.* Enteritidis may not be present in such positive chicken products due to no another specific band of about 1584 was detected. The results of true positive samples from using EMA-RAPD-PCR were identical to the conventional method. No specific bands of 770 and 1584 bp were observed by the EMA-RAPD-PCR analysis in all *Salmonella* spp. negative samples, in which the absence of *Salmonella* spp. was confirmed by the conventional method. Therefore, the false-positive results obtained from dead *Salmonella* cells present in chicken products samples were found to be at zero level. The optimized EMA-RAPD-PCR method

involving 20-h culturing in NB followed by EMA treatment and primer 3-based RAPD-PCR was effective and reliable enough for specific detection of viable *Salmonella* cells in various food samples. Wang and Mustapha (2010) successfully developed a 12-h enrichment step combined with EMA-Real-Time-PCR for detection of low concentrations of viable *Salmonella* cells in artificially contaminated chicken rinses and egg broth. Their method could detect as low as 10 CFU/ml *Salmonella* from the samples. In addition, Nocker and Camper (2006) reported that most of the DNA from dead cells was probably decreased during the DNA extraction procedure by centrifugation due to precipitation with the cell debris. However, 0.01 ng of the remaining DNAs from dead cells could be a template for amplification by PCR technique. EMA with an average binding capacity constant of 2.5×10^5 M can be covalently linked to DNA of dead cells by photoactivation with high yield of up to 75% after intercalating into the DNA of such cells (Bolton and Kearns, 1978). Therefore, EMA can not be completely binded to all DNA from dead cells. Pre-enrichment step in appropriate media and conditions could play an important role in enhancing the numbers of viable cells. Thus, the use of an appropriate pre-enrichment step combined with EMA treatment prior to PCR analysis has the potential to overcome the amplification problem of DNA from dead cells and then prevent false positive results. Compared to PMA, our recent EMA-RAPD-PCR has also shown that amplification of a long amplicon (770 or 1584 bp) completely inhibited the interference of DNA from dead cells. In fact, EMA is known to be more toxic to viable cells and has a lower binding ability than PMA. However, our result indicated that the developed EMA-RAPD-PCR technique was still effective in detection of viable *Salmonella* cells as the condition of EMA-treatment was properly optimized when compared to some studies as described previously. The optimal EMA concentration of 3 μg/ml or 7.14 μmol/l resulted in effective prevention in the false positive from DNA of dead cells without affecting the viable cells. Banihashemi *et al.* (2012) also reported that PMA concentrations at higher than 20 μmol/l caused toxicity to viable cells tested. In addition, 20-h pre-enrichment in NB increased the number of viable *Salmonella* cells into a sufficient level before EMA treatment was done. However, some studies have reported that the PMA-PCR method was incompletely effective in inhibition of amplification of DNA from dead cells (Pan and

Breidt, 2007; Kralik *et al.,* 2010). Pan and Breidt (2007) found that PMA-PCR technique was not fully suppression of the DNA amplification from dead *L. monocytogenes* cells caused by heat treatment. Similar to Banihashemi *et al.* (2012), the developed EMA-RAPD-PCR in this study will be used to detect only viable *Salmonella* cells present in food and environmental samples, regardless of the treatment causing cell death. This research is the first study that combined EMA with RAPD-PCR to inhibit the amplification of DNA from dead cells for detection of *Salmonella* spp. in the food. The optimized EMA-RAPD-PCR technique involving 20-h culturing in NB developed in this study has high efficiency and reliability for viable *Salmonella* detection from chicken product samples. This study indicated that using the EMA-RAPD-PCR technique can prevent false positive results from DNA of the dead *Salmonella* cells. In summary, the overall comparison between the EMA-RAPD-PCR and conventional method for detection of *Salmonella* spp. in food products was shown in Table 7.

Table 7 Comparison between the EMA-RAPD-PCR method and the conventional method.

Method	EMA-RAPD-PCR method	Conventional Method
Time	Rapid No pre-enrichment 1 h Pre-enrichment 24 h	Long (1-2 weeks)
Reliability	High sensitivity No pre-enrichment ≥1.3×10 viable cells Pre-enrichment 1 viable cell	Depending on the number of isolated strains

Table 7 (continued)

Method	EMA-RAPD-PCR method	Conventional Method
Work load	Non laborious	Laborious
Cross contamination	Low	High
Cost per test	200 baht	500-1000 baht
Method	Automate	Manual

When compared to the conventional method (modified ISO 6579: 2002), the developed EMA-RAPD-PCR reduces the time for obtaining the detection result of *Salmonella* spp. present in food products and can be applied for detection of many samples in one time with less labor intensive and low cost consumption. The interpretation is also simple and does not required a highly skilled technician. Scientists need to examine cultures visually to check for microorganisms. Because of our limited vision, humans can see those microbes only when growth reaches a high number of colony-forming units. PCR method typically uses DNA marker that can be detected by an instrument. Skilled technician is needed in order to correctly differentiate and select the suspect colony of *Salmonella* spp. grown on selective plating media. The developed EMA-RAPD-PCR is minimized scale technique which is performed easily with less procedure steps and small volume of reagents under the separated small area such as PCR cabinet. Lower cross contamination is normally observed when compared to the conventional method because of less sample transferring steps during work. The media plates are easily contaminated when the plates are disclosed for colony observation or sub-culture while PCR method is performed in a microtube, no need to sub-culture in many steps. In conventional methods, detection is highly dependent on growth conditions that do not allow all the microbes to proliferate. Rapid methods target metabolic markers, so they can also detect viable but non culturable microorganisms. The workload for cleaning a large numbers of glassware of conventional method is higher than those of EMA-RAPD-

PCR. The developed EMA-RAPD-PCR provides higher sensitivity and specificity than the conventional methods because the technique is directly based on the detection target DNA gene of microorganism, which is related to the presence of specific genetic elements in the microbial cells. Furthermore, EMA-RAPD-PCR may be semi-automated with PCR combined with pre-enrichment step, which offers increased sensitivity of detection, operate in a continuous data-collecting mode and provides significantly reduced time-to-result. The result can be obtained within an hour for the high numbers of microorganism contaminated food sample. For some low numbers of contaminated food sample, the result is completed within one day with the incubation period of 20 h pre-enrichment step added. This method has also been shown to detect slow-growers and/or viable but non-cultural microorganisms as compared with the conventional methods used today due to *Salmonella* DNA detection. Most importantly, this method can be implement in support of sterile or non-sterile manufacturing processes during the monitoring and controlling of critical process parameters in a food factory that can reduce or eliminate process variability. The factory can gain reduction in laboratory overhead and headcount, lower inventories (raw material, in-process material, and finished product), a reduction in warehousing space, and a decrease in repeat testing, deviations, out-of-specification investigations, reprocessing or lot rejection. For the expense of food sample test, most conventional method costs about 500-1000 baht per test while the developed EMA-RAPD-PCR costs approximately 200 baht per test. Significant savings from rapid EMA-RAPD-PCR method also come from the ability to identify, contain, and recover from a contamination event quickly. The financial, supply-chain, and brand benefits of being able to recall affected products from distribution centers before they reach customers are obvious. Therefore, EMA−RAPD−PCR involving 20−h culturing in pre−enrichment medium is an efficient, reliable, and economical procedure to detect only viable *Salmonella* spp. in food samples. This technique has demonstrated effectiveness in preventing the DNA amplification of dead *Salmonella* cells. Moreover, the EMA−RAPD−PCR has the potential for use as a rapid, simple, and reliable monitoring tool of *Salmonella* spp. prevalence along the food production chain.

The results of this part confirmed the hypothesis that ethidium bromide monoazide could limit the disadvantage of primer 3-based RAPD-PCR in that it could not distinguish between DNA originated from viable and dead cells. EMA-RAPD-PCR technique inhibited the DNA amplification of the dead cells in all artificially and naturally *Salmonella* contaminated food samples tested. The developed EMA-RAPD-PCR in this study involving 20-h culturing in pre-enrichment medium is an efficient, reliable, and economical procedure to detect only viable *Salmonella* spp. in the food samples. Moreover, the EMA-RAPD-PCR has the potential for use as a rapid, simple, and accurate monitoring tool of *Salmonella* spp. prevalence along the food production chain.

CONCLUSIONS

The main objective of this research was to develop the single primer-based random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) combined with nucleic acid dye for detection of only viable *Salmonella* spp. in food samples. The research was divided into 3 parts, which composed of the study in the consistency of primer 3-based RAPD-PCR for the detection of various *Salmonella* serotypes, the efficiency of primer 3-based RAPD-PCR for detection of stressed *S.* Enteritidis under various stress treatments *in vitro* and food processing conditions, and the development of primer 3-based RAPD-PCR combined with ethydium bromide monoazide (EMA) for detection of only viable *Salmonella* spp. in food samples. From the results and discussions the following conclusions can be drawn.

Part 1**:** The single primer-based RAPD-PCR using 20-mer oligonucleotide single designed on the basis of *S.* Typhimurium *gatD* gene encoding galactitol-1-phosphate dehydrogenase (namely primer 3) could produce the specific DNA product of approximate 770-bp in all 80 *Salmonella* strains (38 serotypes). No 770-bp DNA band was amplified from any DNA samples of 20 non-*Salmonella* bacteria (13 serotypes). In addition, 770 bp product was generated from DNAs of 5 serotypes of *Salmonella* spp. (Rissen, Braenderup, Panama, Enteritidis, and Corvallis) were detected by 770 bp DNA. The 770 bp DNA band had high specificity and consistency for the detection of various *Salmonella* serotypes.

Part 2**:** Primer 3 -based RAPD-PCR could detect both normal *Salmonella* cells and stressed *Salmonella* cells treated by various stress conditions *in vitro* including UV irradiation 20 min, ethanol (50%) 5 min, hydrochloric acid (pH 4) 5 min, sodium hydroxide (pH 10) 5 min, hydrogen peroxide (0.38 mM) 5 min, sodium hypochlorite (200 ppm) 5 min, mild temperature (52°C) 15 min and low temperature (0 and 4°C) 24 h. The same positive signal of 770-bp DNA product was detected when the stressed *S.* Enteritidis cells from fresh ground chicken meat and the processed foods (chilled, frozen and fermented food) were analyzed by 20-h pre-enrichment RAPD-PCR. All *S.* Enteritidis samples under above stress conditions gave the same positive

signal of 770-bp DNA band as the non-stressed cells. The sensitivity test of RAPD-PCR was 100% when compared to the conventional method. The low number of bacterial cells could be detected by RAPD-PCR combined with 20 h pre-enrichment step in NB.

Part 3**:** Primer 3 -based RAPD-PCR combined with ethidium bromine monoazide (EMA-RAPD-PCR) was developed for the discrimination of viable *Salmonella* cells from dead cells. The minimum amount of EMA was 3 μg/ml. The suitable light exposure time was at least 5 min. The optimum light exposure distance was 20 cm. Detection limit of EMA-RAPD-PCR for viable *Salmonella* cells was not less than 1.3×10^3 cells. EMA-RAPD-PCR could detect *S.* Enteritidis mixed with other bacteria in food. Food material did not affect EMA-RAPD-PCR. The EMA-RAPD-PCR could inhibit the dead cell DNA amplification of all artificially and naturally *S.* Enteritidis contaminated in chicken products. Thus, only viable *S.* Enteritidis cells were amplified by EMA-RAPD-PCR. The false-positive result obtained from dead *Salmonella* cells present in chicken products samples was found to be at zero level.

This research is the first study to combine EMA with RAPD-PCR for inhibition of the amplification of dead cell-derived DNA in order to detect only viable *Salmonella* spp. in foods. The optimized EMA-RAPD-PCR involving 20 h culturing in pre-enrichment medium is an efficient and economical procedure to detect only viable *Salmonella* spp. in food samples. Moreover, the EMA-RAPD-PCR has the potential for use as a rapid, simple, cost effective and reliable monitoring tool with high sensitivity and specificity for *Salmonella* spp. prevalence along the food production chain when compared to the conventional method.

RECOMMENDATION

The developed ethidium bromide monoazide-random amplified polymorphic DNA-polymerase chain reaction (EMA-RAPD-PCR) involving 20-h culturing in pre-enrichment medium is an efficient, reliable, and economical procedure to detect only viable *Salmonella* spp. in food samples. This technique has demonstrated effectiveness in preventing the DNA amplification of dead *Salmonella* cells. Moreover, the EMA-RAPD-PCR has the potential for use as a rapid, simple, and accuracy monitoring tool of *Salmonella* spp. prevalence along the food production chain.

The obtained results from this study confirm the reproducibility, specificity and reliability of EMA-RAPD-PCR technique involving 20-h culturing step in NB for detection of viable *Salmonella* spp. Implementing the developed EMA-RAPD-PCR assay as alternative method for monitoring of *Salmonella* spp. throughout the food production chain is needed. In addition, the specific 770-bp DNA product can be sequenced and determined to possibly use as a probe for specific detection of *Salmonella* spp. Other nucleic acid dyes including PMA should be investigated as the alternative dye for reliable detection of viable *Salmonella* spp. in foods.

LITERATURE CITED

Abee, T. and J.A. Wouters. 1999. Microbial stress response in minimal processing. **Int. J. Food Microbiol.** 50: 65-91.

Agusti, G., F. Codony, M. Fittipaldi, B. Adrados and J. Morató. 2010. Viability determination of *Helicobacter pylori* using propidium monoazide quantitative PCR. **Helicobacter.** 15: 473-476.

Altuvia, S., M. Almiron, G. Huisman, R. Kolter and G. Storz. 1994. The dps promoter is activated by OxyR during growth and by IHF and sigma S in stationary phase. **Mol. Microbiol.** 13: 265-272.

Altuvia, S., A. Zhang, L. Argaman, A. Tiwari and G. Storz. 1998. The *Escherichia coli* OxyS regulatory RNA represses fhlA translation by blocking ribosome binding. **Embo J.** 17: 6069-6075.

Alvarez-Ordonez, A., A. Fernandez, A. Bernardo and M. Lopez. 2009. Comparison of acids on the induction of an Acid Tolerance Response in *Salmonella* Typhimurium, consequences for food safety. **Meat Sci.** 81: 65-70.

Alves, J., V.V. Marques, L.F.P. Pereira, E.Y. Hirooka and T.C.R.M. De Oliveira. 2012. Multiplex PCR for the detection of *Campylobacter* spp. and *Salmonella* spp. in chicken meat. **J. Food Safety.** 32: 345-350.

Andrews, H.L. and A.J. Baumler. 2005. *Salmonella* species, pp. 327-339. *In* P.M. Fratamico, A.K. Bhunia and J.L. Smith, eds. **Foodborne Pathogens: Microbiology and Molecular Biology.** Horizon Scientific Press Ltd., United Kingdom.

AOAC. 2002. **Accreditation Criteria for Laboratories Performing Food Microbiological and Chemical Analyses in Foods, Feeds, and Pharmaceutical Testing**. DIGART International Ltd., Geneva, Switzerland.

Asai, T., T. Namimatsu, T. Osumi, A. Kojima, K. Harada, H. Aoki, S. Toshiya and T. Takahashi. 2010. Molecular typing and antimicrobial resistance of *Salmonella enterica* subspecies *enterica* serovar Choleraesuis isolates from diseased pigs in Japan. **Comp. Immunol. Microb.** 33: 109-119.

Atlung, T. and L. Brondsted. 1994. Role of the transcriptional activator AppY in regulation of the cyx appA operon of *Escherichia coli* by anaerobiosis, phosphate starvation, and growth phase. **J. Bacteriol.** 176: 5414-5422.

Bae, S. and S. Wuertz. 2009. Discrimination of viable and dead fecal Bacteroidales bacteria by quantitative PCR with propidium monoazide. **Appl. Environ. Microbiol.** 75: 2940-2944.

Bangtrakulnonth, A., S. Pornruangwong, T. Chalermchaikit and K. Saitanu. 1993. *Salmonella* Enteritidis infection in Thailand: a public health problem?, pp.175-180. *In* **Proceedings of The 11th International Symposium of The Word Association of Veterinary Food Hygienists Thailand 1993.** 24-29 October 1993, Chulalongkorn University, Bangkok.

Banihashemi, A., M.I. Van Dyke and P.M. HUCK. 2012. Long-amplicon propidium monoazide-PCR enumeration assay to detect viable *Campylobacter* and *Salmonella*. **J. Appl. Microbiol.** 113: 863-873.

Bartlett, J.M. and D. Stirling. 2003. A short history of the polymerase chain reaction. **Methods Mol. Biol.** 226: 3-6.

Bej, A.K., W.Y. Ng, S. Morgan, D.D. Jones and M.H. Mahbubani. 1996. Detection of viable *Vibrio cholerae* by reverse-transcriptase polymerase chain reaction (RT-PCR). **Mol. Biotechnol.** 5: 1-10.

Bell, C. and A. Kyriakides. 2002. ***Salmonella*: A Practical Approach to The Organism and Its Control in Foods.** Backwell Science, London.

Bhunia, A.K. 2008. **Foodborne Microbial Pathogens: Mechanisms and Pathogenesis.** Springer Science Business Media, LLC, New York.

Biotium. 2009. **Ethidium Monoazide, Bromide (EMA) and Propidium Monoazide (PMA)**. Biotium, Inc., Hayward, CA.

Bleve, G., L. Rizzotti, F. Dellaglio and S. Torriani. 2003. Development of reverse transcription (RT)-PCR and real-time RT-PCR assays for rapid detection and quantification of viable yeasts and molds contaminating yogurts and pasteurized food products. **Appl. Environ. Microbiol.** 69: 4116-4122.

Bouchrif, B., B. Paglietti, M. Murgia, A. Piana, N. Cohen, M. Ennaji, S. Rubino and M. Timinouni. 2009. Prevalence and antibiotic-resistance of *Salmonella* isolated from food in Morocco. **J. Infect. Dev.** 28: 35-40.

Boyen, F., F. Haesebrouck, D. Maes, F. Van Immerseel, R. Ducatelle and F. Pasmans. 2008. Non-typhoidal *Salmonella* infections in pigs: a closer look at epidemiology, pathogenesis and control. **Vet. Microbiol.** 130: 1-19.

Brenner, D.J. 1984. Facultatively anaerobic gram-nagative rods, pp. 408-516. *In* N.R. Krieg and J.C. Holt, eds. **Bergey's Manual of Systematic Bacteriology.** 1st ed. Williams and Walkins, Baltimore.

Brescia, C.C., S.M. Griffin, M.W. Ware, E.A. Varughese, A.I. Egorov and E.N. Villegas. 2009. *Cryptosporidium* propidium monoazide-PCR, a molecular Biology-based technique for genotyping of viable *Cryptosporidium oocysts*. **Appl. Environ. Microbiol.** 75: 6856-6863.

Bryan, P.J., R.J. Steffan, A. DePaola, J.W. Foster and A.K. Bej. 1999. Adaptive response to cold temperatures in *Vibrio vulnificus*. **Curr. Microbiol.** 38: 168-75.

Busch, U. and H. Nitschko. 1999. Methods for the differentiation of microorganisms. **J. Chromotogra. B.** 722: 263-278.

Candrian, U. 1995. Polymerase chain reaction in food microbiology. **J. Microbiol. Methods.** 23: 89-103.

Cardinale, E., J.D.P. Gros-Claude, F. Tall, E.F. Gueye and G. Salvat. 2005. Risk factors for contamination of ready-to-eat street-vended poultry dishes in Dakar, Senegal. **Int. J. Food Microbiol.** 103: 157-165.

Carmel-Harel, O. and G. Storz. 2000. Roles of the glutathione and thioredoxin-dependent reduction systems in the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress. **Annu. Rev. Microbiol.** 54: 439-61.

Cawthorn, D.M. and R.C. Witthuhn. 2008. Selective PCR detection of viable *Enterobacter sakazakii* cells utilizing propidium monoazide or ethidium bromide monoazide. **Appl. Environ. Microbiol.** 105: 1178-1185.

CDC. 2005. Outbreaks of *Salmonella* infections associated with eating Roma Tomatoes-United States and Canada. 2004. **MMWR Morb. Mortal. Wkly. Rep.** 54: 325-328.

CDC. 2008. Outbreak of *Salmonella* serotype Saintpaul infections associated with multiple raw produce items United States, 2008. **MMWR Morb. Mortal. Wkly. Rep.** 57: 92-934.

CDC. 2009. Multistate outbreak of *Salmonella* infections associated with peanut butter and peanut butter containing products-United States, 2008-2009. **MMWR Morb. Mortal. Wkly. Rep.** 58: 85-90.

CDC. 2010a. ***Salmonella***. Available Source: http://www.cdc.gov/ *Salmonella/*, December 17, 2011.

CDC. 2010b. Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food-10 states, 2009. **MMWR Morb. Mortal. Wkly. Rep.** 59: 418-422.

Chansiripornchai, N., P. Ramasoota, A. Bangtrakulnonth, J. Sasipreeyajan and B.S. Svenson. 2000. Application of randomly amplified polymorphic DNA (RAPD) analysis for typing avian *Salmonella enterica* subs. *enterica*. **FEMS Immunol. Med. Mic.** 29: 221-225.

Chen, S., F. Wang, J.C. Beaulieu, R.E. Stein and B. Ge. 2011. Rapid detection of viable salmonellae in produce by coupling propidium monoazide with loop mediated isothermal amplification. **Appl. Environ. Microbiol.** 77: 4008-4016.

Christman, M.F., R.W. Morgan, F.S. Jacobson and B.N. Ames. 1985. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella* Typhimurium. **Cell**. 41: 753-62.

CJ, D. 1994. **Genetics of Bacterial Virulence**. Blackwell Scientific, London.

Cogan, T.A. and T.J. Humphrey. 2003. The rise and fall of *Salmonella* Enteritidis in the UK. **J. Appl. Microbiol.** 94: 114-119.

Compton, T. 1990. Degenerate primers for DNA amplification, pp. 39-45. *In* M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White, eds. **PCR Protocols**. Academic Press, New York.

Cox, J.M. and A. Pavic. 2010. Advances in enteropathogen control in poultry production. **J. Appl. Microbiol.** 108: 745-755.

Crump, J.A. and E.D. Mintz. 2010. Global trends in typhoid and paratyphoid fever. **Emerg. Infect.** 50: 241-246.

Csonka, L.N. 1989. Physiological and genetic responses of bacteria to osmotic stress. **Microbiol. Rev.** 53: 121-47.

CSPI. 2005. *Salmonella* outbreaks linked to produce on the rise: produce is primary cause of large *Salmonella* outbreaks. *In* **CSPI Newsroom**. 21 November 2005. Center for Science in the Public Interest, Washington, D.C.

Delarocque, A.E., J.C. Desenclos, P. Bouvet and P.A.D. Grimont. 1998. Risk factors for the occurrence of sporadic *Salmonella enterica* serotype Enteritidis infections in children in France: a national case-control study. **Epidemiol. Infect.** 121: 561-567.

Delgado Viscogliosi, P., L. Solignac and J.M. Delattre. 2009. Viability PCR, a culture independent method for rapid and selective quantification of viable *Legionella pneumophila* cells in environmental water samples. **Appl. Environ. Microbiol.** 75: 3502-3512.

De Medici, D., L. Croci, E. Delibato, S.D. Pasquale, E. Filetici and L. Toti. 2003. Evaluation of DNA extraction methods for use in combination with SYBR Green I real-time PCR to detect *Salmonella enterica* serotype Enteritidis in poultry. **Appl. Environ. Microbiol.** 69: 3456-3461.

de Oliveira, F.A., A.P. Pasqualotto, W.P. da Silva and E.C. Tondo. 2010. **Characterization of *Salmonella* Enteritidis Isolated from Human Samples**. Food Research International, In Press, Corrected Proof.

DeTraglia, M.C., J.S. Brand and A.M. Tometsko. 1978. Characterization of azidobenzamidines as photoaffinity labels for trypsin. **J. Biol. Chem.** 253: 1846-1852.

de Wet, S.C., S.E. Denman, L. Sly and C.S. McSweeney. 2008. An improved method for RNA extraction from carcass samples for detection of viable *Escherichia coli* O157:H7 by reverse-transcriptase polymerase chain reaction. **Lett. Appl. Microbiol.** 47: 399-404.

Ellingson, J.L.E., J.L. Anderson, S.A. Carlson and V.K. Sharma. 2004. Twelve hour real-time PCR technique for the sensitive and specific detection of *Salmonella* in raw and ready-to-eat meat products. **Mol. Cell. Probes.** 18: 51-57.

Ewing, W.H. 1986. The taxonomy of Enterobacteriaceae, isolation of Enterobacteriaceae and preliminary identification. The genus *Salmonella*, pp. 1-91, 181-318. *In* P. Edwards and W.H. Ewing, eds. **Proceeding of the 4th Identification of Enterobacteriaceae**. Elsevier, New York.

FDA/BAM. 1995. **Bacteriological Analytic Manual, 8th Edition.** Available Source: http://www.fda.gov/Food/Science Research/Laboratory Methods/Bacteriological Analytical/, September 13, 2001.

Fittipaldi, M., A. Nocker and F. Codony. 2012. Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification. **J. Microbiol. Methods.** 91: 276-289.

Fittipaldi, M., N.J. Rodriguez, F. Codony, B. Adrados, G. Peñuela and J. Morató. 2010. Discrimination of infectious bacteriophage T4 virus by propidium monoazide real-time PCR. **J. Virol. Methods.** 168: 228-232.

Flekna, G., P. Stefanic, M. Wagner, F.J. Smulders, S.S. Mozina and I. Hein. 2007. Insufficient differentiation of live and dead *Campylobacter jejuni* and *Listeria monocytogenes* cells by ethidium monoazide (EMA) compromises EMA/real-time PCR. **Res. Microbiol.** 158: 405-412.

Foster, J.W. 1991. *Salmonella* acid shock proteins are required for the adaptive acid tolerance response. **J. Bacteriol.** 173: 6896-6902.

Foster, J.W. and H.K. Hall. 1990. Adaptive acidification tolerance response of *Salmonella* Typhimurium. **J. Bacteriol.** 172: 771-778.

Foster, J.W. and M.P. Spector. 1995. How *Salmonella* survive against the odds. **Annu. Rev. Microbiol.** 49: 145-74.

Francis, K.P., P.D. Taylor, C.J. Inchley and M.P. Gallagher. 1997. Identification of the ahp operon of *Salmonella* Typhimurium as a macrophage-induced locus. **J. Bacteriol.** 179: 4046-8.

Fuqua, S.A.W., S.D. Fitzgerald and W.L. McGuire. 1990. A simple polymerase chain reaction method for detection and cloning of low-abundance transcripts. **Biotechnol.** 9: 206-211.

Gahan, C.G. and C. Hill. 1999. The relationship between acid stress responses and virulence in *Salmonella* Typhimurium and *Listeria monocytogenes*. **Int. J. Food Microbiol.** 50: 93-100.

Galan, J.E., C. Ginocchio and P. Costeas. 1992. Molecular and functional characterization of the *Salmonella* invasion gene *InvA*: homology of *InvA* to members of a new protein family. **J. Bacteriol.** 174: 4338-4349.

Gautom, R.K. 1997. Rapid pulse field gel electrophoresis protocol for typing of *Escherichia coli* O157:H7 and other gram-negative organisms in 1 day. **J. Clin. Microbiol.** 35: 2977-2980.

Giannella, R.A. 1981. Pathogenesis of acute bacterial diarrheal disorders. **Annu. Rev. Med.** 32: 341-357.

Gonzalez-Escalona, N., T.S. Hammack, M. Russell, A.P. JacobsonDe, A.J. Jesus, E.W. Brown and K.A. Lampel. 2009. Detection of live *Salmonella sp.* cells in produce by a *Taq*Man-based quantitative reverse transcriptase real-time PCR targeting *invA* mRNA. **Appl. Environ. Microbiol.** 75: 3714-3720.

Graiver, D.A., S.E. Saunders, C.L. Topliff, C.L. Kelling and S.L. Bartelt-Hunt. 2010. Ethidium monoazide does not inhibit RT-PCR amplification of nonviable avian influenza RNA. **J. Virol. Methods.** 164: 51-54.

Graves, D.E., C.L. Watkins and L.W. Yielding. 1981. Ethidium bromide and its photoreactive analogues: spectroscopic analysis of deoxyribonucleic acid binding properties. **Biochemistry-US.** 20: 1887-1892.

Gray, J.T. and P.J. Fedorka-Cray. 2002. *Salmonella*, pp. 55–68. *In* D.O. Cliver and H.P. Riemann, eds. **Foodborne Diseases.** Academic Press, San Diego.

Greenberg, J.T., P. Monach, J.H. Chou, P.D. Josephy and B. Demple. 1990. Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in *Escherichia coli*. **Proc. Natl. Acad. Sci. USA.** 87: 6181-6185.

Grob, U., H. Tschape, I. Bednarek and M. Frosch. 1998. Antibiotic resistance in *Salmonella enterica* serotype Typhimurium. **Eur. J. Clin. Microbiol.** 17: 385-387.

Hanes, D. 2003. Nontyphoid *Salmonella*, pp. 137-149. *In* O. Henegariu, N.A. Heerema, S.R. Dloughy, G. Vance and P.H. Hand-Vogt, eds. **International Handbook of Foodborne Pathogens.** Marcel Dekker, Inc., New York.

Hanning, I.B., J.D. Nutt and S.C. Ricke. 2009. Salmonellosis outbreaks in the United States due to fresh produce: sources and potential intervention measures. **Foodborne Pathog. Dis.** 6: 635-648.

Harris, L.J., J.N. Farber, L.R. Beuchat, M.E. Parish, T.V. Suslow, E.H. Garrett and F.F. Busta. 2003. Outbreaks associated with fresh produce: incidence, growth, and survival of pathogens in fresh and fresh-cut produce**. Compr. Rev. Food Sci. Food Safety.** 2: 78-141.

Harrison, C., C. Quigley, E. Kaczmarski and E. Devlin. 1992. An outbreak of gastro-intestinal illness caused by eggs containing *Salmonella* Enteritidis phage type-4. **J. Infect.** 24: 207-210.

Helmuth, R. and A. Schroeter. 1994. Molecular typing methods for *S*. Enteritidis. **Int. J. Food Microbiol.** 21: 69-77.

Heyde, M. and R. Portalier. 1990. Acid shock proteins of *Escherichia coli*. **FEMS Microbiol. Lett**. 57: 19-26.

Hilton, A.C., J.G. Banks and C.W. Penn. 1996. Random amplification of polymorphic DNA (RAPD) of *Salmonella*: strain differentiation and characterization of amplified sequences. **J. Appl. Bacteriol.** 81: 575-584.

Hixon, S.C., W.E.JR. White and K.L. Yielding. 1975. Selective covalent binding of an ethidium analog to mitochondrial DNA with production of petite mutants in yeast by photo affinity labeling. **J. Mol. Biol.** 92: 319-329.

Hu, L. and D.J. Kopecko. 2003. Typhoid *Salmonella*, pp 151-165. *In* M.D. Millotis and J.W. Bier, eds. **International Handbook of Foodborne Pathogens**. Marcel Dekker, Inc., New York.

ICMSF. 1978. **Microorganism on Food. I. Their Significance and Methods of Enumeration**. 2nd ed. University of Toranto Press, Toronto.

ICMSF. 1996. Salmonellae. *In* **International Commission on Microbiological Specification for Foods, Microorganism in Foods, Microbiology Specification of Food**. Blackie Academic & Professional, New York.

Innis, M.A. and D.H.Gelfand. 1990. Optimization of PCRs, pp. 3-12. *In* **PCR Protocols.** Academic Press, New York.

Invitrogen. 2010. **Molecular Probes: The Handbook; Section 5.2: Chemical Cross-Linking Reagents.** California Invitrogen Corporation, Carlsbad.

ISO. 2002. Microbiology of food and animal feeding stuffs-Horizontal method for the detection of *Salmonella* spp. (ISO 6579: 2002). *In* **International Organization for Standardization.** Geneva, Switzerland**.**

Jacobsen, C.S. and W.E. Holben. 2007. Quantification of mRNA in *Salmonella* sp. seeded soil and chicken manure using magnetic capture hybridization RT-PCR. **J. Microbiol. Methods.** 69: 315-321.

Jasson, V., L. Jacxsens, P. Luning, A. Rajkovic and M. Uyttendaele. 2010. Alternative microbial methods: an overview and selection criteria. **Food Microbiol.** 27: 710-730.

Jay, J.M., M.J. Loessner and D.A. Golden. 2005. **Modern Food Microbiology**. 7th ed. Springer Science Business Media, Inc., New York.

.

Jolivet-Gougeon, A., S. David-Jobert, Z. Tamanai-Shacoori., C. Menard and M. Cormier. 2000. Osmotic stress induced genetic rearrangements in *Escherichia coli* H10407 detected by randomly amplified polymorphic DNA analysis. **Appl. Environ. Microbiol.** 6: 5484-5487.

Jones, P.G., R.A. VanBogelen and F.C. Neidhardt. 1987. Induction of proteins in response to low temperature in *Escherichia coli*. **J. Bacteriol.** 169: 2092-2095.

Khoodoo, M.H.R., M.I. Issack and Y. Jaufeerally-Fakim. 2002. Serotyping and RAPD profiles of *Salmonella enterica* isolates from Mauritius. **Lett. Appl. Microbiol.** 35: 146-152.

Kim, B.H., I.S. Bang, S.Y. Lee, S.K. Hong, S.H. Bang, I.S. Lee and Y.K. Park. 2001. Expression of cspH, encoding the cold shock protein in *Salmonella* enterica serovar Typhimurium UK-1. **J. Bacteriol.** 183: 5580-5588.

Klein, P.G. and V.K. Juneja. 1997. Sensitive detection of viable *Listeria monocytogenes* by reverse transcription-PCR. **Appl. Environ. Microbiol.** 63: 4441-4448.

Kobayashi, T., S. Kensaku, T. Tetsuo, R. Sekine, V.P. Kelly, K. Kamata, S. Nishimura and S. Yokoyama. 2005. Structural basis of nonnatural amino acid recognition by an engineered aminoacyl-tRNA synthetase for genetic code expansion. **PNAS.** 102: 1366-1371.

Kolmodin, L.A. and J.F. Williams. 1997. PCR, pp. 3-15. *In* B.A. White, ed. **Method in Molecular Biology. vol. 67.** Humana Press, Inc., Newjersey, USA.

Kralik, P., A. Nocker and I. Pavlik. 2010. *Mycobacterium avium* subsp. *paratuberculosis* viability determination using F57 quantitative PCR in combination with propidium monoazide treatment. **Int. J. Food Microbiol.** 141: S80-S86.

Krawetz, S.A., R.T. Pon and G.H. Dixon. 1989. Increased efficiency of the *Taq* polymerase catalysed polymerase chain reaction. **Nucleic Acids Res.** 17: 819-825.

Lawyer, F.C., S. Stoffel, R.K. Saiki, S.Y. Chang, P.A. Landre, R.D. Abramson and D.H. Gelfand. 1993. High-level expression, purification, and enzymatic characterization of full length *Thermus aquaticus* DNA polymerase and a truncated form deficient in 5' to 3' exonuclease activity. **PCR Methods Appl.** 2: 275-287.

Lee, J.L. and R.E. Levin. 2006. Selection of universal primers for PCR quantification of total bacteria associated with fish fillets. **J. Microbiol. Methods.** 67: 456-462.

Lindquist, S. 1986. The heat-shock response. **Annu. Rev. Biochem.** 55: 1151-1191.

Lu, Y., W. Yang, L. Shi, L. Li, M.J. Alam and S. Guo. 2009. Specific detection of viable *Salmonella* cells by an ethidium-monoazide-loop mediated isothermal amplification (EMA-LAMP) method. **J. Health Sci.** 55: 820-824.

Malorny, B., J. Hoorfar, M. Hugas, A. Heuvelink, P. Fach, L. Ellerbroek, C. Bunge, C. Dorn and R. Helmuth. 2003. Interlaboratory diagnostic accuracy of a *Salmonella* specific PCR-based method. **Int. J. Food Microbiol.** 89: 241-249.

Maripandi, A., S.S. Raja, P. Ponmurugan and G. Gurusubrmanian. 2007. Random amplification of polymorphic DNA (RAPD) of *Salmonella* Enteritidis isolated from chicken samples. **Biotechnol.** 6: 278-282.

Masters, C.I., J.A. Shallcross and B.M. Mackey. 1994. Effect of stress treatments on the detection of *Listeria monocytogenes* and enterotoxigenic *Escherichia coli* by the polymerase chain reaction. **J. Appl. Bacteriol.** 77: 73-79.

McClelland, M., F. Mathieu-Daude and J. Welsh. 1995. RNA Fingerprinting and display Using Arbitrary Primed PCR. **Trends Genet.** 11: 242-246.

McKillip, J.L., L.A. Jaykus and M. Drake. 1998. rRNA stability in heat-killed and UV-irradiated enterotoxigenic *Staphylococcus aureus* and *Escherichia coli* O157:H7. **Appl. Environ. Microbiol.** 64: 4264-4268.

Mead, P.S., L. Slutsker, V. Dietz, L.F. McCaig, J.S. Bresee, C.Shapiro, P.M. Griffin and R.V. Tauxe. 1999. Food-related illness and death in the United States. **Emerg. Infect. Dis.** 5: 607-625.

Minami, A., W. Chaicumpa, M. Chongsa-Nguan, S. Samosornsuk, S. Monden, K. Takeshi, S. Makino and K. Kawamoto. 2010. Prevalence of foodborne pathogens in open markets and supermarkets in Thailand. **Food Control.** 21: 221-226.

Miyamoto, T., H.Z. Tian, T. Okabe, S. Trevanich, K. Asoh, S. Tomoda, K.I. Honjoh and S. Hatano. 1998. Application of random amplified polymorphic DNA analysis for detection of *Salmonella* spp. in foods. **J. Food Protect.** 61: 785-791.

Molla, D., D. Alemayehuand and W. Salah. 2003. Sources and distribution of *Salmonella* serotypes isolated from fooda, slaugter hause personnel and retail meat products in ethiopia: 1997-2002. **Ethip. J. Health Dev.** 17: 63-70.

Montville, T.J. and K.R. Matthews. 2008. **Food Microbiology: An Introduction.** 2nd ed. ASM Press, Washington.

Morin, N.J., Z. Gong and X.F. Li. 2004. Reverse transcription-multiplex PCR assay for simultaneous detection of *Escherichia coli* O157: H7, *Vibrio cholerae* O1 and *Salmonella* Typhi. **Clin. Chem.** 50: 2037-2044.

Munoz, N., M. Diaz-Osorio, J. Moreno, M. Sanchez-Jimenez and N. Cardona-Castro. 2010. Development and evaluation of a multiplex real-time polymerase chain reaction procedure to clinically type prevalent *Salmonella enterica* serovars. **J. Mol. Diagn.** 12: 220-225.

Mustapha, A. and Y. Li. 2006. Molecular detection of foodborne bacterial pathogens, pp. 69-90. *In* J. Maurer, ed. **PCR Methods in Foods.** Springer, New York.

Newkirk, K., W. Feng, W. Jiang, R. Tejero, S.D. Emerson, M. Inouye and G.T. Montelione. 1994. Solution NMR structure of the major cold shock protein (CspA) from *Escherichia coli*: identification of a binding epitope for DNA. **Proc. Natl. Acad. Sci. USA.** 91: 5114-5118.

Newell, D.G., M. Koopmans, L. Verhoef, E. Duizer, A. Aidara–Kane, H. Sprong, J. V.D. Giessen and H. Kruse. 2010. Food-borne diseases-the challenges of 20 years ago still persist while new ones continue to emerge. **Int. J. Food Microbiol.** 139: S3-S15.

Ngwai, Y.B., C. Wambebe and Y. Adachi. 2007. Survival ability of *Salmonella* Typhimurium L1388 and *Salmonella* Enteritidis L1225 under stressful growth conditions. **Online J. Health Allied Scs.** 6:1-10.

Nocker, A. and A.K Camper. 2006. Selective removal of DNA from dead cells of mixed bacterial communities by use of ethidium monoazide. **Appl. Environ. Microbiol.** 72: 1997-2004.

Nocker, A. and A.K. Camper. 2009. Novel approaches toward preferential detection of viable cells using nucleic acid amplification techniques. **FEMS Microbiol. Lett.** 291: 137-142.

Nocker, A., A. Mazza, L. Masson, A.K. Camper and R. Brousseau. 2009. Selective detection of live bacteria combining propidium monoazide sample treatment with microarray technology. **J. Microbiol. Methods.** 76: 253-261.

Nocker, A., T. Richter, F. Schuren, R. Montijn and R. Kort. 2010. Live/dead discrimination in 454 pyrosequencing-based bacterial community analysis of environmental water samples. **Int. Microbiol.** 13: 59-65.

Nocker, A., P. Sossa-Fernandez, M.D. Burr and A.K. Camper. 2007. Use of propidium monoazide for live/dead distinction in microbial ecology. **Appl. Environ. Microbiol.** 73: 5111-5117.

Nogva, H.K., S.M. Dromtorp, S.H. Nissen and K. Rudi. 2003. Ethidium monoazide for DNA based differentiation of viable and dead bacteria by 5′-nuclease PCR. **Biotechniques.** 810: 812-813.

Nunoshiba, T., T. DeRojas-Walker, S.R. Tannenbaum and B. Demple. 1995. Roles of nitric oxide in inducible resistance of *Escherichia coli* to activated murine macrophages. **Infect. Immun.** 63: 794-798.

Oliveira, S.D., L.R. Santos, D.M.T. Schuch, A.B. Silva, C.T.P. Salle and C.W. Canal. 2002. Detection and identification of *Salmonella* from poultry-related samples by PCR. **Vet. Microbiol.** 87: 25-35.

Omiccioli, E., G. Amagliani, G. Brandi and M. Magnani. 2009. A new platform for Real-Time PCR detection of *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157 in milk. **Food Microbiol.** 26: 615-622.

Omwandho, C.O.A. and T. Kubota. 2010. *Salmonella enterica* serovar Enteritidis: a mini-review of contamination routes and limitations to effective control. **JARO-JPN. Agr. Res. Q.** 44: 7-16.

Osiriphun, S., A. Pongpoolponsak and K. Tuitemwong. 2004. Quantitative risk assessment of *Salmonella* spp. in fermented pork sausage (Nham). **Kasetsart J. (Nat. Sci.).** 38: 52-65.

Padungtod, P. and J.B. Kaneene. 2006. *Salmonella* in food animals and humans in northern Thailand. **Int. J. Food Microbiol**. 108: 346-354.

Pan, Y. and F.JR. Breidt. 2007. Enumeration of viable *Listeria monocytogenes* cells by real time PCR with propidium monoazide and ethidium monoazide in the presence of dead cells. **Appl. Environ. Microbiol.** 73: 8028-8031.

Parry, C.M. 2006. Epidemiological and clinical aspects of human typhoid fever, pp. 1-18. *In* P. Matroeni and D. Maskell, eds. ***Salmonella* Infections: Clinical, Immunological and Molecular Aspects.** Cambridge University Press, New York.

Patel, P.D. 1994. **Rapid Analysis Techniques in Food**. Chapman and Hall, London.

Paukatong, K.V. and S. Kunawasen. 2001. The hazard analysis and critical control points (HACCP) generic model for the production of Thai pork sausage (Nham). **Berl. Munch Tierarztl.** 114: 1-4.

Pavlov, A.R., N.V. Pavlova, S.A. Kozyavkin and A.I. Slesarev. 2004. Recent developments in the optimization of thermostable DNA polymerases for efficient applications. **Trends Biotechnol.** 22: 253-260.

Pavlov, A.R., N.V. Pavlova, S.A. Kozyavkin and A.I. Slesarev. 2006. Thermostable DNA polymerases for a wide spectrum of applications: comparison of a robust hybrid Topo*T*aq to other enzymes, pp. 241-257. *In* J. Kieleczawa, ed. **DNA Sequencing II: Optimizing Preparation and Clean up**. Jones and Bartlett Publishers, Burlington, MA.

Penteado, A.L. and M.F.F. Leitão. 2004. Growth of *Salmonella* Enteritidis in melon, watermelon and papaya pulp stored at different times and temperatures**.** **Food Control.** 15: 369-373.

Piknova, L., A. Stefanovica, H. Drahovska, M. Sasik and T. Kuchta. 2002. Detection of *Salmonella* in food, equivalent to ISO 6579, by a three days polymerase chain reaction-based method. **Food Control.** 13: 191-194.

Poole, K. 2012. Stress responses as determinants of antimicrobial resistance in Gram-negative bacteria**. Trends Microbiol**. 20: 227-34.

Portillo, F.G. 2000. Molecular and cellular biology of *Salmonella* pathogenesis, pp. 3-34. *In* J.W. Cary and D. Bhatnagar, eds. **Microbial Foodborne Diseases: Mechanisms of Pathogenesis and Toxin Synthesis**. Technomic Publishing Company, Inc., USA.

Pui, C.F., W.C. Wong, L.C. Chai, E. Nillian, F.M. Ghazali, Y.K. Cheah, Y. Nakaguchi, M. Nishibuchi and S. Radu. 2011. Simultaneous detection of *Salmonella* spp., *Salmonella* Typhi and *Salmonella* Typhimurium in sliced fruits using multiplex PCR. **Food Control.** 22: 337-342.

Rabsch, W., H. Tschäpe and A.J. Bäumler. 2001. Non-typhoidal salmonellosis: emerging problems. **Microbes Infect.** 3: 237-247.

Rahn, K., S.A. De Grandis, R.C. Clarke, S.A. Mcewen, J.E. Galan and C. Ginocchio. 1992. Amplification of an *invA* gene sequence of *Salmonella* Typhimurium by polymerase chain reaction as specific method for detection of *Salmonella*. **Mol. Cell Probes.** 6: 271-279.

Randall, L.P. and M.J. Woodward. 2001. Multiple natibiotic resistance locus in *Salmonella enterica* serotype Typhimurium DT 104. **Appl. Environ. Microbiol.** 67: 1190-1197.

Rawsthorne, H., C.N. Dock and L.A. Jaykus. 2009. PCR-based method using propidium monoazide to distinguish viable from non-viable *Bacillus subtilis* spores. **Appl. Environ. Microbiol.** 75: 2936-2939.

Rimler, R.B. 1990. Comparisons of *Pasteurella multocida* lipopolysaccharides by sodiumdodecyl sulfate-polyacrylamide gel electrophoresis to determine relationship between group B and E Hemorrhagic Septicemia strains and serologically related group A strains. **J. Clin. Microbiol.** 28:654-659.

Rogers, G.B., F.A. Stressmann, G. Koller, T. Daniels, M.P. Carroll and K.D. Bruce. 2008. Assessing the diagnostic importance of nonviable bacterial cells in respiratory infections. **Diagn. Microbiol. Infect. Dis.** 62: 133-141.

Rudi, K., B. Moen, S.M. Dromtorp and L. Holck. 2005. Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples. **Appl. Environ. Microbiol.** 71: 1018-1024.

Rudi, K., K. Naterstad, S.M. Dromtorp and H. Holo. 2005. Detection of viable and dead *Listeria monocytogenes* on gouda-like cheese by real-time PCR. **Lett. Appl. Microbiol.** 40: 301-306.

Rychlik, W., W.J. Spencer and R.E. Rhoads. 1990. Optimization of the annealing temperature for DNA amplification *in vitro*. **Nucleic Acids Res.** 18: 6409-6412.

Sakai, T. and T. Chalermchaikit. 1996. The major sources of *Salmonella* Enteritidis in Thailand. **Int. J. Food Microbiol.** 31: 173-180.

Salcedo, S.P., M. Noursadeghi, J. Cohen and D.W. Holden. 2001. Intracellular replication of *Salmonella* Typhimurium strains in specific subsets of splenic macrophages *in vivo*. **Cell Microbiol.** 3: 587-597.

Sambrook, J. and W.D. Russel. 2001. **Molecular Cloning: A Laboratory Manual**. 3rd ed. Cold Spring Harbor, N.Y.

Sampathkumar, B., G.G. Khachatourians and D.R. Korber. 2003. High pH during trisodium phosphate treatment causes membrane damage and destruction of *Salmonella* enterica serovar Enteritidis. **Appl. Environ. Microbiol.** 69: 122-129.

Sanchez, G., P. Elizaquivel and R. Aznar. 2012. Discrimination of infectious hepatitis A viruses by propidium monoazide real-time RT-PCR**. Food Environ. Virol.** 4: 21-25.

Scharff, R.L. 2010. **Health-Related Costs from Foodborne Illness in The United States**. Produce Safety Project at Georgetown University, Washington, D.C.

Schnirring, L. 2007. **Peanut Butter Maker Says Moisture Triggered *Salmonella* Contamination**. Minneapolis, MN.

Schrader, C., A. Schielke, L. Ellerbroekand and R. Johne. 2012. PCR inhibitors. **J. Appl. Microbiol**. 113: 1014-1026.

Sheridan, G.E.C., C.I. Masters, J.A. Shallcross and B.M. Mackey. 1998. Detection of mRNA by reverse transcription-PCR as an indicator of viability in *Escherichia Coli* cells. **Appl. Environ. Microbiol.** 64: 1313-1318.

Singh, S., A.S. Yadav, S.M. Singh and P. Bharti. 2010. Prevalence of *Salmonella* in chicken eggs collected from poultry farms and marketing channels and their antimicrobial resistance**. Food Res. Int.** 43: 2027-2030.

Soejima, T., F. Schlitt-Dittrich and S. Yoshida. 2011. Rapid detection of viable bacteria by nested polymerase chain reaction via long DNA amplification after ethidium monoazide treatment. **Anal. Biochem.** 418: 37-43.

Soto, S.M., N. Martinez, B. Guerra, M.A. Gonzalez-Hevia and M.C. Mendoza. 2000. Usefulness of genetic typing methods to trace epidemiologically *Salmonella* serotype Ohio. **Epidemiol. Infect.** 125: 481-489.

Taitt, C.R., Y.S. Shubin, R. Angel and F.S. Ligler. 2004. Detection of *Salmonella enterica* serovar Typhimurium by using a rapid, array-based immunosensor. **Appl. Environ. Microbiol.** 70: 152-8.

Takayama, K. and S. Kjelleberg. 2000. The role of RNA stability during bacterial stress responses and starvation. **Environ. Microbiol.** 2: 355-365.

Tamanai-Shacoori, Z., A. Jolivet-Gougron, C. Me'nard, M. Bomnaure-Mallet and M. Cormier. 2005. Peracetic acid stress-induced genetic rearrangements in *Escherichia coli* H10407 detected by RAPD and RFLP analyses. **Microbiol. Res.** 161: 164-168.

Tenover, F.C., R.D. Arbeit and R.V. Goering. 1997. How to select and interpret molecular strain typing methods for epidemiological studies of bacterial infections: a review for healthcare epidemiologists. **Infect. Cont. Hosp. Ep.** 18: 426-439.

Theiringer, H.A., P.G. Jones and M. Inouye. 1998. Cold shock and adaptation. **Bioassays.** 20: 49-57.

Towner, K.J. and A. Cockayne. 1993. **Molecular Methods for Microbial Identification and Typing.** Chapman & Hall, London.

Trevanich, S., S. Tiyapongpattana and T. Miyamoto. 2010. Application of an optimized 18h method involving one step culturing and single primer-based PCR assay for detection of *Salmonella* spp. in foods. **Food Control.** 21: 593-598.

Trkov, M., I. Majerikova, B. Jerasek, A. Stefanovicova, N. Rijpens and T. Kuchta. 1999. Detection of *Salmonella* in food over 30 h using enrichment and polymerase chain reaction. **Food Microbiol.** 16: 393-399.

USDA-ERS. 2009. **Foodborne Illness Cost Calculator for *Salmonella***. Washington, D.C.

Vaitilingom, M., F. Gendre and P. Brignon. 1998. Direct detection of viable bacteria, molds, and yeasts by reverse transcriptase PCR in contaminated milk samples after heat treatment. **Appl. Environ. Microbiol.** 63: 1157-1160.

Van der Zee, H. 1994. Conventional methods for the detection and isolation of *Salmonella* Enteritidis. **Int. J. Food Microbiol.** 21: 41-46.

Vesper, S., C. McKinstry, C. Hartmann, M. Neace, S. Yoder and A.Vesper. 2008. Quantifying fungal viability in air and water samples using quantitative PCR after treatment with propidium monoazide (PMA). **J. Microbiol. Methods.** 72: 180-184.

Wachsmuth, I.K., J.A. Kienhbauch, C.A. Bobb, D.N. Cameron, N.A. Strockbine, J.G. Wells and P.A. Blake. 1991. The use of plasmid profiles and nucleic acid probes in epidemiologic investigations of foodborne, diarrheal diseases. **Int. J. Food Microbiol.** 12: 77-90.

Wagner, A.O., C. Malin, B.A. Knapp and P. Illmer. 2008. Removal of free extracellular DNA from environmental samples by ethidium monoazide and propidium monoazide. **Appl. Environ. Microbiol.** 74: 2537-2539.

Wang, L., Q. Zhong and Y. Li. 2012. Ethidium monoazide-loop mediated isothermal amplification for rapid detection of *Vibrio parahaemolyticus* in viable but non-culturable state. **Energy Procedia.** 17: 1858-1863.

Wang, L.X. and A. Mustapha. 2010. EMA-Real-Time PCR as a reliable method for detection of viable *Salmonella* in chicken and eggs. **J. Food. Sci.** 75: 134-139.

Wang, S.J. and D.B. Yeh. 2002. Designing of polymerase chain reaction primers for the detection of *Salmonella* Enteritidis in foods and faecal samples. **Lett. Appl. Microbiol.** 34: 422-427.

Wang, S.S. and R.E. Levin. 2006. Discrimination of viable *Vibrio vulnificus* cells from dead cells in real-time PCR. **J. Microbiol. Methods.** 64: 1-8.

Warner, J.M. and J.D. Oliver. 1998. Randomly amplified polymorphic DNA analysis of starved and viable but nonculturable *Vibrio vulnificus* cells. **Appl. Environ. Microbiol.** 64: 3025-3028.

Way, J.S., K.L. Josephson, S.D. Pillai, M. Abbaszadegan, C.P. Gerba and I.L. Pepper. 1993. Specific detection of *Salmonella* spp. by multiplex polymerase chain reaction. **Appl. Environ. Microbiol.** 59: 1473-1479.

WHO. 2005. **Fact Sheet No. 139 (Revised April, 2005).** Available Source: http://www. who.int/mediacentre/factsheets/fs139/en/prnt.html, December 18, 2011.

WHO. 2009. **FAO/WHO Expert Meeting on *Salmonella* and *Campylobacter* in Chicken Meat. 4-8 May 2009.** Available Source: http://www.who.int/foodsafety/micro/jemra/meetings/may09/en/index.html (accessed June 6, 2011), December 18, 2011.

Williams, J.G.K, A.R. Kubelik, J.K. Livak, J.A. Rafalski and S.V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic Markers. **Nucleic Acid Res.** 18: 6531-6535.

Woese, C.R. 1987. Bacterial evolution. **Microbiol. Rev.** 51: 221-271.

Wolffs, P.F.G., K. Glencross, B. Norling and M.W. Griffiths. 2007. Simultaneous quantification of pathogenic *Campylobacter* and *Salmonella* in chicken rinse fluid by a flotation and real-time multiplex PCR procedure. **Int. J. Food Microbiol.** 117: 50-54.

Wong, D.M.A., T. Hald, P.J.V.D. Wolf and M. Swanenburg. 2002. Epidemiology and control measures for *Salmonella* in pigs and pork. **Livest. Prod. Sci.** 76: 215-222.

Wong, K.K., R.M. Wong, K.E. Rudd and M. McClelland. 1994. High-resolution restriction map for a 240-kilobase region spanning 91 to 96 minutes on the *Salmonella* Typhimurium LT2 chromosome. **J. Bacteriol.** 176: 5729-5734.

Yan, S.S., M.L. Pendrak, B.A. bela-Rider, W.J. Punderson, P.D. Fedorko and S.L. Foley. 2004. An overview of *Salmonella* typing public health perspectives. **Clin. Appl. Immunol. Rev.** 4: 189-204.

Yang, B., D. Qu, X. Zhang, J. Shen, S. Cui, Y. Shi, M. Xi, M. Sheng, S. Zhiand and J. Meng. 2010. Prevalence and characterization of *Salmonella* serovars in retail meats of market place in Shaanxi, China. **Int. J. Food Microbiol.** 141: 63-72.

Yeh, K.S., T.H. Chen, T.H.C.W. Liao, C.S. Chang and H.C. Lo. 2002. PCR amplification of the *Salmonella* Typhimurium *fim*Y gene sequence to detect the *Salmonella* species. **Int. J. Food Microbiol**. 78: 227-234.

Yoke-Kqueen, C., L. Learn-Han, A.S. Noorzaleha, R. Son, S. Sabrina, S. Jiun-Horng and K. Chai-Hoon. 2008. Characterization of multiple-antimicrobial resistant *Salmonella enterica* subsp. *enterica* isolated from indigenous vegetables and poultry in Malaysia. **Lett. Appl. Microbiol.** 46: 318-324.

Yousef, A.E. and C. Carlstrom. 2003. *Salmonella*, pp. 167-205. *In* A.E. Yousef and C. Carstrom, eds. **Food Microbiology: A Laboratory Manual.** John Wiley & Sons, Inc., New Jersey.

Yousef, A.E. and V. K. Juneja. 2002. **Microbial stress adaptation and food safety**. CRC Press LLC, Florida.

Zhao, S., A.R. Datta, S. Ayers, S. Friedman, R.D. Walker and D. G. White. 2003. Antimicrobial-resistant *Salmonella* serovars isolated from imported foods. **Int. J. Food Microbiol.** 84: 87-92.

Zheng, M., B. Doan, T.D. Schneider and G. Storz. 1999. OxyR and SoxRS regulation of fur. **J. Bacteriol.** 181: 4639-43.

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LIST OF PUBLICATIONS:

- **Saiyudthong, S.** 1998. Review of foodborne diseases. Food. 28(4): 296-298.
- **Saiyudthong, S**. and M. Bunyaratanakornkit. 1999. Microbiological quality of hamburger. Food. 29(4): 250-262.

- Maleehuan, T. and **S. Saiyudthong.** 1999. Food safety of Thai fermented food. Food. 29(4): 277-282.
- **Saiyudthong, S.** 2000. Chili: the herb plant. Food. 30(1): 59-62.
- **Saiyudthong, S**. and J. Japakaset. 2001. Prevalence of *Aeromonas* spp. in foods. Food. 31(4): 257-267.
- Karuwanna, P. and **S. Saiyudthong**. 2003. The effect of Thai plant on wine production. Food. 33(3): 204-210.
- **Saiyudthong, S**. 2005. The standard quality of spirit product. Food. 37(2): 135-138.
- Stonsaovapak, S. and **S. Saiyudthong**. 2010. Antilisterial effects of ethanolic extracts of some edible Thai plants on refrigerated cooked pork. Maejo Int. J. Sci. Technol. 4(3): 540-546.
- **Saiyudthong, S.** and S. Trevanich. 2013. An optimized EMA-RAPD-PCR for a reliable detection of viable *Salmonella* spp. in chicken products. J. Food Safety. x(x): x-x. (In press for publication)

CONFERENCES AND ACADEMIC PRESENTATIONS

- **Saiyudthong, S.** and S. Trevanich. 2008. Effect of various stresses on efficiency of RAPD-PCR for specific detection of antimicrobial drug-resistant *Salmonella* Enteritidis. *In* Evolving Microbial Food Quality and Safety: Proceedings of the 21st International ICFMH Symposium, Food Micro, 1-4 Sep 2008. Aberdeen, Scotland. (Poster presentation)
- **Saiyudthong, S**., S. Buates and S. Trevanich. 2010. Factors affecting PCR-EMA technique for *Salmonella* Typhimurium detection. *In* Proceedings of the 5th Central European Congress on Food, 19-22 May 2010. Bratislava, Slovak Republic. (Oral presentation)
- **Saiyudthong, S.** and N. Prapasuwannakul. 2010. Microbiological safety and quality of red wine from different grape varieties planted in Thailand. *In* Proceedings of the 5th Central European Congress on Food, 19-22 May 2010. Bratislava, Slovak Republic. (Oral presentation)

- **Saiyudthong, S**. 2011. The effect of different yeasts on the fermentation of Thai shiraz wine. *In* Proceedings of The 4th International Conference on Fermentation Technology for Value Added Agricultural Products (FerVAAP2011), 25-26 Aug 2011. Khon Kaen, Thailand. (Poster presentation)