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THESIS

DEVELOPMENT OF HYBRIDIZATION TECHNIQUES FOR THE DETECTION OF SALMONELLA SPP. IN PORK

DAMRONGSAK ARLAI

A Thesis Submitted in Partial Fulfillment of
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Damrongsak Arlai 2015 : Development of Hybridization Techniques for the Detection of *Salmonella* spp. in Pork. Doctor of Philosophy (Agricultural Research and Development), Major Field: Agricultural Research and Development, Faculty Agriculture at Kamphaeng Saen. Thesis Advisor: Assistant Professor Sujate Chaunchom, Ph.D. 129 pages.

The dissertation newly modifies the fluorescence in *situ* hybridization technique (FISH) to detect for *Salmonella spp*. in the pork industry. Herewith the study shows the method of the rapid detection of *Salmonella* in pork sample which was conducted by using optimization of cell wall permeabilizing conditions for Sal3 probe labeled at 3′-end with terminal transferase by FISH. The study results unveil that the optimum condition for cell permiation is using lysozyme at the concentration of 1 mg/ml dissolved in 10mM Tris-HCl, 5mM EDTA, pH 8.0, and incubation proceeds at 37 C^{o} for 3 minutes. Furthermore the dissertation newly classifies the quality of *Salmonella* detection in pork by FISH into five levels, namely, 0 - 4 as poor to very good, respectively. The optimal condition gave the average score for FISH signal at level 4. Comparison to the results between the standard method ISO 6579 (2002) and the FISH method evaluated by the Kappa statistics equaled to 0.46. The results derived from both techniques were corresponding and accepted in the moderate range of standard values. The sensitivity (93.5%) and the specificity (66.6%) were achieved by FISH compared with culture. While, the results of FISH experiment were applied to *Salmonella* detection that contaminated levels higher than 3×10^{6} cfu/ml onto pork, detection in this study is less time consuming (only 8 hour) and convenience for many samples testing compared to the bacterial culture method. Addtionally, the dissertation firstly implements the method for detecting low numbers of *Salmonella* cells in pork samples by integrating “Catalyse Reporter Deposition or CARD” and the dot blot hybridization. The result of this method can detect 7.47 µg/ml of *Salmonella* DNA (expected cells concentration <3 cfu/ml) which is better than conventional methods of dot blot hybridization. And the study recommends these purposed methods are appropriate to apply in a conventional lab.

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Student’s signature Thesis Advisor’s signature

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Damrongsak Arlai

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LIST OF ABBREVIATIONS

DIG	=	Digoxigenin
DAB	=	Diaminobenzedine
TBST	=	Tris-base buffer saline
PBS	=	Phosphate buffer saline
BSA	=	Bovine serum albumin
µl	=	microliter
ml	=	milliliter
µg	=	microgram
mg	=	milligram
bp	=	base pair
mM	=	milimolar
M	=	molar
DNA	=	Deoxyribonucleic acid
RNA	=	Ribonucleic acid
dUTP	=	Deoxyuridine triphosphate
TSA	=	Tyramide signal amplification
ABC	=	Avidin–biotinylated enzyme complex
rpm	=	round per minute
°C	=	Degree Celsius
OD	=	Optical Density
HRP	=	Horseradish peroxidase
AP	=	Alkaline phosphatase
EM	=	Effective microorganisms
FISH	=	Fluorescence in situ hybridization
CARD	=	Catalyzed reporter deposition
BPW	=	Buffered peptone water

DEVELOPENT AN *IN SITU* HYBRIDIZATION FOR SALMONELLA DETECTION IN PORK PRODUCT

INTRODUCTION

The presence of microbial pathogens in food is among the most serious for public health worldwide. Particularly in developing counties in Asia and Africa, due to the bad general hygiene conditions, but it is also largely widespread in developed countries. In the latter, 95% of recorded clinical cases are foodborne (Forbes *et al,* 1998, Liu *et al.,* 2011), the increasing rate of morbidity and mortalbility of children and old aged people is significant. It is has been estimate that 70% of the 3.2 million deaths of children under 5 worldwide, are attributed to foodborne diarrheal diseases (Gadgil and Gadgil, 2006). Especially in developing countries like Thailand, pathogen contaminated foods and the resulting health and economic impacts are significant. Factors that have been attributed to the increased risk for foodborne disease include new feeding practices, changes in animal husbandry, changes in agronomic process, increase in international trade, changes in food technology, increase in susceptible populations, increase in travel and changes in lifestyle and consumer demands (WHO, 2007).

Salmonella is a common foodborne pathogen in humans and animals, and one of the leading causes of foodborne illness (Tirado and Schmidt, 2001). This disease is typical indicative onset of salmonellosis. The route of infection is usually found from contaminated food ingestion. The economic impact of this zoonose in commercial food production is also substantial and the control of *Salmonella* is becoming more challenging with the trend towards cheaper and faster food. Globally, millions of cases of salmonellosis in humans are reported annually (Rhen, 2007). Including unreported cases, in 1995 nontyphoidal salmonellosis affected an estimated 1.3 billion humans and caused three million deaths (Pang *et al.,* 1995). The World Health Organization (WHO) reports that the incidence and severity of cases of salmonellosis have increased significantly (WHO, 2010). Strains resistant to a range of

antimicrobials emerged in the 1990s and constitute a serious additional concern for public health (WHO, 2010). According to EFSA epidemiological data (2011), in the European Union (EU) *Salmonella* is the second cause of foodborne disease after *Campylobacter* and it is still first in many EU States. While, The Centers for Disease Control and Prevention (CDC, 2008) reports approximately 40,000 cases of salmonellosis in the United States each year. The OzFoodNet sites reported 9,533 cases of *Salmonella* infection in Australia, a rate of 43.6 cases per 100,000 population (OzFoodNet, 2010). In Asia, *Salmonella* Enteritidis has also emerged as the most common human serotype in Japan, the Republic of Korea and Thailand (Galanis E *et al.,* 2006). In Thailand during 1991-1995, the morbidity markedly increased each year form 1991-1995, with 105, 307, 471, 659 and 877 cases, especially Bangkok was mostly predominant with *Salmonella* infection and then in southern, northeastern, central and northern regions, respectively (Boriraj *et al.,* 1997). In 1998, the morbidity rate of salmonellosis in Thailand increased from 76-1,057 per 100,000 population per year (Chalermchaikit, 2001). The information of salmonellosis reports have shown the relationship between salmonella in food of animal origin and public health problems salmonella spp. are commonly found in chicken eggs and pork meats at market (Sasipreeyajan *et al.,* 1996; Boonmar *et al.,* 1998a).

In the past, the *Salmonella* outbreaks are often related with the consumption of poultry or beef. Pork meat is not usually considered a major source of Salmonella spp. infections in humans (Steinbach *et al.,* 1999). In contrast to nowadays, the overall prevalence of *Salmonell*a outbreaks in eggs and poultry meat that is decreasing. But, the overall of pork meat increased in cases of human salmonellosis (EFSA, 2011). In the US, the number of human cases of salmonellosis related to the consumption of pork has been estimated at 100 000 cases per year (Miller *et al.,* 2005). According to EFSA, 10-20% of human infections with salmonella in the EU may be attributed to the pig reservoir. Source attribution studies have been performed for four EU member states, estimating the proportion of pork-associated cases acquired domestically. The results obtained were 0.1-0.3% for Sweden, 3.4–3.7% for the United Kingdom, 3.6–9.7% for Denmark and 7.6–15.2% for the Netherlands (Pires & Hald, 2010). The entry of *Salmonella*-infected pigs into the abattoirs plays a crucial role as a source of

contamination of carcasses and as an important point of introduction of *Salmonella* into the food chain and it has persisted for long periods in swine (Silva *et al.,* 2006), resulting in contamination of the slaughterhouse (Gray *et al.,* 1995). Swine can carry *Salmonella* in several tissues, especially those of the digestive tract and the associated lymph nodes, thus representing a potential risk for consumers which must be properly identified and controlled (Jung *et al.,* 2001; Castagna *et al.,* 2004; Silva *et al.,* 2006). Due to disease severity has made it a motivation for appropriated preventive measure such as the Commission Regulation (EC) No 2073/2005 on microbiological criteria specifiying the *Salmonella* for carcasses. Sampling rules of 25 grams of meat should not be carriying salmonella in the epidemic situated salmonellosis.

Pork has been indicated as the contamination in a wide range of meat samples in Thailand. It was associated with the slaughter house during the time of meat processing through to the consumer hands (Angkititrakul *et al.,* 2005). Most of the consumers in developed countries are increasingly becoming concerned about pig rearing conditions and pork eating quality. In Thailand, pork is the meat product that has been controlled under food safety policies. Moreover Thailand has high potential capacity in pork exportation. However, *Salmonella* contamination is still reported in contaminated samples from the butcher shop. Good management eventually points to slaughter house sanitation levels and importance of butcher meat hygienic. Meat inspection as the surveillance tool would serve the prevention program effectively, in accordance with the hygiene and sanitized method through all the procedures involved. Thereafter the guaranteed or certified legal document should be given to the salmonella free exported meat products. Derived from the meat inspection aspect, the agent identification is mainly concerned. Bacterial culture method is the gold standard with accurate results even though the limitation of this method is time consuming (at least 4-6 days). In this research the In *situ* hybridization technique (ISH) is developed for *Salmonella* spp. detection in the pork industry of Thailand for food safety and good quality exported meat purpose and importance of meat products in human diet requires systems that ensure meat products safety. Although food safety plans such as good hygiene practice (GHP), good manufacturing practice (GMP), and implementing hazard analysis critical control points (HACCP) along

the whole food chain have been established by both regulatory authorities and industry all over the world, the success of these approaches in decreasing the incidence of human salmonellosis has been minor because of improper use and/or incomplete implementation. It is important to focus our efforts towards the real risks in the population. The challenge is therefore to use a multidisciplinary approach to identify the best mitigation strategies along the food-chain to prevent foodborne disease, especially at the primary production level, and then implement appropriate prevention programs. The most appropriate method to achieve this goal is through the use of the risk assessment process which links pathogens in food to the public health problem.

OBJECTIVES

1. To determine the specificity of Sal3 probe for salmonella spp.

2. To develop *In situ* hybridization for rapid and accurate detection of Salmonella detection.

3. To develop signal amplification technology based on catalyzed reporter deposition (CARD) for detecting low numbers of *Salmonella* cells

LITERATURE REVIEW

1. Overview of *Salmonella*

1.1 The genus *Salmonella*

The genus *Salmonella* is a member of the family *Enterobacteriaceae* and it has been known to cause illness for over 100 years. Because, these bacteria are the most prevalent agents causing food-borne diseases in both developed and developing countries (Glynn *et al.,* 1998; Duijkeren *et al.,* 2003). They were discovered by Dr. Daniel E. Salmon. Genus *Salmonella* has more than 2,500 serotypes and all serotypes can cause illnesses in humans (WHO, 2005). But only about 80 are frequently involved in animals and human diseases worldwide (de Freitas Neto *et al.,* 2010). *Salmonella* often cause very large multistate outbreaks of food infection, shows that this proves the greater resistance of this pathogen in the external environment and in food. In developed countries the main source of salmonellosis is still today food of animal origin, particularly livestock production such as raw meats, eggs, poultry, raw milk, chocolate and vegetables (WHO, 2002; Malkawi and Gharaibeh, 2004; Owlia *et al.,* 2007, WHO, 2007). Also fresh fruits and vegetables and undrinkable water, can convey the bacteria to humans. *Salmonella* is quite resistant to adverse conditions and this allows them to persist in the environment and spread along the food chain, from the animals to the food of animal origin, or to plants that are fertilized with animal manure (Giaccone *et al.,* 2012). Nowadays, *Salmonella* are still reported that the incidence and severity of cases of *Salmonellosis* have increased significantly and remain a major problem of public health worldwide and in all animals (Ayana and Surekha, 2008; WHO, 2010).

Salmonella can cause human illness when it is ingested, and can lead to *Salmonella* infection through various modes of transmission, including through food

and water sources, animal-to-human contact, and person-to-person contact. One study found that 87% of all confirmed cases of *Salmonella* were foodborne, with 10 percent from person-to-person infection and 3% caused by pets (Jean and Roberts, 2009). The dangers for human health mainly arise from food contaminated with *Salmonella enterica*, which is often present in the intestines of livestock, without causing any infection to the animals. This may be a potential hazard to food hygiene, if the healthy carriers are the people involved in producing and handling the food. Usually, animal carrier eliminates *Salmonella* in their faeces for several months after the episode of gastroenteritis through which they became carrier. In the case of *Salmonella typhimurium*, it has been demonstrated that humans can be asymptomatic carriers of the bacterium for decades (Weill, 2009). From the intestinal contents of livestock, *Salmonella* can contaminate fresh meat, raw milk and egg shells. If the necessary hygienic precautions are not taken in the early stages of the production line (slaughter, milking, egg collecting), there is a risk that the *Salmonella* may then spread along each of these production chains, even polluting products such as cured meats, dairy products and egg-based dishes if they were made using raw milk or unpasteurized eggs. Moreover, through the faeces of animals and man, *Salmonella spp*. can contaminate farmland, surface water flow and vegetables if they are fertilized with animal manure or dung that is not properly fermented. Therefore, vegetables can be a source of disease to humans just like fresh meat, milk, shell eggs and by-products. Besides in animals, *Salmonella* can adhere well to work surfaces, and from there spread to other foodstuffs by cross-contamination (Møretrø *et al.,* 2011). Generally, forms of gastroenteritis caused by non-typhoid *Salmonella* are moderately serious diseases with a quick recovery and without the need to resort to specific therapies. Although in some cases such as young children, elderly, or immunodeficiency (HIV) subjects that are affected; salmonellosis may also lead to the patient's death (Pathan *et al.,* 2010).

The severity of *Salmonella* infections can also become more pseualent by the fact that in recent years more and more *Salmonella* strains have been spreading to the entire world. They are resistant to one or more of the antibiotics for human treatment such as fluoroquinolones and third generation cephalosporins. In addition to the Typhimurium serotype, *Salmonella* strains which are multiresistant to many

antibiotics have also been detected in the Agona, Anatum, Choleraesuis, Derby, Dublin, Heidelberg, Kentucky, Newport, Pullorum, Schwarzengrund, Senftenberg, and Uganda serotypes (Yan *et al.,* 2010). In most cases, human infection appears itself through diarrhoea, persistent fever and stomach pain which appear 12 to 72 hours after the infection. The disease is self-limiting and clears up within 4-7 days, but it has rather significant side effects: it takes months for the patient to regain proper bowel function and they can remain healthy carriers for months. In addition, chronic complications may occur such as widespread polyarthritis (Reiter's syndrome), ocular and urinary disorders, and even occasional cases of endocarditis and appendicitis. All these diseases are hard to treat even with antibiotics (Castillo *et al.,* 2011).

Each year, many people are suspected of getting have getting *Salmonella* infection. This is a significant cause of morbidity, mortality in death worldwide (WHO, 2005). The US Centers for Disease Control and Prevention (CDC) reported, *Salmonella* is the leading cause of bacterial foodborne illnesses in the United States, and is responsible for approximately 1.4 million cases and each year results in 15,000 hospitalizations and 400 deaths each in the United States (CDC, 2008). An estimated 95% of these Salmonellosis cases are associated with the consumption of contaminated of food products (Mead et al., 1999). European Union reported 5,311 case of Salmonellosis outbreaks in 2005, 64% were due to *Salmonella* spp. The most common serovars involved were Enteritidis (64%) and Typhimurium (2.5%). All other serovars showed an incidence of less than 1% (EFSA. 2008). Previous studies, the *Salmonella* investigations have proven to be quite variable and are affected by changes in human demographics and lifestyles, human behavior, changes in industry and technology, changes in travel and commerce, the shift toward global economy, microbial adaptation, the basics of health policy in each country, and the lack of knowledge on food safety and handling practices about hygiene and sanitation among consumers (Knabel, 1995; Altekruse *et al.,* 1997; Hall, 1997). Another study, in the tropical zone as seen in studies by Brent *et al* (2006) and Kariuki *et al* (2006a) who found the peak non-typhoidal salmonella bacteremia cases occurred during the rainy season. Meanwhile, this is different from studies in non-tropical zone, as seen in studies by Kazemi *et al* (1974) and Olsen *et al* (2001) who found the majority of cases occurred during summer months, is related with society costs like medical costs, the

value of time lost from work, the value of premature death and others (Hotes, 2011, Ball *et al.,* 2011). In 1996, the USDA, Economic Research Service reported that the total costs for medical care and lost productivity, resulting from Salmonellosis value was between 0.6 – 3.5 billion dollars annually. Other costs associated with *Salmonella* include various direct expenses producers face as a consequence of *Salmonella* infection in their flocks. Control measures such as biosecurity practices, cleaning and disinfecting of facilities, rodent control programs, vaccination, and testing all can significantly increase production costs. Moreover, Salmonella contamination of food products can significantly reduce consumer demand and affect producer profits. (San Myint, 2004) In addition, *Salmonella* have an enormous economic impact (Roberts *et al.,* 2003; Voetsch *et al.,* 2004), and today, they are the most important foodborne pathogens in terms of deaths caused (Adak *et al.,* 2002; Kennedy *et al.,* 2004; Mead *et al.,* 1999).

1.2 Characteristics of Salmonella

Salmonella is a genus of gram-negative, facultative anaerobic, rod-shaped bacteria that can infect people, birds, reptiles, and other animals. They are non-lactose fermenting, non-spore forming and most are motile. The optimum condition of growth is at temperature 37^{o}C and pH 6.5-7.5 and can grow in both aerobic and anaerobic conditions and have ability to utilize a variety of substrates (Gray and Fedorka-Cray, 2002). These zoonoses are known to be hardy and ubiquitous bacteria that multiply at 7-45ºC. Persistence of *Salmonella* in the environment for long periods (months or even years) is possible in the presence of suitable organic subtracts (Schwartz, 1999). However, the organisms cannot growth at the temperature below 7^{o}C and pH < 3.8. These organisms can be eliminated by cooking and pasteurization of milk (71.7^{o}C, 15 seconds) and fruit juices (70-74^{o}C, ≤20 seconds) (Gray and Fedorka-Cray, 2002). Currently the genus Salmonella is divided into two species: Salmonella enterica and Salmonella bongori. The species Salmonella enterica consist of six subspecies: *S.* enterica, *S.* salamae, *S.* arizonae, *S.* diarizonae, *S.* houtenae and *S.* indica whereas no subspecies has been assigned to Salmonella bongori (Su and Chiu, 2007). Based on the combination of bacterial surface-antigens the genus Salmonella is subdivided into 2,541 serovars. For convenience the serovars are denominated by genus and serovar only (e.g. *Salmonella* enterica, subspecies enterica, serovar Typhimurium is called *Salmonella*

Typhimurium). According to Popoff *et al.* (2004) 1,504 serovars belong to *Salmonella* enterica, subspecies enterica. Most zoonotic serovars is better adapted than the latter to live in the intestine of man and warm-blooded animals and associated with human illness are in this group. , whereas *S. bongori* resistance in the external environment and is detectable in the intestinal contents of warm-blooded animals, so it is rare for it to be found in food for human consumption. (Giaccone *et al.,* 2012). All *Salmonella* serovars are considered potentially pathogenic for humans, but the degree of host adaptation varies, which affects the pathogenicity (M.E.E. *et al,* 2011).

Figure 1 Salmonella bacterium, the long stringy structures protruding from the bacteria are called flagella, which the bacterium uses to move.

Source: Brands (2006)

Salmonella growth may still occur in a wide pH range (4.5 to 9.5) depending on the surrounding conditions. The temperature range at which *Salmonella* has been growing is 2 ^{o}C to 54 ^{o}C (*S.* Typhimurium). Regarding available moisture, growth inhibition has been reported for water activity (a_w) values below 0.93 (Doyle *et al.,* 2001). A salt content of 3-4% generally inhibits the growth of *Salmonellae*, but

increasing temperature is increase salt tolerance. However, a salt content above 8% is bactericidal for salmonellae (Jay *et al.,* 2005). Except the rare non-motile *Salmonella* serovars such as *S.* Gallinarum and *S.* Pullorum, the vast majority of *Salmonella* is motile and propelled by peritrichous flagella. The motile *Salmonella* may lose their ability to develop flagella under the effect of sublethal ”stress”, caused by external physicochemical influence such as refrigeration or high temperatures (Krieg and Holt, 1984., Doyle *et al.,* 2001). Salmonellae catabolize D-glucose and other carbohydrates with the production of acid and gas. They are oxidase negative and catalase positive, grow on citrate as a sole carbon source, generally produce hydrogen sulfide, 4 decarboxylate lysine and ornithine, and do not hydrolyze urea. Many of these traits have formed the basis for the presumptive biochemical identification on *Salmonella* isolates, as shown in table 1.

Table 1 Biochemical characteristics of Salmonella

Biochemical characteristic	Reaction
Indole	-
Methyl	+
Voges-proskauer	-
Citrate	+
Oxidase	-
Catalase	+
Ureasa	-
Phenylalanine adaminase	-
Hydrogen sulphide	+
Lysine decarboxylase	+
Ornithine decaboxylate	+
Motility ($36^{o}C$)	+
Acid produced form lactose	-
Acid produced form glucose	+

+ = Positive reaction; **- = Negative**

Source: Quinn *et al* (1994)

Salmonella are also classified by three specific types of antigens including O antigens represents a *Salmonella* specific polysaccharide, H antigens represents the filamentous portion of the bacterial flagella, and Vi antigens represents the capsular. Antigens have been used to isolate and identify more than 2500 serotypes of Salmonella (Popoff *et al.,* 2004; Grimont and Weil, 2007). There are two species of

Salmonella, namely S. bongori and S. enterica. S. enterica is divided into six subspecies including enterica, salamae, arizonae, diarizonae, houtenae, and indica (Table 2). The subspecies can be further divided into serotypes, also called serovars, differentiated from each other based on the presence of somatic (O) and flagellar (H) antigens. The majority (99.5%) of strains of salmonella isolated from humans and warm-blooded animals belong to subspecies I (Grimont, 2007). This subspecies is numerically the most significant and causes approximately 99% of Salmonella infections in humans (Uzzau *et al.,* 2000), while the other five subspecies II-V and *S bongori* are primarily associated with cold-blooded animals and are only infrequently isolated from mammals (Foti *et al.,* 2009).

Table 2 Current *Salmonella* Nomenclature

Taxonomic position (writing format) and nomenclature				**No. of serotypes in each species or subspecies**
Genus (capitalized, italic)	**Species** (italic)	**Subpecies** (italic)	**Serotypes (or serovars)** (capitalized, not italic)*	
Salmonella	*enterica*	*enterica* (or subspecies I)	Choleraesuis, Enteritidis, Paratyphimurium, typhimurium	1,504
		salamae(or subspecies II)	9,46:z:z39	504
		arizonae(or subspecies IIIa)	43:z29:-	95
		diarizonae(or subspecies IIIb)	6,7:1,v:1,5,7	333
		houtenae(or subspecies IV)	21:m,t:-	72
		indica(or subspecies VI)	59:z36:-	13
	bongori subterranea	subpecies V	13,22:z39:-	22

*Some selected serovars are listed as examples.

Source: Lin-Hui Su *et al* (2007)

1.3 Reseviors of *Salmonella spp*.

The organisms are shed in high numbers in the faeces of infected individuals. Domestic animals are most likely infected following exposure to faeces, contaminated feed, chronic carriers introduced into the population, rodents, or sometimes by contact with infected workers (Giovannacci *et al.,* 2001). With, S. *enterica* subspecies I mainly isolated from birds and mammals. It has been suggested that the reason that subspecies I is found more often in the food supply is also the reason why it is isolated more often from foods and clinical isolations and is associated with foodborne disease. *Salmonella* serovars can cause disease in food animals, however these animals can often be asymptomatic carriers, shedding salmonellae via the fecal route, which allows for transmission of the disease from animal to animal, or via contamination of feed, water, and equipment (Aleksic *et al.,* 1996; Olsen *et al.,* 2000; Hurd *et al.,* 2001; Crump *et al.,* 2002; Andrews and Bäumler, 2005). It is considered that *Salmonella* will continue to be a feature of humans, animals and the general environment and that effort should be directed to controlling its introduction and spread into the agricultural and food chains (Murray, 2000). In the past, the main animal reservoir outbreaks are often related with the consumption of poultry or beef. Pork meat is not usually considered a major source of *Salmonella* spp. infections in humans (Steinbach *et al.,* 1999). In contrast to nowadays, the overall prevalence of *Salmonella* outbreaks in eggs and poultry meat that is decreasing. But, the overall of pork meat increased up of case human salmonellosis. For example, In EU up to 27% of cases (EFSA, 2011). Meanwhile, In the United States reported the higher *Salmonella* prevalence from two large commercial pork processing plants. Salmonella contamination on carcasses at each of these sampling points was 91.2% (before scalding), 19.1% (pre-evisceration), and 3.7% (chilled final), respectively (Schmidt *et al.,* 2012). In the north of Thailand that was investigated of salmonella contamination in 55.5% of freshly cut pork, 70.5% of transported pork, and 34.5% of retail products from the slaughterhouse (Sanguankiat *et al.,* 2010). So, pork meat as a source of human salmonellosis has caused an increasing number of investigations worldwide (Fraser, 2006, Kich *et al.,* 2007, Backus and King. 2008, Duggan *et al.,* 2009, Hopkins *et al.,* 2010). One study found that *Salmonella* may survive in the calf rearing

units between four months to two years clearly indicating that many cleaning and disinfection procedures were inadequate (McLaren and Wray, 1991). The investigation on the levels of host adaptation, *Salmonella* serotypes may generally be classified as being:

a) Highly adapted to an animal host such as *S.* Abortusovis in sheep, *S.* Dublin in cattle, *S.* Pullorum, *S.* Gallinarum in poultry, *S.* Choleraesuis in pigs.

b) Common, non host adapted, that cause food borne illness such as *S.* Typhimurium, *S.* Enteritidis.

c) Adapted to humans and not usually pathogenic to animals, which cause severe disease with septicaemic-typhoid syndrome such as *S.* Typhimurim, *S.* Paratyphimurium (European Commission, 2000).

2. *Salmonella spp*. in human

Human salmonellosis caused by *Salmonella*, it is divided into two major symptoms. The first symptom is well known as "non-typhoid or gastroenteritis". The salmonella serotypes are associated such as *S.* Typhimurium and *S.* Enteritidis which is a localized infection of gastrointestinal tract. While, the second symptom is systemic infection "typhoid or enteric fever" and these serotypes are associated such as *S.* Typhi and *S.* Paratyphi, *S.* Choleraesuis. (Miller *et al.,* 1995; Gray and Fedorka-Cray, 2002). The highest risk groups of people for salmonellosis include immune compromised individuals (elderly, newborns and infants), included the patient of gastric hypoacidity and frequent or recent users of antibiotics (Crum-Cianflone, 2008, Montville and Matthews, 2005). Due to these groups delayed or weak immune response, *Salmonella* is able colonize the intestine, causing severity illnesses like septicemia, aseptic reactive arthritis, Reiter's syndrome, and ankylosing spondylitis (Montville and Matthews, 2005). The European Food Safety Authority (EFSA) was published; all *Salmonella* serovars are considered to be of public health significance (Anon., 2006). In 2008, a total of 131,468 human salmonellosis cases were confirmed in EU. The top five serovars associated with confirmed human cases were: *Salmonella* (*S.*) Enteritidis (58.0%), *S.* Typhimurium (21.9%), *S.* Infantis (1.1%), *S.* Virchow

(0.7%) and *S.* Newport (0.7%) (Anon., 2010d). However, some serovars are less likely to cause infection in humans. While, in Thailand, between from 1993 to 2002. A total of 70,235 isolate of food poisoning that the resulting 44,087 isolation of *Salmonella enteric* (A total of 118 serotypes were identified). The distribution of *Salmonella* serotypes in Thailand during 1993-2002 by different source of reservoirs. The reported of the most common *Salmonella* serotype, which was *Salmonella enterica* serotype Weltevreden. (Bangtrakulnonth *et al.,* 2004). Another period that reported between from 2003 to 2006, a change of serotype distribution to other serotypes such as *S.* Enteritdis and *S.* Stanley infection in humans was observed. These serotypes were previously rare, but now belong to the most commonly isolated serotypes as shown in Figure 2. Many reports represented that *Salmonella* were isolated in various food-producing animals such as pigs, cattle and chickens (Gray and Fedorka-Cray, 2002). These data resulted in an important possible transmission of these pathogens to Thai peoples (Bangtrakulnonth *et al.,* 2003; 2005; 2006).

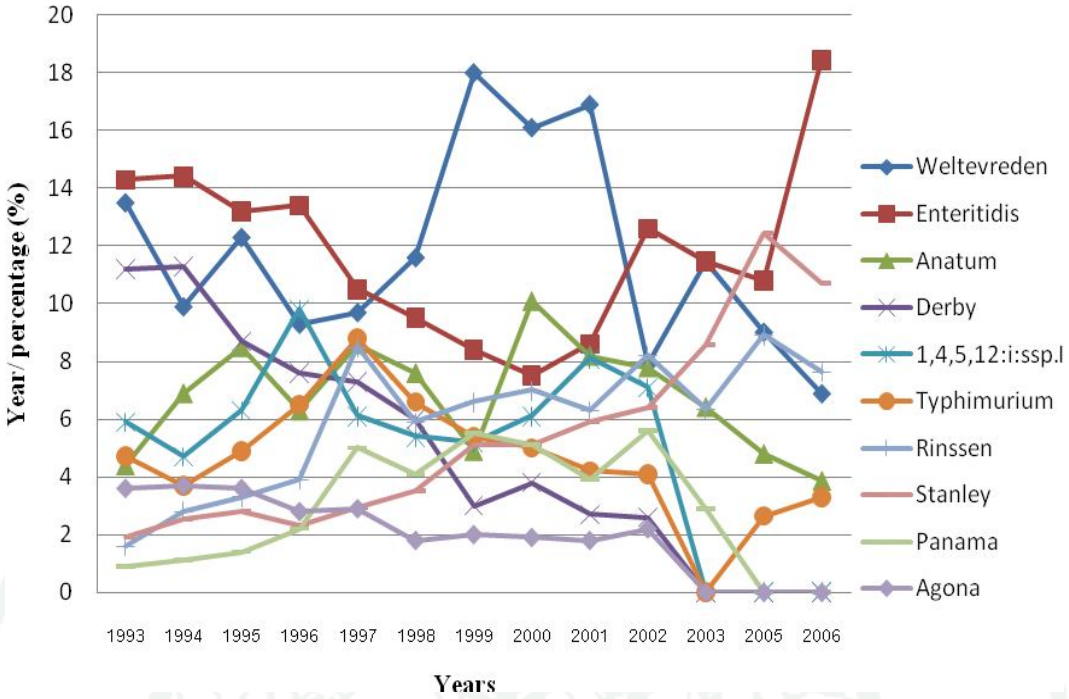


Figure 2 The common serotypes of Salmonella isolates from humans in 1993 to 2006, Thailand

Source: Bangtrakulnonth *et al* (2003, 2004, 2005, 2006)

The severity of salmonella infection depends on serotype, type and immune status of host, and dose of ingestion. In general, the clinical signs of food-borne illnesses are gastroenteritis, enteric fever, septicemia and asymptomatic carrier. In case of asymptomatic carrier is very important, because of the pathogens can be survive in this host for long periods and result in the potential source for distribution of these pathogens. Typically symptom of gastroenteritis in humans begins 24-48 hours after ingestion with fever, nausea and vomiting, follows with abdominal pain and diarrhea (Gray and Fedorka-Cray, 2002). However, these illnesses are considered as self-limiting infection. The infectious dose of *Salmonella* spp. was thought to be in excess of 10,000 cells but a number of outbreaks have been reported where the infectious dose was found to be very low (10-100 cells), depending on the type of food, strain type, the physiological state of bacteria and characteristics of the host. The

establishment of *Salmonella* infection depends on the ability to survive the environment outside of human digestive system, the ability to resist the gastric acid of the human stomach and the ability of the pathogen to attach (colonize) and enter (invade) intestinal cells. For the latter, *Salmonella* must compete with indigenous gut microorganisms for suitable attachment sites. Diarrhoea associated with salmonellosis is thought to appear in response to bacterial invasion of intestinal cells rather than the action of enterotoxins. A main difference with other bacterial intestinal pathogens like *Shigella* and *E. coli,* who are replicating within the cytoplasma of host cells, is that *Salmonella* is confined to endocytotic vacuoles in which bacterial replication takes place. The infected vacuoles move and release *Salmonella* cells into the tissue. Prior to invasion of intestinal cells, *Salmonella* has to encounter and attach to these cells. This involves several types of fimbriae or pili. Genes coding for these fimbriae are located on the chromosome and on plasmids. Other virulence factors of *Salmonella* include siderophores (to retrieve essential iron from the host) and enterotoxins (Darwin and Miller, 1999).

The primary therapeutic scheme for *Salmonella* gastroenteritis is supportive therapy with fluid and electrolyte replacement (Gray and Fedorka-Cray, 2002). Due to non-typhoid salmonellosis results in a self-limiting diarrhea, hence, antimicrobial therapy is not essential for treatment in this situation. However, severe invasive disease or prolonged illness in suppressed immunucompromise patients can occur. Moreover, in case of new-borne, infant, children and elderly who are at risk of septicemia, antimicrobial therapy is still required (Lee *et al.,* 1994). For humans, the treatment of choice for enteric fever and septicemia is often cetriaxone (a third generation of cephalosporins). While, ampicillin and ciprofloxacin should be used in chronic carrier patients (Gray and Fedorka-Cray, 2002).

3. Pork production in Thailand

Pig production in Thailand increased by 3.5 percent per year between 1992 and 2008, reaching the quantity of approximately nine million pigs each year

(Kiratikarnkul, 2010). From situation of pig production in 2006, the bird flu outbreak caused a decrease of chicken consumption. Therefore, pork has been become the second protein source in Thai consumption (Ngasaman, 2007). The central region of Thailand (especially the part of it in the vicinity of Bangkok) has been the main pork producing area of the country, while the South has the smallest number of pigs (Kiratikarnkul, 2010) (Figure 3). Most of the pigs produced in Thailand are consumed domestically because of the presence of foot-and-mouth disease. Some of the producing areas have in limited export markets for fresh meat to Hong Kong, Vietnam and Singapore, while processed pork-based products are more widely exported (FAO RAP, 2002a, Ngasaman, 2007). In 2009, Apart from the impact of an economic downturn, the biggest change in the demand pattern has to be that Thai people nowadays are more health conscious than in the past and they understand more about food safety. Consumer purchasing behavior has changed from being based largely on the price or quantity to a level where quality is also taken into consideration. They are especially willing to pay more for food considered to be safer. While, the Thai government does not provide marketing support directly, it has made food safety a central factor of policy for all food commodities. The product of hygiene foods, including pig meat, will be endorsed by the government's agencies. Department of livestock development have a programme to encourage the consumer to buy chicken and pork from hygienic, certified producers. The producers gain from the recognition while consumers are assured of the safety of products. The government has also established a new agriculture standards act to control the production, inspection and certification of agricultural product, including food of animal origin. A national food committee act was responsible for harmonizing overall food control systems in Thailand and for setting the policies and strategies regarding food safety and food security of the country (Limlamthong, 2009).

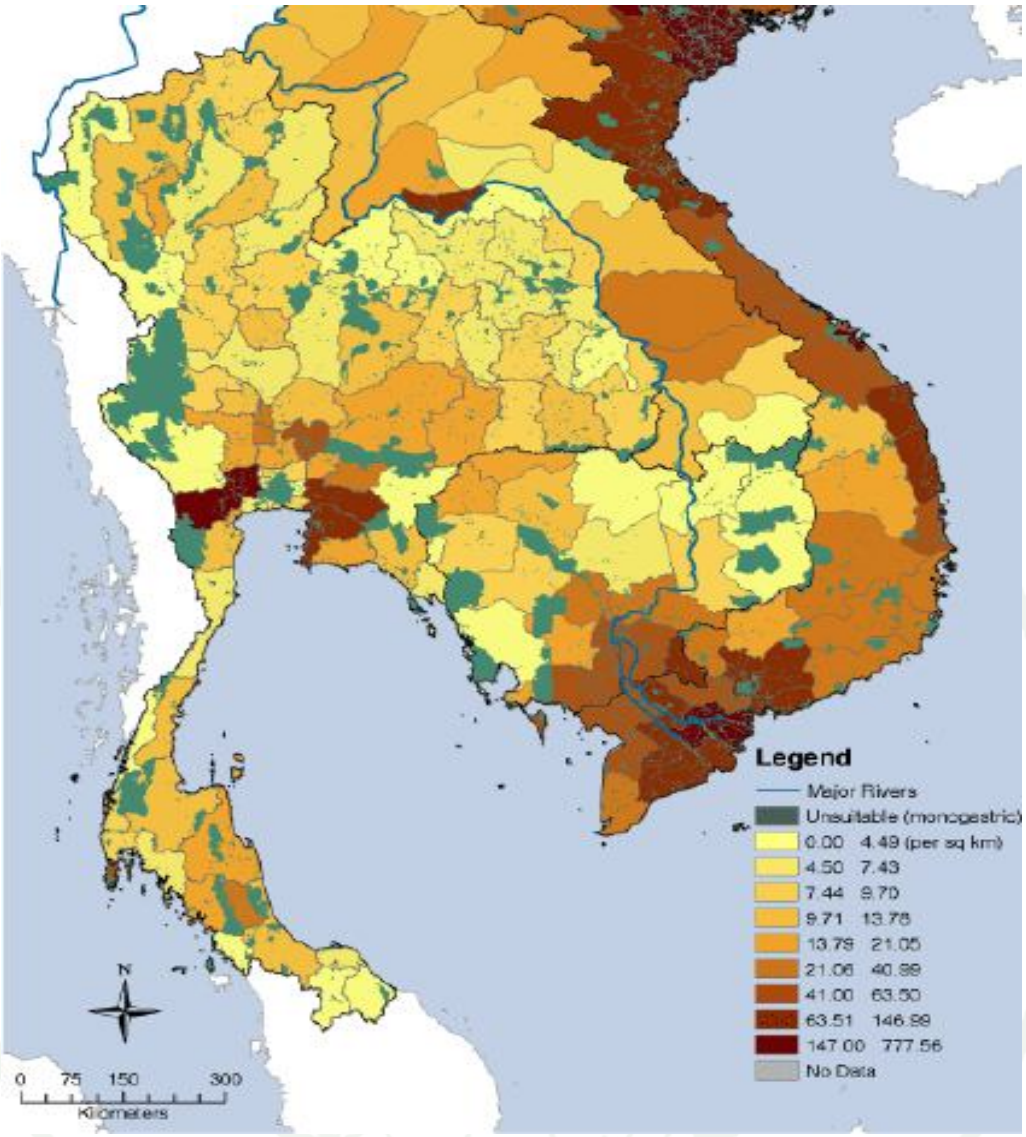


Figure 3 Density of pig in the Mekong countries

Source: Knisp (2004)

In Thailand, Pork production has changed from a farming system of small scale production to larger scale commercial operations. Nowadays, 80% of total pig production of Thailand are obtained from large farms and feed mill companies. Modern pig farm style will not to take a long period to combine crop production with animal husbandry. The general style of pig production systems consists of two groups. The first group is the breeder farm which includes of breeding, gestating, and

farrowing units. The second group is the fattening farm which including nursery and fattening units. The breeder farm provides weaned piglets and distributes them to the fattening farms. Animal husbandry is now organized as fattening farms, specialized from the private company for increaseing the productivity of fattening farms. Assisted by research and development, livestock production efficiency has risen rapidly and the quality of output is also considerably higher than from traditional farming methods. After finishing pigs, the farmers sell them back to the private company and the animals are transported to the slaughter house. The slaughter pigs are kept in the holding pen for a few hours with water supply for resting and calming down. The pigs are slaughtered; carcasses are processed further and transported to retail in supermarkets (Kiratikarnkul, 2010, Ngasaman, 2007). With intensive pig farming, a growing concern over diseases in recent times has led to a wider use of vaccines and drugs which has further increased production costs; over half of the total production cost can be attributed to feed costs (Quirke *et al.,* 2003). Moreover, most of the waste from intensive farming systems (e.g., solid and liquid waste, foul odours and pathogens) has become externalities for the farming sector as well as the rest of society. Among the problems which contribute to ecological imbalances are: severe eutrophication of surface water, leaching of the underground water table, and deposits of heavy metals which create pathogens harmful to humans and animals. These problems are more severe in the case of intensive pig production than for other kinds of intensively produced livestock (Kiratikarnkul, 2010).

4. *Salmonella* in pig

With the consumption of pork in the world, there is a risk that there may have been *Salmonellla* infection in human. Not only do these pose the risk of causing salmonellosis, but also the risk of transmitting antimicrobial resistant *Salmonella* spp. to consumers (Bangtrakulnonth *et al.,* 2006, Isenbarger *et al.,* 2002). From this the issue about the food safety has been considered and contributed to the *Salmonella* control at all stages of the pig production. Especially, the export countries develop standards for swine production. Therefore, in order to minimize the probability of contracting human salmonellosis, it would be necessary to aim at reducing or totally

eliminating herds with high *Salmonella* prevalence (Alban and Stark, 2005). Many countries have regulations to control the *Salmonella* and efforts to reduce the incidence of *Salmonella* at farm level through to retail markets, including Thailand.

There is a prevalence of *Salmonella* from slaughterhouses in Thailand, such as Khon Kaen province that has indicated a high prevalence of *Salmonella* contamination in all sample types from three different slaughterhouses. Isolation of similar *Salmonella* serovars from different type of samples within the slaughterhouse may be due to cross-contamination during the slaughtering processes. The overall percentages of *Salmonella* isolated from pigs, pig carcasses, water and workers were 27.14%, 36.67%, 19.51% and 10.71%, respectively. The most prevalent serovars from all samples in each slaughterhouse were *S. enterica* subsp. ser *enterica* 4,5,12:i:- (48.15%) for slaughterhouse A, *S.* Rissen (35.44%) for slaughterhouse B and *S.* Rissen (44.44%) for slaughterhouse C. (Sithigon and Sunpetch, 2009). These are the common serovars of *Salmonella* found in pigs of Thailand that could be important sources of *Salmonella* for human. Those frequently found in edible pork products and diarrheal patients in the northeastern region including also other parts of Thailand (Bangtrakulnonth et al., 2004, Angkitrakul *et al.,* 2005 and Vaeteewootacharn *et al.,* 2005) (Bangtrakulnonth *et al.,* 2004). Serovars of *Salmonella* in samples that were collected from animals and their environment were similar that, even if the animal immune response is able to clear the infection, the animal is probability of reinfection remained high (Dorn-in *et al.,* 2009).

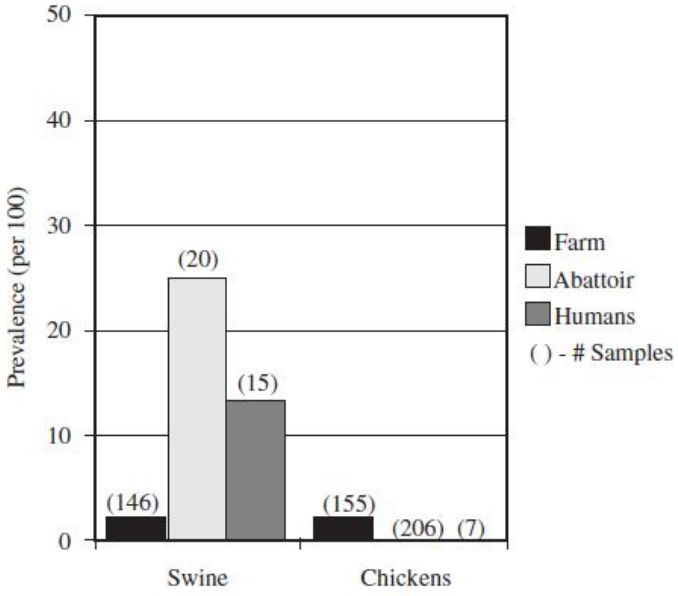


Figure 4 Prevalence of Salmonella isolated from swine, chickens and worker from farm and slaughterhouse in north Thailand

Source: Hanson *et al* (2002)

According to the study of *Salmonella* prevalence isolated from farms and slaughterhouses in north Thailand that are shown, the overall prevalence of *Salmonella* was 8% and the prevalence rate in swine (15%) was significantly higher than in chickens (1%). More *Salmonella* were isolated from swine at the slaughterhouse (25%) than from swine at the farm (2.1%) Figure 4. Moreover, *Salmonella* organisms were isolated from swine slaughterhouse samples at a higher percentage than from swine farm samples (Hanson *et al.,* 2003). While the overall of Salmonella prevalence that was isolated in individual pig level from healthy pigs at 7% (Hong *et al.,* 2006; Tran *et al.,* 2004; Padungtod and Kaneeene, 2006). In another earlier study using a similar method but combined with ELISA a much higher prevalence of 55% at pig level could be shown (Patchanee *et al.,* 2002)

Management of *Salmonella* shedding in pig farms, herd size is a one factor that was associated with *Salmonella* infection that pigs from large herds have been shown to be more likely to be infected with *Salmonella* (Dorn-in *et al.,* 2009, Mejía *et al.,* 2006). Increased number of pigs per pen should increase the frequency of contacts

among pigs, which having snout contacts increased the odds of finding *Salmonella* (Lo Fo Wong *et al.,* 2004) and hence pen density leading to a fast rate of transmission (Giesecke, 1994). But, another study found that small or moderate herd sizes have a higher chance of being *Salmonella* infected. Because, large herd size have more resources for support of effective biosecurity measures, and the specialist will have a good manufacturing practice scheme (Stege *et al.,* 2000; Van der Wolf *et al.,* 2001). To establish adequate and cost-effective intervention strategies at herd level, risk factors for the occurrence of *Salmonella* in grower and finishing pigs have to be investigated. In Thailand, most farmers have smaller herds and pigs were mostly raised under an open housing system. But, farmers usually take better care of their animals. So, herd size was not a factor to increase the number of *Salmonella* in pigs in Thailand (Dorn-in *et al.,* 2009).

The overall biosecurity and the introduction of animals into the herds may be more important in preventing *Salmonella* entering pig herds (Zheng *et al.,* 2007; Nollet *et al.,* 2004). From the study of potential managerial factors was associated with *Salmonella* in pig herds or the presence of antibodies have been investigated in Europe and North America. Types of feeds, whether wet or dry, were found to be associated with *Salmonella* in pigs in Europe (Boloeil *et al.,* 2004; Leontides *et al.,* 2003; Lo Fo Wong *et al.,* 2004) and America (Hanson *et al.,* 2006). For example, open sources of water may be contaminated with *Salmonella* when using well water instead of public or open sources of water increased the odds of finding *Salmonella*, e.g. in Spain (Mejia *et al.,* 2006) and the US (Hanson *et al.,* 2006). Meanwhile, the Thailand Department of livestock development (DLD) has the guidelines of water treatment (30 ppm Chlorine). However, DLD certified farms may not always monitor chlorine level in their water hence chlorine was not effective for preventing *Salmonella* from getting in the water. So, *Salmonella* can still be found in pig herds (Dorn-in *et al.,* 2009). Another, management suggested having bird proof nets, use effective microorganisms (EM) or feed acidified or fermented byproducts which fed to finishers could reduce the level of *Salmonella* infection (Van der Wolf *et al*, 1999; Callaway *et al.,* 2004; Mejia *et al.,* 2006). Also the results of Kranker *et al*, (2001) demonstrate that dry feed for sows has a significant effect on seroprevalence (meat

juice at slaughterhouse). In addition, housing and hygienic practices were found to be associated with the occurrence of *Salmonella* in pig herds in France (Boloeil *et al.,* 2004), Spain (Mejia *et al.,* 2006), Belgium (Nollet *et al.,* 2004) and the US (Van der Wolf *et al.,* 2001).

A reduction of *Salmonella* infection in pigs can help reduce the number of human *Salmonellosis* cases (Wierup, 1994). A reduction in on-farm infection can only be realised by understanding the transmission of *Salmonella* on-farm and applying control measures to reduce transmission. In practice, serologically negative swine herds are sometimes found to still produce pigs that are bacteriologically positive in the gut and associated lymph nodes at slaughter (Nollet *et al.,* 2005). It has been suggested that these pigs were recently infected, so that the serological response was not fully developed at the time of sampling. However, if some *Salmonella* strains are truly able to actively decrease the immunological response, the current monitoring programmes, which usually are based on serology, may show inadequate in these cases (Boyen *et al.,* 2008). Pigs are usually infected after weaning and rarely show any clinical signs. *Salmonella* excretion is intermittent and occurs mainly in the first half of the fattening phase, peaking at approximately 60 days of age. Faecal-oral transmission is a major route of *Salmonella* transmission (Wray, 2001), both from pig-to-pig contact or from a contaminated environment. Snout contacts, aerosol and dust transmission are also possible routes (Fedorka-Cray *et al.,* 1995; Fedorka-Cray *et al.,* 1994). Depending on the inoculation dose, oral experimental infection of pigs with *Salmonella* Typhimurium may result in clinical signs and faecal excretion of high numbers of bacteria (Boyen *et al.,* 2008). After infection of pigs, *Salmonella* can excrete high numbers of *Salmonella* in faeces (10^7 *S.* Typhimurium/g faeces) within 2-6 h (Fedorka-Cray *et al.,* 1994; Hurd *et al.,* 2001), but usually excretion is intermittent with low levels of bacteria (Kranker *et al.,* 2003; Nielsen et al., 1995). Experimentally, large doses are required to induce disease in pigs ($>10^7$), but in field situations the initial infective dose is expected to be much lower, as a result of impaired intestinal motility and immunity, disruption of intestinal flora and increased gastric pH (Schwartz, 1999).

Salmonella pathogenesis is characterised by three phases: the initial colonisation of the intestine, the invasion of enterocytes, and finally the dissemination to lymph nodes and other organs (Darwin and Miller, 1999). Furthermore, recent results have indicated that following oral exposure of pigs to *S.* Typhimurium, the bacterium may be isolated from caecal contents within 4-6 hours, much more rapidly than previously expected (Fedorka-Cray *et al.,* 1995; McDowell *et al.,* 2007). To successfully cause disease in a host, *Salmonella* has to overcome several host defence mechanisms. The low pH in the stomach significantly reduces the number of bacteria that reaches the intestine. In the intestinal lumen, peristalsis further prevents colonisation of the intestinal wall and the gut-associated lymphoid tissue by *Salmonella* bacteria. Furthermore, the normal intestinal flora inhibits colonisation by bacterial interference producing inhibitory substances, competing by tissue adhesion sites and limiting nutrients. Other host defense factors include the detergent-like effect of bile, the decreasing oxygen supply and the cationic antimicrobial peptides present on the surface of epithelial cells. If *Salmonella* successfully invade the intestinal mucosa and multiply in the gut-associated lymphoid tissue, it spreads to the regional lymph nodes. If macrophages are able to limit *Salmonella* from spreading, infection is limited to the intestine and the gut-associated lymphoid tissue and this might result in acute gastroenteritis. Non host-adapted serovars, such as *S.* Typhimurium, usually cause a localised infection in pigs. Affected pigs might present watery, yellow diarrhoea, inappetence, lethargy and fever. Even though mortality is low, morbidity can be high. If *Salmonella* invasion is not limited by the first cellular defence, the bacteria spreads to the thoracic duct into the vena cava, giving rise to a systemic disease. *Salmonella* might disseminate to multiple organs via mononuclear phagocytes. Systemic disease is usually caused by the host-adapted serovar *S.* Cholerasuis. However, in immuno-compromised pigs, all *Salmonella* serovars are potentially able to cause systemic disease. Septicaemic pigs might present inappetence, lethargy and fever, along with respiratory signs. Usually, diarrhoea is not a common feature of septicaemic *Salmonella* infection. Infection in pigs is frequently subclinical and *Salmonella* can be carried in the tonsils, the intestines and the gut-associated lymphoid tissue (Baumer *et al.,* 2000; Fedorka-Cray *et al.,* 2000; Rychlik and Barrow, 2005). In response to a *Salmonella* infection, pigs present a humoral immune response which is affected by the route and quantity of the infectious *Salmonella* dose, serovar virulence and age. Experimental studies with *S.*

Cholerasuis showed that immunoglobulin concentrations peak one week following infection and remain high for two to three months (Gray *et al.,* 1996). Following reinfection, antibody response is rapidly triggered. A cellular immune response might also be detected depending on the antigenic stimulation. Upon recovery, a pig can remain in the carrier state (Schwartz, 1999). Carriers are a major reservoir of *Salmonella* and pose an important threat to animal and human health. Carrier pigs can shed *Salmonella* and spread it in the environment infecting other animals and/or contaminating carcasses, which might ultimately result in human exposure (Fedorka-Cray *et al.,* 2000).

The typical symptoms of *Salmonellosis* in pigs and, as such, records of clinical cases are a poor indicator of the overall prevalence of disease. Specific studies undertaken to estimate prevalence are therefore indicated and can serve a number of purposes including predicting the risk of contaminated food products entering the food chain and as a baseline estimate by which to judge subsequent trends or the success of surveillance and control programmes. Monitoring *Salmonella* in pig herds can be done by culturing pooled fecal samples or by serologic detection of antibodies (Davies *et al.,* 2003) using either serum or meat juice samples (Nielsen et al., 1998). The proportion of animals positive on caecal culture compared to those positive on serology may reflect genuine differences in the sensitivities of the two techniques but may also reflect the occurrence of recent, and possibly very recent infection, in at least a percentage of animals.

4.1 *Salmonella* in pork products

In the present, pork is the major source of protein intake in many countries of the world. But, the number of reported cases of *Salmonella* contaminated in pork has grown dramatically all over the world. (Cravens, 2000; Wray, 2001 Wegener and Baggesen, 1996;Berends *et al.,* 1998; Fedorka-Cray *et al.,* 2000; Alban *et al.,* 2002). Many studies have focused on the role of pork as a significant source of Salmonella, from eggs and poultry meat (Anon., 2008e; Anon., 2010d). Approximately 20% of the human salmonellosis cases in EU were attributed to the pig reservoir (Fares et al.,

2010). In Denmark, approximately 15-20% of human cases are considered to be related to pork and pork production (Wegener *et al.,* 1994). Among pork products, ground pork was found to be the most likely contaminated by *Salmonella* (16%) with the lowest *Salmonella* contamination in pork chops (1% to 3.3%) (Foley *et al.,* 2008).

Finishing pigs have been recognized as the major source of *Salmonella* contaminations of carcasses and pork products at later stages in the food chain (Boloeil *et al.,* 2004) including Thailand. From previous studies in several parts of Thailand have demonstrated the presence of *Salmonella* in pigs herds, slaughterhouses and pork products at markets (Padungtod and Kaneeene, 2006 and Vaeteewootacharn *et al.,* 2005). Also the risk of transmitting antimicrobial resistant *Salmonella* spp. to consumer is present (Bangtrakulnonth *et al.,* 2006 and Isenbarger *et al.,* 2002). Since this *Salmonella*-carrier state in animals like pigs that are latently contaminated, who under normal conditions do not shed *Salmonella*. These organisms may remain alive for months in wet, warm areas such as in feeder pig barns or in water dugouts (Owlia *et al.,* 2007). The prevalence of infection differs amongst species and countries and is much higher than the incidence of clinical disease, which is frequently caused by stressful situations such as sudden deprivation of feed, parturition, drought, crowding, transportation, poor handling, their immune status and the administration of some drugs (Nietfeld *et al.,* 1999, Asghar *et al.,* 2002; Prendergast *et al.,* 2008; Wales *et al.,* 2009). Every action is stimulated by strongly stress to carrier animals, because this may be associated with the release of catecholamines by major stressed animals which in turn can stimulate the growth of *S.* Enteritidis and *S.* Typhimurium (Nietfeld *et al.,* 1999; Wang *et al.,* 2007) and *Salmonella* can be excreted with the faeces, increasing the risk of cross-contamination between shipping equipment and holding areas, resulting in pre-slaughter transmission of *Salmonella* to non-infected pigs during transport and in the slaughterhouse (Berends *et al.,* 1997; Marg *et al.,* 2001; Isaacson *et al.,* 1999; Boughton *et al.,* 2007). Although the mechanism of this stress-induced excretion is not known, there are some indications that catecholamines may play a role (Wang *et al.,* 2007). It is assumed that approximately 70% of *Salmonella* carcass contaminations in slaughterhouses are caused by contamination from animals that are carriers themselves and 30% from other carrier animals (Berends *et al.,* 1997). In

general, between 5% and 30% of the produced carcasses can contain *Salmonella* spp. (Berends *et al.,* 1997; Botteldoorn *et al.,* 2003). Tonsils, lymph nodes, faeces and the digestive tract are the most likely locations where Salmonella can be expected after contamination (Blaha, 2001; Swanenburg *et al.,* 2001b). Thus, pork belongs to the major sources of food-associated Salmonellosis-the most important microbiological contamination of meat - in humans. At the highest risk-level are countries where pork is consumed raw or not thermally treated. Besides the risk of endogenous contamination, one has to take into account the risk of cross-contamination during slaughter and processing (Prendergast *et al.,* 2008; Wales *et al.,* 2009).

The possibility of lairage contamination as a potential source of *Salmonella* positive isolates in caecal contents is also supported by a recent risk factor study, which reported that in pigs which spent 3–6 hours in lairage the odds of *Salmonella* in caecal contents was 3.3 times that of pigs that spent less than 3 hours, while for pigs that spent more than 6 hours, the odds were 13.1 (McDowell *et al.,* 2007). *S.* Typhimurium is reported to be the most common serotype associated with swine. Pigs are likely to be infected with *Salmonella* at the farm, during transportation or while waiting in the lairage prior to slaughter (Vieira- Pinto *et al.,* 2006). Swine can carry *Salmonella* in several tissues. The palatine tonsils are often heavily infected in pigs and should, therefore, not be underestimated as a source of *Salmonella* contamination during slaughter (Wood *et al.,* 1989). It is generally accepted that *Salmonella* can spread throughout an organism using the blood stream or the lymphatic fluids and infect internal organs, although this has not yet been studied in swine. The colonisation of the mesenteric lymph nodes, spleen and liver can result in prominent systemic and local immune responses (Dlabac *et al.,* 1997). From investigation in Midwest U.S. (Bahnson *et al.,* 2006), risk factors were identified for harboring *Salmonella enterica* among slaughter-weight pigs. Samples were collected on farms (feces) and at slaughter (distal colon content, cecal content and ileocolic lymph nodes). The mean individual pig prevalence was 5% for feces, 4% for distal colon content, 15% for ileocolic lymph nodes, and 17% for cecal contents. The five most common serotypes were *S.* Agona, *S.* Derby, *S.* Schwarzengrund, *S.* Typhimurium, and *S.* Senftenberg. Berend *et al*. (1997) estimated that in general between 5-30% of

the carcasses produced may contain *Salmonella*. Risk factors were inadequately cleaned polishing machines, inadequate during evisceration, i.e. faulty evisceration and hygiene practices. An estimated 5-15% of carcasses contamination occurred 55-90% during polishing during evisceration practice and 5-35% during processing. The digestive tract and lymph nodes that were representing a potential risk for consumers which must be properly identified and controlled (Jung *et al.,* 2001; Castagna *et al.,* 2004; Silva *et al.,* 2006).

In Thailand, reports have shown the relationship between *Salmonella* spp. in foods of animal origin and public health problems (Rasrinual *et al.,* 1988; Boriraj *et al.,* 1997; Boonmar *et al.,* 1998a). During 1993 to 2002: In Khon Kaen province, S. Anatum is the most common serotype isolated from rectal swab of diarrheal cases submitted to a public hospital, pork from retail markets (46.1%), supermarkets (32.6%), swine farms (25%) and slaughterhouses (70.59%) (Angkititrakul, 2008) and Patchanee *et al.* (2002) reported that the prevalence of *Salmonella* increased to 82.5 % at slaughterhouse compared to 69.5 % at the farm level. In the studies of *Salmonella* in pork chain, the prevalence in retail pork products decreased to 34.5 % (Sanguankait, 2005) compared with the prevalence at slaughterhouse in lymphnodes of 64.1 % (Chantong, 2005) and the prevalence at fattening farm level in feces of 62.9 % (Dorn-in, 2005). *Salmonella* is widespread in pigs with an average prevalence between 6 and 82.5 % (Table 3) (Padungtod *el al*., 2006; Angkititrakul *et al.,* 2005); therefore the chance of infection is relative high. There are various serotypes (Table 4). However, the most common serotypes in pigs were *S.* Rissen and *S.* Typhimurium (Sanguankait, 2005; Chantong, 2005; Dorn-in, 2005). These also belong to the all common *Salmonella* infections in Thai people (Bangtrakulnonth *et al*, 2006).

Table 3 The prevalence of Salmonella in pig

Year	Pre slaughter pigs	slaughter pigs	Pig product
1999	69.5% Patchnee et al., 1999	82.5% Patchnee et al., 1999	-
2005	62.9% Dorn-in, 2005	64.1% Chantong, 2005	34.5%
2006	6% Padungtod and Kaneene, 2006	28% Padungtod and Kaneene, 2006	29% Padungtod and Kaneene, 2006

Source: ngasaman *et al* (2007)

Table 4 Distribution of Salmonella serotypes in pig

Serotype	Pre slaughter pigs	Slaughter pigs	Pig product
S. Rissen	45.4%	45.9%	43.3%
S. Typhimurium	18.6%	10.8%	16.3%
S. Stanley	11.2%	11.7%	6.3%
S. Krefeld	3.1%	-	10.6%
S. Lagos	-	-	6.0%
S. Waltevreden	3.7%	-	-
S. Anatum	2.4%	-	-

Source: ngasaman *et al* (2007)

The number of *Salmonella* organisms on the surfaces of carcasses of pigs may be reduced as a result of careful slaughter procedures, such as scalding individually, careful removal of intestines (Oosterom and Notermans, 1983; Berends *et al.,* 1997., a plastic bag over the rectum (Nesbakken *et al.,* 1994; Sørensen *et al.,* 1999a., and a decontamination step after slaughter (Snijders et al, 1985; Snijders, 1988).

Berends *et al.,* (1998) Suggested to introduce logistic slaughter, or separate slaughter of pigs free from a certain pathogen, to avoid introduction of certain bacterial zoonoses into the slaughterline and to avoid cross-contamination between herds during slaughter. However, the effects of logistic slaughter on the level of *Salmonella*-contaminated pork after slaughter have never been tested. It is not known if separate slaughter of pigs from *Salmonella*-free farms, under practical conditions, will result in *Salmonella*-free pork. To reduce contamination at the slaughterhouse, a reduction of Salmonella carriage at the herd level is needed.

5. *Salmonella* detection

Salmonella control is therefore necessary at all the key steps of food production. Surfaces that come into contact with food are an important component of any integrated program to ensure the safety of foods throughout the food supply chain (López-Campos *et al.,* 2012) and safe products for consumers (Hedberg *et al.,* 1992). This control requires rapid and reliable methods in the detection, isolation, characterization and quantification of *Salmonella*. It is essential that methods for detection of *Salmonella* in foods have the ability to detect low levels of pathogens that are healthy, as well as those that are stressed/injured due to conditions in the food and/or during food processing. The detection of low numbers of cells is particularly important for *Salmonella* spp., since epidemiological evidence suggests that low doses of certain *Salmonella* strains can cause disease in a significant proportion of the consumers (Hedberg *et al.,* 1992). Microbiological analysis is also an essential tool for carrying out tests in accordance with the microbiological criteria established for each food type, as well as being essential for evaluating the actions of different management strategies based on the Hazard Analysis and Critical Control Points (HACCP) system (Stannard 1997; Jasson et al. 2010). But, microbiological analysis of foods is based on the detection of microorganisms by preenrichment, selective enrichments and plating on selective agar media followed by biochemical and serological tests, which requires 5 to 7 days for completion (US FDA/CFSAN, 2006; Okamura *et al.,* 2008) and are labor-intensive. Tremendous efforts are on-going to develop novel detection technologies with high sensitivity and speed. Also, the

implementation of preventive systems such as the HACCP has greatly improved food safety, but it will not be fully effective until better methods of analysis are developed. These new detection methods are the necessary technologies that will substantially improve our food safety once integrated in the HACCP (Bhunia, 2008).

5.1 ISO 6579:2002

Current isolation of *Salmonella* from suspected samples following the ISO standard protocol: 6579:2002 is generally accepted as the gold standard culture method for classifying the Salmonella-on farm and food from animal origin (Davies *et al.,* 2000; Rajic *et al.,* 2005; Rene *et al.,* 2012). In most cases, they are traditional culture methods that use selective liquid or solid culture media, to grow, isolate, and enumerate the target microorganism and simultaneously prevent the growth of other microorganisms present in the food (Jasson *et al.* 2010).

The world health organization (WHO) requires at least a sampling of 25 grams of meat to not be carrying any trace of salmonella. The ISO 6579 standard protocol includes two enrichment steps: - a preenrichment step to allow injured cells to resuscitate and a selective enrichment step to favor the growth of *Salmonella* cells. Pre-enrichment of *Salmonella* in buffered peptone water (BPW) is commonly used for most foods, though some foods require a more specific medium (European Committee for standardization, 1997). Selective enrichment aims at increasing the number of *Salmonella* in samples, while at the same time reducing the non-*Salmonella* population. In the first step, a nonselective but nutritious medium is used (buffered peptone water); in the second step, the selective medium contains selective agents to suppress the growth of accompanying microflora. Two different selective media are used in the second step because the culture media have different selective characteristics against the numerous *Salmonella* serovars (Forsythe, 2000).Time and temperature of incubation during the preenrichment and selective enrichment steps play a significant role in the selectivity of the media. One of the selective media used in the second enrichment step has historically been a selenite cystine broth that contains a very toxic substance (sodium biselenite), and for this reason its use has

been replaced by other media such as a Muller-Kauffmann tetrathionate/novobiocin (MKTTn) broth. Rappaport-Vassiliadis soya peptone (RVS) broth is the standard Rappaport-Vassiliadis (RV) broth but with tryptone substituted by soya peptone because it has shown better performance than the standard broth (de Boer, 1998). The next step is plating of the samples on selective differential agars containing selective agents such as bile salts and brilliant green, which have various diagnostic characteristics (e.g., lactose fermentation, H_2S production, and motility) to differentiate *Salmonell*a spp. from the other microflora such as *Proteus* spp., *Citrobacter* spp., and *E. coli*. The Oxoid Biochemical Identification System (OBIS) *Salmonella* test (Oxoid, Basingstoke, U.K.) is a rapid test to differentiate *Salmonella* spp. from *Citrobacter* spp. and *Proteus* spp. The principle of the test is based on the determination of pyroglutamyl aminopeptidase (PYRase) and nitrophenylalanine deaminase (NPA) activity, to which *Salmonella* spp. is negative, *Citrobacter* spp. is PYRase-positive and NPA-negative, and *Proteus* spp. NPA-positive and PYRase-negative. Selective agars differ in their selectivity toward *Salmonella,* and for this reason a number of media are used in parallel (xylose lysine desoxycholate; XLD) or xylose lysine tergitol-4 (XLT-4) and phenol red/brilliant green agar). The last steps include biochemical and serological confirmation of suspected *Salmonella* colonies to confirm the identity and to identify the serotype of the isolates (de Boer, E. 1998; Gray and Fedorka-Cray, 2002). *Salmonella* spp. is lactose-negative, H_2S-positive, and motile. However, lactose-positive strains have been isolated from human infections, and an additional selective medium agar may therefore be needed. Bismuth sulfite agar is considered as the most suitable medium for such strains (de Boer, 1998; D'Aoust et al., 1992; Ruiz et al., 1996). The most frequently isolated serovars from foodborne outbreaks are *S. typhimurium* and *S. enteritidis*. Traditional phenotypic methods such as biotyping, serotyping, and phage typing of isolates, as well as antimicrobial susceptibility testing, provide sufficient information for epidemiological purposes. The ISO standard protocol for detecting and identifying *Salmonella* spp. is depicted in table 5 and in Appendix B.

Table 5: Principles and media for conventional culturing of Salmonella

Steps	Commonly used components
1. Non-selective pre-enrichment	- Buffer Peptone Water (BPW)
2. Selective enrichment	- Rappaport Vassiliadis broth (RV)
	- Rappaport Vassiliadis Soya broth (RVS)
	- Modified Semisolid Rappaport Vassiliadisagar (MSRV)
	- Selenite broth
	- Selenite Brilliant Green broth
	- Tetrathionate broth
	- Tetrathionate Brilliant green broth
3. Plating on solid agars	- Brilliant green agar (BGA)
	- Desoxy Cholate Citrate agar (DCA)
	- Brilliant green Phenol Red Lactose Sucrose (BPLS)
	- Xylose Lysine Deoxycholate (XLD)
	- Xylose Lysine –tergitol 4 (XLT4)
4. Verification	- Biochemistry
5. Further identification steps	- Serotyping

Source: modified from ISO 6579(2002)

Conventional culture methods have limitations from both quantitative and qualitative data. These methods allow the detection of a single bacteria cell in complex samples by pre-enrichment step of a single cell growth into a colony on a plate. Moreover, four to six days that is time-consuming and labour intensive for isolation and identification because of the need for culture on selective media and confirmation of the suspect isolate by biochemical and immunological tests. Besides being time consuming, the conventional culture method has also been reported to show poor sensitivity for low-level contamination in samples and problems in interpreting the results of different laboratories might occur (D'Aoust, 1992b; Andrew *et al.,* 2003; Uyttendaele *et al.,* 2003; Settanni and Corsetti, 2007). In spite of its importance, the microbiological analysis of food has many limitations. Uncertainty of the analytical result must be considered when establishing microbiological criteria, including the variance associated with the sampling plan, method of analysis, and laboratory performance (Betts and Blackburn, 2009). The culture method of food remains a challenging task for virtually all assays and technologies, especially for particular pathogenic species (Feng, 2007). The problems may be due to 1). The

complexity of food matrices and composition. 2). the heterogeneous distribution of low levels of pathogens. 3). the stress suffered by the microorganisms during the processing of foods. 4). the presence of bacteria from the normal microbiota, especially in raw foods. When applied to the detection of pathogens in the environment, culture-based protocols may be inaccurate due to the selective nature of the media which requires the use of a specific medium and specific culture conditions for each microorganism. Because, the bacterial cells present in any given environmental samples can be cultured with these techniques. Salmonella detection and identification in food has the probability to increase the chances of preventing diseases caused by this pathogen, but the correlation between culturability and infectivity has not been properly determined, this technique remains questionable, especially in the light of increasing numbers of Salmonella cases worldwide.

Since food regulatory agencies have established strict control programs in order to avoid food pathogens entering the food chain, official laboratories should be managed to process-rapidly and efficiently-a high number of samples. According to these requirements, more accurate detection, eliminate the need for cell culture, can be achieved by the use of molecular techniques. These methods are commonly used in microbial ecology studies as cultivation independent tools to analyze the structure of microbial communities or to detect specific organisms, such as pathogens in complex samples (Amann *et al.,* 1995; Baudart *et al.,* 2002; Bayardelle and Zafarullah, 2002; Bej *et al.,* 1991; Cooper and Danielson, 1997; Field *et al.,* 2003; Josephson *et al.,* 1993; Mittelman *et al.,* 1997; Reynolds *et al.,* 2001; Tsai *et al.,* 1993). Rapid isolation and identification of Salmonella in food will increase the chances of preventing diseases caused by this pathogen. Fluorescence in situ hybridization (FISH) is one of the molecular methods that relies on at least one enrichment step and detection of specificity of nucleic acid target by oligonucleotide probe (Chen *et al.,* 1997)., when compared to conventional methods.

5.2 *In situ* hybridization

Detection of *Salmonella* in food is generally performed by traditional methods such as ISO 6579. This process is laborious and time consuming, and is rarely carried out in practice (FAO/WHO, 2009). These methods take an average of 4-6 days to be completed for negative and positive samples, respectively (Rijpens *et al.,* 1998). Several researchers have developed new screening methods for alternative methods of detection and enumerate pathogens in food, for example *In situ* hybridization (ISH) method with rRNA-targeted oligonucleotide probes. This is one of the most rapid techniques for acceptable monitoring of the real risks of bacterial numbers alteration or the possibility of pathogenic bacteria in food contamination. (Mereno *et al.,* 2001; Garcia-Armisen and Pierre, 2004; Schmid *et al.,* 2005; Vieira-Pinto *et al.,* 2008; Bisha, 2009). However, rather than diminish the importance of *in situ* hybridization, the now widespread use of screening technologies has increased the need to temporally and spatially localize the distribution of mRNA expression. Many scientific reports concerning the application of the ISH technique, it is broadly applied in food, microbiology of environment, histopathology, histoimmunology, cytogenetics. Initially, it was developed in order to identify and to determine the number of bacterial cells in water ecosystem environments (Skowrońska & Zmysłowska, 2006), deposits, rhizosphere and soil. Also, this technique may be useful in several respects, including the diagnostic medicine, the detection of pathogens within human and animal tissues (Moter and Gobel 2000), the estimation of total biomass, the detection of specific organisms, genes and estimations of species diversity (Ian and Tim, 2006).

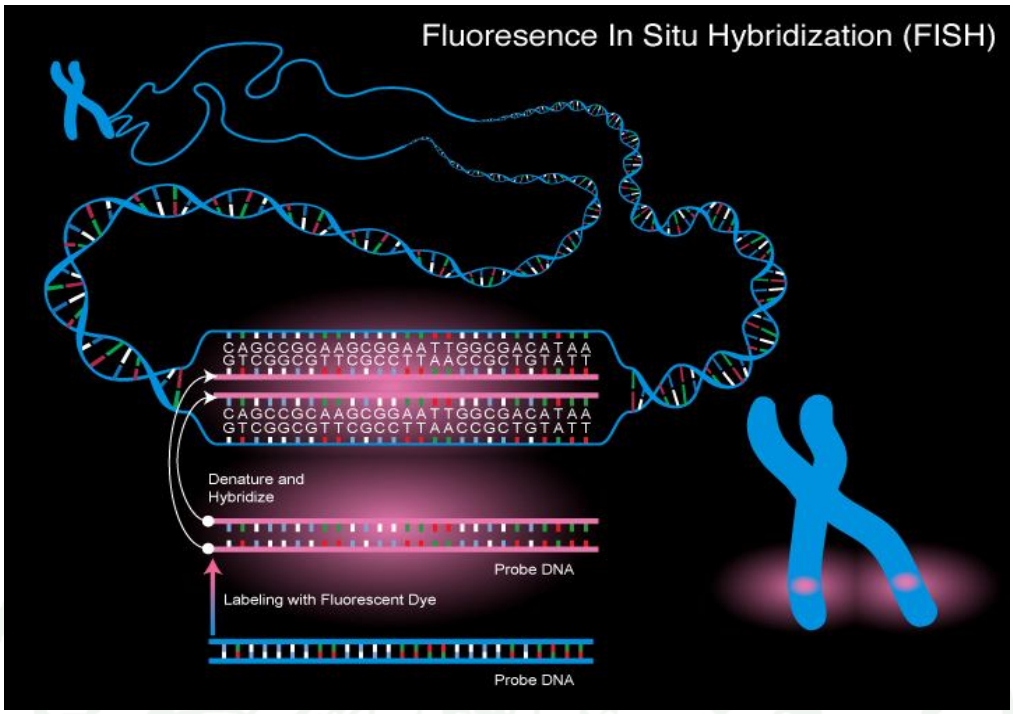


Figure 5 Fluorescence *in situ* hybridization

Source: Leja (2012)

In particular, Fluorescence *in situ* hybridization (FISH) has become a powerful tool for phylogenetic identification of bacteria at the level of single cells without previous cultivation (Amann *et al.,* 1995, Vieira-Pinto *et al.,* 2008; Bisha, 2009). This technique is using oligonucleotide probes with a short fragment of the nucleic acid, which is synthesized and labeled that are most often marked on one or on both ends with a fluorescent dye. The design of oligonucleotides can be hybridized or are paired with complementary sequences of DNA or RNA from microorganisms (Figure 5), such as a strain, a group of similar strains, an entire domain, and all life. The sequence of the probe determines the specificity and sensitivity of rRNA in ribosomes of the target cells, identifying them on various taxonomic levels (Örmeci and Karl, 2008). FISH enables the detection of most bacteria, even in samples where the proportion of cultivable bacteria among the total microbial population is relatively low, and in samples. A large number of FISH has been applied in the investigation of microbial symbiosis, the analysis of microbial diversity in environmental samples, the evaluation of the presence of bacteria in wastewater treatment plants (Amann *et al.,*

2001), the identification of bacteria relevant in diagnostic medicine, the detection of pathogens within human and animal tissues (Moter and Gobel 2000), and the enumeration and identification of specific contamination sources in factory processes, e.g., factory plants (Gunasekera *et al.,* 2003). A standard FISH method consists of five main parts (Figure 6): sample preparation, fixation, permeabilization of cells, hybridization, washing off the excess unbound probe and observation under a fluorescent microscope. Fixation increases the permeability of the cell to allow probe penetration and protects cells from lysis during hybridization. During permeabilization of cells, the cells are typically applied on microscope slides and dehydrated in a series of ethanol solutions. The hybridization step involves the penetration of probe to the cells and specific binding of the labeled oligonucleotide to the complimentary sequences of nucleic acid in the sample. The washing step removes unspecific bound between probes and rRNA-target. Finally, the bound probe is visualized under a fluorescent microscope. (Ootsubo *et al.,* 2003; Örmeci and Karl, 2008; Jasson *et al.,* 2010).

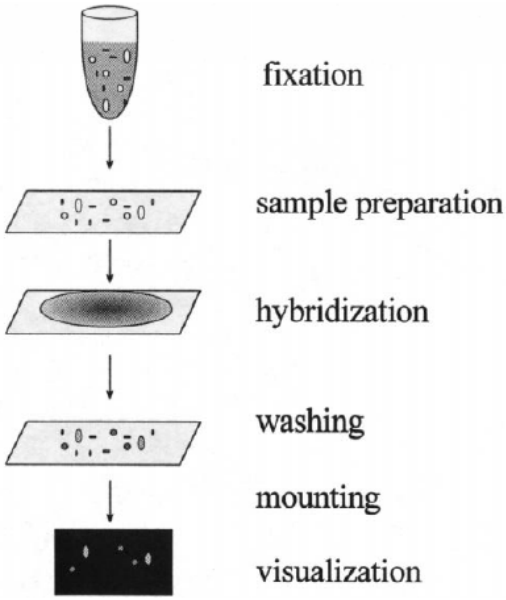


Figure 6 Flow chart of a typical FISH procedure.

Source: Moter *et al* (2000)

According to Amann *et al.* (1995), the *in situ* hybridization with oligonucleotide probes targeting rRNA seems to be a powerful tool for rapid and specific identification of individual microbial strains in pure culture, on colonies and in their environment. Some advantages of FISH over culture methods were already described by Moreno *et al.* (2001), Blasco *et al.* (2003) and Fang *et al.* (2003). These include: decreased proneness to inhibitory substances; identification of viable but not cultivable cells; rapid availability of quantitative results; the possibility of simultaneous identification of different species in the same sample; and relatively low cost per experiment. Although the application of FISH technique in food samples has already been described for detection of foodborne pathogens such as *Staphylococcus* (Gory *et al.* 1999), *Escherichia coli* (Regnault *et al.* 2000), *Salmonella* (Fang *et al.* 2003; Oliveira *et al.* 2003) and *Campylobacter* (Moreno *et al.* 2001). The purpose of this research was to evaluate the applicability of the FISH technique for the rapid detection of *Salmonella* in pork slaughter and to compare it with the standard culture method actually used for detecting *Salmonella* in pork meat.

MATERIALS AND METHODS

1. Probe synthesis and labelling

Preparation and synthetic oligonucleotide probe from Sal3 primer (5′-AATCACTTCACCTACGTG-3′), designed by according to Nordentoft *et al.,* 1997 (Figure 7).

1. Add 100 pmol of Sal 3 primers and sterile double distilled water to a final volume of 9 µl to a reaction vial.
2. Add the following on ice:

Reaction buffer (vial 1)	volume	4 µl
$CaCl_2$-Solution (vial 2)	”	4 µl
DIG-dUTP Solution (vial 3)	”	1 µl
dATP Solution (vial 4)	”	1 µl
400U Terminal transferase (vial 5)	”	1 µl

3. Mix and centrifuge briefly.
4. Incubate at 37^{O}C for 15 mins, then place on ice.
5. Stop reaction by adding 2 µl 0.2 M EDTA (pH 8.0).

To compare the similarity of Sal3 sequence with the Genbank database by using BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). An 18-mer oligonucleotide probe sequence complementary to the region from 23S rRNA positions was selected as being specific for *Salmonella* serovars representing all subspecies and negative strain. The theoretical specificity was checked by using the Clustalw2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

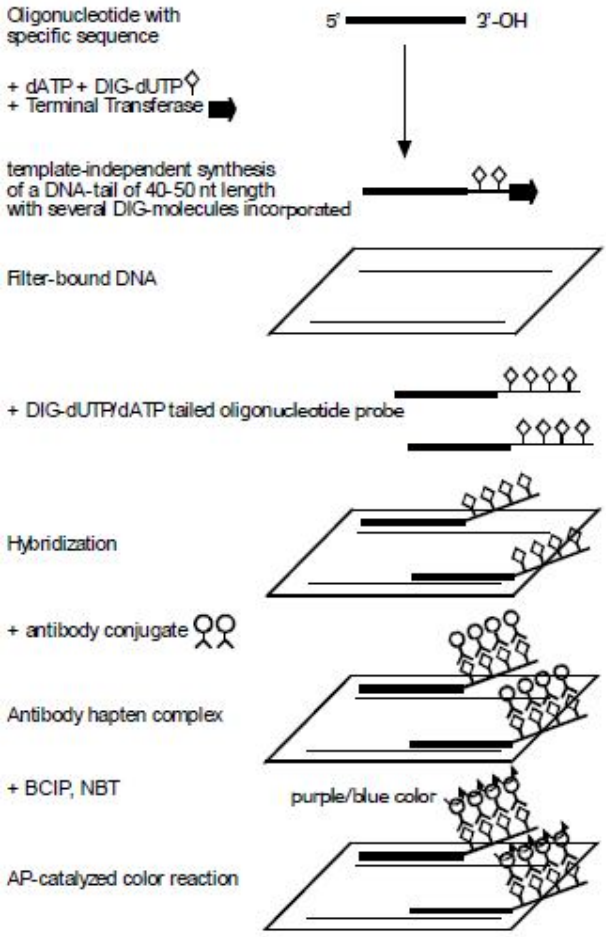


Figure 7 Non-radioactive oligonucleotide tailing and signal amplification

Source: www.roch-applied-science.com.DIG.

2. The evaluation of specificity of Sal3 probe

2.1 Reference stains and culture

Pure culture of *S.* enteritidis, *S.* typhimurium, *S.* paratyphimurium and another *Salmonella spp*. 57 serovars, those were selected and isolates identified from the WHO Nation Salmonella and Shigella Center, Department of Medical Sciences, Ministry of Public Health, Thailand. All strains identified as *S. enterica* were serotyped according to the Kauffman- White serotyping scheme (*Salmonella* antisera (S & A Reagent Laboratory LMT, Bangkok, Thailand) were used for serotyping. And negative strain for this study: *Actinomyces* spp., *Campylobacter jejuni*., *Corynebacterium* spp., *Escherichia coli., Klebsiella* spp., *Pseudomonas* spp.,

Staphylococcus aureus., *Streptococcus agalactiae* and *Streptococcus suis*, those were selected from bacterial laboratory, Department of veterinary *public health*, Faculty of veterinary medicine, Kasetsart university. The reference strain of Salmonella spp. and negative bacteria that were streaked into MAC agar plates for over-night culture at 37 °C for activating bacteria, picked 1-2 colony of each bacterial and inoculate each into a 2 mL nutrient broth and grow overnight at 37° C with Orbital Shaker model OS-20 (BIOSAN, LATVIA) before cell harvest at logarithmic phase to obtain cells with high ribosome content for DNA extract.

2.2 DNA extraction protocol

Collected pellets of each bacterium sample were used for DNA extraction using the phenol-chloroform extraction method described by Sambrook and Russell (2001). Briefly, 100 µl of sample was resuspended in deionized water, mixed with 500 µl of D-solution (4 M guanidiumthiocyanate, 50 mM Tris-HCl, 20 mM EDTA, pH 8.0) and incubated at room temperature for 5 minutes. Subsequently, 200 µl each of phenol and chloroform were added, vortexed and centrifuged at 13,000 rpm for 5 minute. Total DNA was precipitated with 700 µl of absolute ethanol and was washed with 75% ethanol. Finally, the DNA pellet was air-dried and resuspended in 20 µl of TE buffer (pH 8.0). The DNA pellets were stored at -20 ° C until used.

2.3 Oligonucleotide probe

A specific oligonucleotide probe for fluorescence in situ hybridization was used Sal3 primer (5'-AATCACTTCACCTACGTG-3'), is specific to target the 23S rRNA for Salmonella spp. (Nordentoft et al., 1997; Olivera & Bernado, 2002; Vieira-Pinto *et al.,* 2005; Örmerci & Karl, 2008; Ghosh & Santosh, 2009; Almeida *et al.,* 2010) This oligonucleotide probe sequence was tailed with digoxigenin at the 3'-end using the Oligonucleotide Tailing Kit, 2nd Generation (Roche Diagnostics, Germany) following the standard protocol described in the package insert. Briefly, the kit utilizes terminal transferase to corporate DIG-dUTP base at the 3'-end of the probe which enables immunologic detection using an anti-digoxigenin antibody. Based on information provided in the Roche insert, tailing using the standard protocol results in a probe with a hypothetical average 3' tail length of 50 nucleotides (Goebl *et al.,*

2007). Test specificity of Sal3 probe determined by sequence & alignment sal3 probe and Dot-blot hybridization. Prehybridization and hybridization solutions contained 0.1 mg/ml^{-1} of Poly-A to prevent non-specific binding of the tailed probes.

2.4 Dot-blotting analysis

The DNA pellet of each bacterium sample from 60 serovars of *Salmonella* reference and 9 strain of negative bacteria that each DNA of bacteria were diluted to a concentration of 100 µg/ml, DNA boiling for 5 minutes at 100 C° and place on ice 10 minutes. Three µl volumes of each DNA sample was dotted with a nitrocellulose membrane. After dotted DNA, the nitrocellulose membrane was allowed to dry at 80 C° for 2 hour in an oven. There are treated with prehybridization and hybridization solution as mentioned before. Meanwhile, Digoxigenin labeling of the probe was performed as described previously in step 1.

Dot-blot hybridization in this study followed with procedure of Keller and Manak (1989). Nitrocellulose membranes were sealed in a polypropylene bag with, per cm^2, 200 µl of a prehybridization mixture containing 20xSSC (3M NaCl, 0.3M Na-Critrate), 50x Denhardt's solution, yeast tRNA, 1M $Na_2HPO_4.7H_2O$, 10% Dextran sulfate, 0.1mg/ml poly(A)). The membranes were incubated at 48.5°C for 2 h in water bath. The prehybridization mixture was removed and replaced with mixed probe/hybridization buffer solution (1.5 µl: 400 µl), per cm^2 of membrane, 100 µl of hybridization mixture containing (50%formamide, 20xSSC (3M Nacl, 0.3M Na-Critrate), 50x Denhardt's solution, yeast tRNA, 1M $Na_2HPO_4.7H_2O$, 10% Dextran sulfate, 0.1mg/ml poly(A)). The membranes were then incubated in water bath at 48.5°C for 18 to 20 h. The membranes were carefully washed away with consecutive washes in wash buffer 1 (1xSSC, 0.1%SDS, pH 7) at 48.5°C for 5 minutes, wash buffer 2 (0.1xSSC, 0.2%SDS, pH 7) at 48.5°C for 5 minutes and in wash buffer 3 (0.5x SSC, pH 7) at 48.5°C for 1 hr. As a blocking reagent, skim milk (5% in 1xTris-buffered saline, TBS) was applied to the membrane for 1 hr at room temperature and the membrane washed with 1xTBS for 5 minutes at room temperature. The membranes were incubated for 1 hr at room temperature with sheep anti-Dig Fab fragments, conjugated to alkaline phosphatase (Roche Diagnostics, German), and diluted 1:100 in 3% bovine serum albumin. Wash three times with 1xTBS, pH 7, each

time for 5 minutes. The alkaline phosphatase substrate was added. The alkaline phosphatase substrate is NBT/BCIP (nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate, thermoscientific). Development of the dark-blue color reaction was allowed to proceed for 20 minutes. The membranes were washed for 5 minutes with 1xTBS buffer, pH 8, air dried, and stored in a polypropylene bag.

3. Development fluorescence *in situ* hybridization *Salmonella* detection in pork product

Fluorescence in situ hybridization (FISH) on slides

FISH assay was performed as previously described by Nordentoft et al., (1997), Vieira-Pinto et al. (2008) with the following modifications.

3.1 Cell fixation

Cells were in an exponential growth phase (OD600=0.5). The cells were harvested by centrifugation (3 minutes, 13000 rpm), and washed twice with 1 ml PBS solution (137mM NaCl, 8.10 mM $Na_2HPO_4.12H_2O$, 2.68 mM KCl, and 1.47 mM KH_2PO_4; pH 7.4), Cells were fixed in 4% paraformaldehyde for 2 hours at 4°C, centrifuged, and washed three times with a PBS solution (137mM NaCl, 8.10 mM $Na_2HPO_4.12H_2O$, 2.68 mM KCl, and 1.47 mM KH_2PO_4; pH 7.4) Cells were then resuspended in 50% ethanol in PBS and stored at -20 °C until further processing.

3.2 Enzymatic permeabilization of fixed cells prior to FISH using fluorescence labeled oligonucleotide

Ten well Teflon slides (Heinz Herenz, Hamburg, German) were used as hybridization supports. The slides were pre-cleaned in ethanol, and were coated with 2% 3-triethoxysilylpropylamine solution (Merck, Darmstadt, Germany) in acetone for 1 minute, twice in acetone for 1 minute and washed in distilled water. (Vieira-Pinto *et al.,* 2008) then, were air-dried and successively dehydrated in 50, 80, 96% (v/v) ethanol (3 minutes each).

Hybridization with Sal3 probe was used as a positive control and to evaluate the influence of different lysozyme concentrations on permeabilization and hybridization efficiency for *Salmonella spp*. and the other bacteria used as the negative controls included *Actinomyces* spp., *Campylobacter jejuni*., *Corynebacterium* spp., *Escherichia coli., Klebsiella* spp., *Pseudomonas* spp., *Staphylococcus aureus*., *Streptococcus agalactiae* and *Streptococcus suis*. Ten microliters of fixed cells were spotted on the well and air dried, after which were dehydrated with ethanol at 50, 80 and 96% levels, during 3 minutes for each concentration. After being air-dried, the permeabilization of the salmonella cell-wall had two different enzymatic pre-treatment protocols tested, employing two different conditions. Firstly, cell smears were covered with 10 µl of different lysozyme solution (0.1, 0.5, 1, 5 and 10 mg/ml Sigma lysozyme in buffer containing 100 mM Tris-HCl, 50 mM EDTA pH 8.0). The temperature and times tested during lysozyme incubation were 25°C for 5 and 20 minutes (Blasco *et al.,* 2003). Secondly, cell smears were covered with 10 µl of different lysozyme at concentrations of 0.1, 0.5, 1, 5 and 10 mg/ml Sigma lysozyme in buffer containing 10 mM Tris-HCl, 5 mM EDTA pH 8.0. Temperatures and times tested during lysozyme incubation were 37 °C for 1, 3 and 5 minutes (Hogardt *et al.,* 2000). Enzymatic treatments were stopped by rinsing the slides thoroughly with 18 MΩ water. Slides were dried and dehydrated by successive immersion in 50, 80, 96% ethanol (3 minutes each).

3.3 Hybridization of Cells and Counting

For whole-cell hybridization, dilution of the Sal3 probe 1:200 in hybridization buffer (0.9M NaCl, 20mM Tris-HCl pH 7.2, 0.1mg/ml poly(A), 5 µg /ml poly(dA), 0.01%SDS). 10 µl of Sal3 probe dilution were added to each hybridization well on Teflon-coated slides. The slides were incubated in a humid hybridization chamber at 45°C for 3 hours. After incubation, the hybridization mixture was gently removed with several millilitres of the washing buffer (0.9 M NaCl, 20mM Tris-HCl pH 7.4, 0.01% SDS). The slides were immersed for 15 minutes in 50 ml of washing buffer. The signal was subsequently detected by incubation with anti-digoxigenin-fluorescein conjugates. (Roche Diagnostics) Dilute this to 1:800 in PBS with 3%

bovine serum albumin in the dark room for 1 hour. After incubation, the slides were washed in buffer solution (0.9 M NaCl, 20mM Tris-HCl pH 7.4, 0.01% SDS) at 46°C for 15 minutes in the dark with agitation, and then rinse with 1X PBS.

The slides were mounted with antifade solution (2.3% DABCO (Sigma), 90% glycerol in PBS). This was applied under a coverslip and immediately viewed with a fluorescence microscope (model Olympus BX51) with a fluorescein filter. Fifteen visual fields were analyzed on each slide. The ranges of fluorescence signal were expressed as the average percentages that are divided into 5 levels: Level 0: Incomplete, the average of the percentage of cells fluorescence signals that was less than 5%. Level 1: Poor, the average percentage of fluorescent cells signal that ranged from 6 to 25%. Level 2: Fair, the average percentages of fluorescent cells signal that ranged from 26 to 50 %. Level 3: Good, the average percentage of fluorescent cells signal that ranged from 51 to 75 %. Level 4: Very good, the average percentage of fluorescent cells signal that ranged from 76 to 100%.

3.4 Detection limit for salmonella detection on pork sample

When the condition of the salmonella cell-wall was appropriate, and the detection limit of FISH procedure was tested on sterile pork (process guarantees that ensuring salmonella-free from charoen pokphand foods company, Thailand). *S.* enteritidis from cells stock were produced with bacteria grown in 5 ml of TSB at 37^{O}C for 16 h. The cells were harvested and diluted in distilled water until the turbidity of the 1 McFarland standards using the McFarland densitometer (Grantbio, U.K.), this resulted in a suspension containing approximately 3×10^{8} CFU/ml. that was diluted in tenfold steps. Then, so spiked with 1 ml from each serial dilutions of *Salmonella enterica* to 25 g of sterile pork. Each sample was mixed with 25 ml of Buffer peptone water (BPW) with 0.1% Tween 80 and homogenized using stomacher (Stomacher 400) at high speed for 90s., the bacteria were collected by filtration onto 33-µm pore-size nylon screen mesh (diameter, 25 mm) and harvested the bacteria suspension by centrifuged at 7000 rpm for 20 minutes, and then collected sample by one milliliter of the mixture was used for determining bacterial counts using plate count agar. To another 1 ml of the mixture was centrifuged for 10 minutes at 13,000 rpm, and cell

pellet was resuspened in 1 ml sterile water that was subjected to detection limit of FISH according to fixed sample and FISH protocol.

3.5 Application of salmonella detection in local pork meat by FISH

A total of 35 samples of pork meat were purchased locally from one commercial abattoir, and stored at -20° C until use. Each sample was split into 2 pieces; 25g of pork meat that hydrated in 25 ml BPW with 0.1% Tween 80, and homogenized using stomacher at high speed for 90s., before bacterial testing was conducted. While, another 25 g of pork is hydrated in 25 ml BPW with 0.1% Tween 80, and homogenized using stomacher at high speed for 90s. The collected meat water 25 ml was filtered with 33-µm pore-size nylon screen mesh and harvested the bacteria suspension by centrifuged at 7000 rpm for 20 minutes. Wash twice with 1 ml PBS, centrifuged at 13000 rpm for 5 minutes and fixed sample, which was subjected to detection according by FISH protocol. After which they were tested by the ISO 6579:2002 microbiological and FISH methods.

3.6 Statistical analysis

Cohen's *Kappa statistic* test performed to determine the agreement between Culture method (ISO 6579: 2002) and FISH technique, demonstrating good inter-rater reliability that were applied by matched samples using WIN EPISCOPE 2.0 software (Win Episcope 2.0). Differences were considered significant at the level of 95% (p-value <0.05).

4. Comparision of conventional dot blot hybridization and signal amplification technology based on catalyzed reporter deposition (CARD) its application in Dot-blot hybridization assay with biotinylated sal3 probe for the detection of Salmonella DNA in samples.

The cells were harvested and diluted in distilled water until the turbidity of the 1 McFarland standards using the McFarland densitometer (Grantbio, U.K.), this resulted in a suspension containing approximately 3×10^8 CFU/ml. that was diluted in tenfold steps. *Salmonella enterica* -positive reference were used undiluted and diluted

up to 10 in all the experiments to analyze the sensitivity of the assay. Each concentration of bacteria sample was performed as DNA extract described previously in step 2.2 and measurement of DNA concentration was determined by UV absorbance analysis wave length 260-280 nm (SmartSpec^{TM} Plus spectrophotometer, BioRAD).

The DNA pellet of each bacterium samples were boiled for 5 minutes at 100 C° and placed on ice for 10 minutes. Three µl volumes of each DNA sample were dotted with a nitrocellulose membrane. After dotting the DNA, the nitrocellulose membrane was allowed to dry at 80 C° for 1 hr in an oven. Of those treated with prehybridization and hybridization solution as mentioned before, CARD-Dot-blot hybridization assay of *Salmonella* DNA was performed on membranes. To improve the comparability to conventional dot-blot hybridization: the sensitivity of dot-blot hybridization assay using tyramide signal amplification (TSA) was compared to dot-blot hybridization assay with indirect detection of biotinylated probe and digoxigenin labeled probe. To detect the low number of bacteria in the sample, this experiment tested types for probe-labeled for dot blot hybridization:

Method A: Alkaline phosphatase detection

Using digoxygenin-labeled probes (labeled with DIG at 3′- tail labeling DIG hybrids) in combination with anti DIG-alkaline phosphatase (AP), detection with NBT/BCIP, labeled and hybridization according to step 2.2 and step 2.4

Method B: Diaminobenzidine detection

Using biotinylate-labeled probe (labeled with biotin at 3′- tail labeling) in combination with streptavidin-HRP, detection with 3,3'-Diaminobenzidine (DAB), labeled and hybridization according to step 2.2 and step 2.4

Method C: Tyramide signal amplification

CARD System, using probe labeled with biotin at 3′-tail labeling biotin hybrids /1^{o}streptavidin-HRP /2^{o}streptavidin-HRP, system tyramide signal

amplification (TSA), detection with DAB, Meanwhile, Biotin labeling of this probe was performed as preparation and synthetic biotinylated probe preparation from Sal3 primer (5′-AATCACTTCACCTACGTG-3′), designed by Nordentoft *et al.,* 1997. Prehybridization and hybridization solutions contained 0.1 mg/ml of poly-A to prevent non-specific binding of the tailed probes.

1. Add 100 pmol of Sal 3 primers and sterile double distilled water to a final volume of 9 µl to a reaction vial.

2. Add the following on ice:

Reaction buffer (vial 1)	volume	4 µl
$CoCl_2$-Solution (vial 2)	”	4 µl
Biotin-16-dUTP solution (vial 3)	”	1 µl
dATP Solution (vial 4)	”	1 µl
400U Terminal transferase (vial 5)	”	1 µl

3. Mix and centrifuge briefly.

4. Incubate at 37^{O}C for 15 minutes, then place on ice.

5. Stop reaction by adding 2 µl 0.2 M EDTA (pH 8.0)

Amplification methods to increase the sensitivity of dot-blotting with biotinylated Sal3 probe. Dot-blot hybridization in this study was modifying procedure of Keller and Manak (1989). Nitrocellulose membrane were sealed in a polypropylene bag with, per cm^2, 200 µl of a prehybridization mixture containing 20xSSC (3M NaCl, 0.3M Na-Critrate), 50x Denhardt's solution, yeast tRNA, 1M $Na_2HPO_4.7H_2O$, 10% Dextran sulfate, 0.1mg/ml poly(A)). The membranes were incubated at 48.5°C for 1 h in water bath. The prehybridization mixture was removed and replaced with mixed biotinylated probe/hybridization buffer solution (1.5 µl: 400 µl), per cm^2 of membrane, 100 µl of hybridization mixture containing (50%formamide, 20xSSC (3M Nacl, 0.3M Na-Critrate), 50x Denhardt's solution, yeast tRNA, 1M $Na_2HPO_4.7H_2O$, 10% Dextran sulfate, 0.1mg/ml poly(A)). The membranes were then incubated in water bath at 48.5°C for 18 to 20 h. The membranes were carefully washed away with consecutive washes in wash buffer 1 (1xSSC, 0.1%SDS, pH 7) at 48.5°C for 5

minutes, wash buffer 2 (0.1xSSC, 0.2%SDS, pH 7) at 48.5°C for 5 minutes and in wash buffer 3 (0.5x SSC, pH 7) at 48.5°C for 1 hr. As a blocking reagent, skim milk (5% in 1xTris-buffered saline, TBS) was applied to the membrane for 1 hr at room temperature and wash membrane with 1xTBS for 5 minutes at room temperature.

Detection of hybridized probes

The membranes were subsequently hybridized with a biotinylated sal3 probe. Signal amplification was performed by consecutive application of streptavidin-HRP complex, biotinyl tyramide, and streptavidin-HRP complex. Visualization of positive hybridization signals was performed by incubation with the chromogenic substrate diaminobenzidine. The presence of hybridization signals within the DNA of *Salmonella* cells was regarded as positive for the presence of *Salmonella*. A schematic representation of the procedure for the Sal3 probe for dot-blot hybridization method is according to the manufacturer's procedure (Figure 8).

Step 1, *Salmonell*a DNA is hybridized with the Biotin-conjugated Sal3 probe.

Step 2, HRP-labelled primary streptavidin is reacted, the primary streptavidin-HRP concentrate should be diluted 1:100 in the primary streptavidin-HRP. Prepare approximately 150µL of primary streptavidin-HRP solution per membranes. After stringent wash, rinse membranes several times in TBS with 0.1% tween 20 wash buffer. Tap off excess TBS with 0.1% tween 20 wash buffer. Apply 3–4 drops of the diluted primary streptavidin-HRP solution to cover the specimen and incubate at room temperature for 15 minutes. Rinse membranes in TBS with 0.1% tween 20 wash buffer and place in three fresh TBS with 0.1% tween 20 wash buffer baths for 5 minutes each to remove residual primary streptavidin-HRP solution.

Step 3, biotinylated tyramine is reacted with HRP in the presence of H_2O_2. Tap off excess TBS with 0.1% tween 20 wash buffer. Apply 3–4 drops of biotinyl tyramide to cover the specimen and incubate at room temperature for 15 minutes. Rinse membranes in TBS with 0.1% tween 20 wash buffer and place in three fresh TBS with 0.1% tween 20 wash buffer baths for 5 minutes each to remove residual biotinyl tyramide solution.

Step 4, HRP-labelled secondary streptavidin is reacted with biotin, Tap off excess TBS with 0.1% tween 20 wash buffer. Apply 3–4 drops of secondary streptavidin-HRP solution to cover the specimen and incubate at room temperature for 15 minutes. Rinse membranes in TBS with 0.1% tween 20 wash buffer and place in three fresh TBS with 0.1% tween 20 wash buffer baths for 5 minutes each to remove residual secondary streptavidin-HRP solution.

Step 5, with detection, HRP-conjugated streptavidin followed by the 3,3′-diaminobenzidine tetrahydrochloride (DAB) reaction is used for light microscopy. Dilute the DAB chromogen concentrate 1:50 in the DAB chromogen. Prepare approximately 150µL of chromogen per membranes. Tap off excess TBS with 0.1% tween 20 wash buffer. Apply 3–4 drops of diluted DAB chromogen to cover the specimen and incubate at room temperature for 5 minutes. The development of a dark-brown coloring is a positive reaction; stop the chromogen reaction by immersing membranes in water for 1 minute. Rinse membranes with several changes of water to remove residual DAB. Finally, the membranes were air-dried at room temperature and stored in a polypropylene bag.

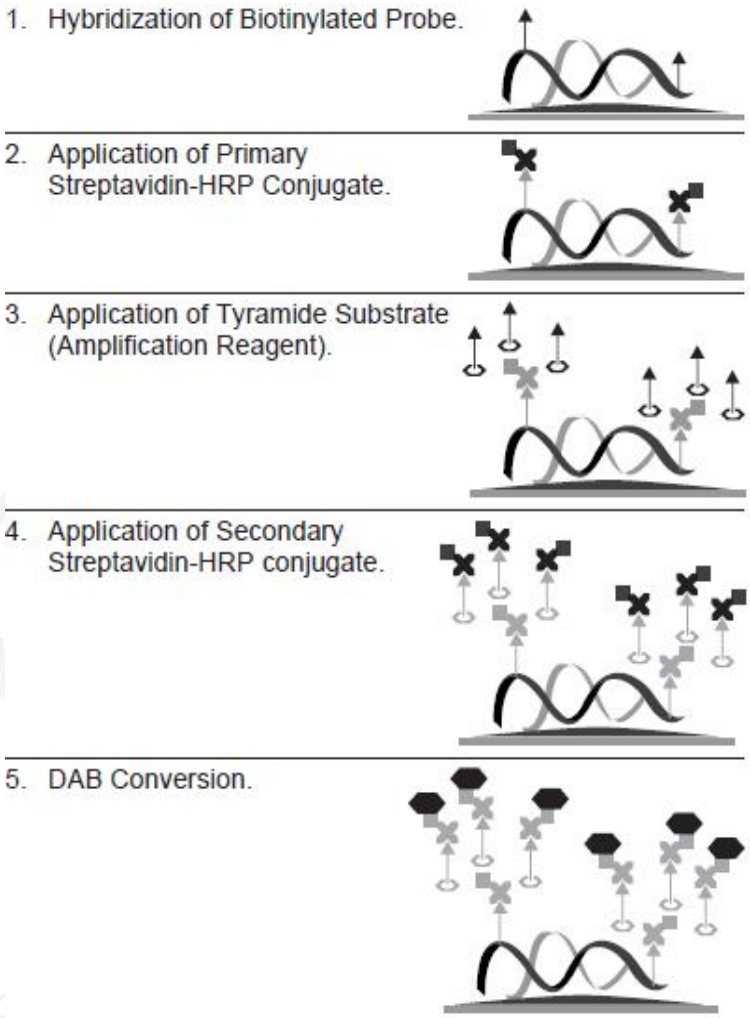


Figure 8 DAKO GenPoint™ procedure**.**

Source: http://www.dako.com/dist/ar42/p107850/prod_products.htm

All methods were developed to detect *Salmonella* DNA. Serial dilutions from Initial concentration~ 3×10^8 cfu/ml up to -8 of dilution factor and each dilution were extracting DNA and measured concentration (Table 10) and then, DNA were dotted 3 µl on nitrocellulose membrane and hybridized with Sal3 probe.

RESULTS AND DISCUSSIONS

Reaults

1. Sequence & Alignments of Sal3 probe

The BLAST network service was used to further determine the uniqueness of the Sal3 probe sequences. Accession numbers and the alignment for the target sequences are given in Figure 9 and Table 6. We found that the Sal3 primers (5′-AATCACTTCACCTACGTG -3′) designed by Nordentoft *et al.* (1997) had specific site of 23S rRNA of *Salmonella enterica* stain, including *S.* typhimurium, *S.* paratyphimurium, *S.* enteritidis. So, these serovars are important to the cause of Salmonellosis in human. The similarity of Sal3 probe compared with three serovars had 100% homology in *Salmonella enteica* nucleotide alignment.

The study of complementary to the region from 23S rRNA positions was selected as being specific for *Salmonella* serovars representing some subspecies (*S.* typhimurium , *S.* paratyphimurium, *S.* enteritidis, *S.* dublin, *S.* agona, *S.* schwarzengrund, *S.* newport , *S.* choleraesuis, *S.* heidelberg and *S. 1,4,[5],12,:i:1,2*) and negative strain (*Actinomyce*s spp., *Campyrobacter jejuni*., *Corynebacterium* spp., *Escherichia coli., Klebsiella* spp., *Pseudomonas* spp., *Staphyrococcus aureus*., *Streptococcus agalactiae* and *Streptococcus suis*) with Clustalw2 program that was present in the following results. The target sequences of all bacteria in this study that reported sequence in the Genbank database, a Sal3 probe, approximately 18 base pairs were matching the sequences of all *Salmonella* subspecies was selected. While, Sal3 probe there were multiple mismatches (at least five mismatches) for bacteria negative strain were selected. A list of the base compositions within the target area is presented in Table 7. Preliminary data from a study which showed that Sal3 probe is specific for *Salmonella enterica* species are found in warm-blooded animals and a group with contamination in pork production industry. Thus, we had chosen to use in the synthesis of probes to test the specificity of the probes, with Salmonella detection in the next step.

Figure 9 Descriptions of Sal3 sequence was aligned with homologous sequences from GenBank database.

Descriptions

Legend for links to other resources: UniGene GEO Gene Structure Map Viewer PubChem BioAssay

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
CP003416.1	Salmonella enterica subsp. enterica serovar Heidelberg str. B182, complete genome	36.2	281	100%	1.1	100%
CP003386.1	Salmonella enterica subsp. enterica serovar Typhimurium str. 798, complete genome	36.2	281	100%	1.1	100%
CP003278.1	Salmonella enterica subsp. enterica serovar Typhi str. P-stx-12, complete genome	36.2	281	100%	1.1	100%
CP003047.1	Salmonella enterica subsp. enterica serovar Gallinarum/pullorum str. RKS5078, complete genome	36.2	281	100%	1.1	100%
CP002614.1	Salmonella enterica subsp. enterica serovar Typhimurium str. UK-1, complete genome	36.2	281	100%	1.1	100%
CP002487.1	Salmonella enterica subsp. enterica serovar Typhimurium str. ST4/74, complete genome	36.2	281	100%	1.1	100%
FR775243.1	Salmonella enterica subsp. enterica serovar Weltevreden str. 2007-60-3289-1 complete genome, contig 56	36.2	36.2	100%	1.1	100%
AP011957.1	Salmonella enterica subsp. enterica serovar Typhimurium str. T000240 DNA, complete genome	36.2	281	100%	1.1	100%
FQ312003.1	Salmonella enterica subsp. enterica serovar Typhimurium SL1344 complete genome	36.2	281	100%	1.1	100%
HM007612.1	Salmonella enterica subsp. enterica serovar Typhimurium strain 5275 23S ribosomal RNA gene, partial sequence	36.2	36.2	100%	1.1	100%
HM007608.1	Salmonella enterica subsp. enterica serovar Enteritidis strain 13 23S ribosomal RNA gene, partial sequence	36.2	36.2	100%	1.1	100%
CP001363.1	Salmonella enterica subsp. enterica serovar Typhimurium str. 14028S, complete genome	36.2	281	100%	1.1	100%
FN424405.1	Salmonella enterica subsp. enterica serovar Typhimurium str. D23580 complete genome	36.2	281	100%	1.1	100%
CP000857.1	Salmonella enterica subsp. enterica serovar Paratyphi C strain RKS4594, complete genome	36.2	281	100%	1.1	100%
AE006468.1	Salmonella enterica subsp. enterica serovar Typhimurium str. LT2, complete genome	36.2	281	100%	1.1	100%
AM933172.1	Salmonella enterica subsp. enterica serovar Enteritidis str. P125109 complete genome	36.2	281	100%	1.1	100%
AM933173.1	Salmonella enterica subsp. enterica serovar Gallinarum str. 287/91 complete genome	36.2	281	100%	1.1	100%
CP001144.1	Salmonella enterica subsp. enterica serovar Dublin str. CT_02021853, complete genome	36.2	281	100%	1.1	100%
CP001138.1	Salmonella enterica subsp. enterica serovar Agona str. SL483, complete genome	36.2	281	100%	1.1	100%
FM200053.1	Salmonella enterica subsp. enterica serovar Paratyphi A str. AKU_12601 complete genome, strain AKU_12601	36.2	281	100%	1.1	100%
CP001127.1	Salmonella enterica subsp. enterica serovar Schwarzengrund str. CVM19633, complete genome	36.2	275	100%	1.1	100%

Table 6 A lists of the base compositions within 23S rRNA genes of all bacteria in this study

Probe or species and serovar (subsecies)	GeneBank accession	Nucleotide sequences	region gene
Sal3 probe		**3'gtgcatccacttcactaa5'**	
Salmonella enterica		**5'cacgtaggtgaagtgatt3'**	23S rRNA
serovar Paratyphi A	FM200053.1	… … … … … …	23S rRNA
serovar Paratyphi B	CP000886.1	… … … … … …	23S rRNA
serovar Paratyphi C	CP000857.1	… … … … … …	23S rRNA
serovar Typhimurium	AE006468.1	… … … … … …	23S rRNA
serovar Enteritidis	AM933172.1	… … … … … …	23S rRNA
serovar Dublin	CP001144.1	… … … … … …	23S rRNA
serovar Agona	CP001138.1	… … … … … …	23S rRNA
serovar Schwarzengrund	CP001127.1	… … … … … …	23S rRNA
serovar Newport	CP001113.1	… … … … … …	23S rRNA
serovar Choleraesuis	AE017220.1	… … … … … …	23S rRNA
serovar Heidelberg	CP001120.1	… … … … … …	23S rRNA
serovar 1,4,[5],12,:i:1,2	U77920.1	… … … … … …	23S rRNA
Campyrobacter jejuni	AY249920.2	…a.c.ac.gg.a.g..	23S rRNA
Pseudomonas aeruginosa	Y00432.1	……a….c.c.g.a	23S rRNA
Staphyrococcus aureus	X68425.1	.t.aa..a…t.a.g..	23S rRNA
Actinomyces suis	M85092.1	..a.a- - - - t…….c.tgt [a]	23S rRNA
Klebsiella pneumoniae	X87284.1	ag…c…a.g…..a	23S rRNA
Streptococcus suis	AY585198.1	….c…a.- - c..c.g.. [a]	16S-23S rRNA
Corynebacterium kutscheri	AF536505.1	.g..ct.a.t.ca…a.	16S-23S rRNA
Streptococcus agalactiae	L31412.1	….c…a.- - c..c.n.. [a]	16S-23S rRNA
Escherichia coli	EF527445.1	….at……tc.att	16S-23S rRNA

[a.] _ , gap in the sequence made by a deletion in the gene.

2. The evaluation of specificity of Sal3 probe

To verify the specificity and availability of the probe was tested by hybridization with Dot-blot hybridization assay (Figure 10, 11, 12 and 13). For this study, Sal3 probe was tested against *Salmonella* DNA 60 serovars. By using stringency hybridization and washing conditions, the probe hybridized and yielded a strong signal for 60 *Salmonella* tested (Table 8). No cross hybridization was observed to any of 9 other representatives of negative bacteria which might occur in the intestinal tract of pigs and humans. According to predicted from the alignment of the sequence in the target area (Table 7), the probe hybridized to the sequences of the tested serovars of subspecies. No hybridization was obtained with the nine strains of negative control, which all had multiple mismatches.

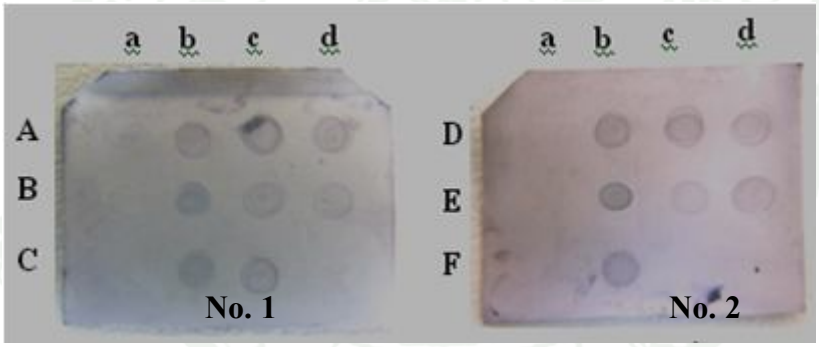


Figure 10 Dot-blot hybridization assay of *Salmonella* DNA on Nitrocellulose membrane no. 1 and no 2.

No 1:- A a: *Actinomyces* spp., **B a:** *Pseudomonas aeroginosa*., **C a:** no DNA**., A b:** *S. stanley*.,**B b:** *S. rissen*., **C b:** *S. lexington*., **A c**:*S. kedougon*.,**B c:** *S. worthington*.,**C c:***S. montevideo.,* **A d:** *S. I 4,5,12: i:- .,* **B d:** *S. anatum* **.,** ;**C d:** no DNA

No 2:- D a: *Klebsiella* spp., **E a:** no DNA., **F a:** no DNA**., D b:** *S. paratyphimurium B***.,** **E b:** *S. i4,12: i-;* **F b:** *S. bangkok*., **D c**: *S. thompson*., **E c:** *S. senftenberg*., **F c:** no DNA*.,* **D d:** *S. soerenga*., **E d:** *S. panama*., **F d:** no DNA

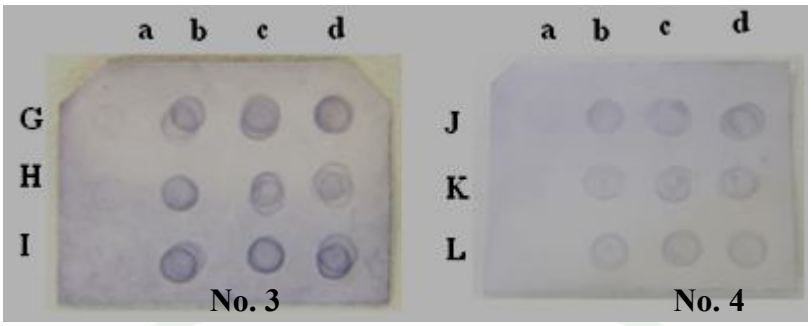


Figure 11 Dot-blot hybridization assay of *Salmonella* DNA on Nitrocellulose membrane no. 3 and no 4.

No. 3:- G a: *Escherichia coli*., **H a:** *Campyrobacter jejuni*., **I a:** no DNA**.,** **G b:** *S. corvalis.,* **H b:** *S. newport***.,** **I b:** *S. orion*., **G c**: *S. welterreden*., **H c:** *S. give*., **I c:** *S. albany.,* **G d:** *S. livingstone.,***H d:** *S. kentuckey* **.,** ;**I d:** *S. bovismorbificans*

No. 4:- J a: *Corynebacterium* spp., **K a:** no DNA., **L a:** no DNA**., J b:** *S. I4,12:d:-***., K b:***S. typhimurium-;* **L b:** *S. mbadaka*., **J c**: *S. enteritidis*., **K c:** *S. blockley*., **L c:** *S, hadar.,* **J d:** *S. schwarzengrund*., **K d:** *S. virginia*., **L d:** ***S.*** *virchow*

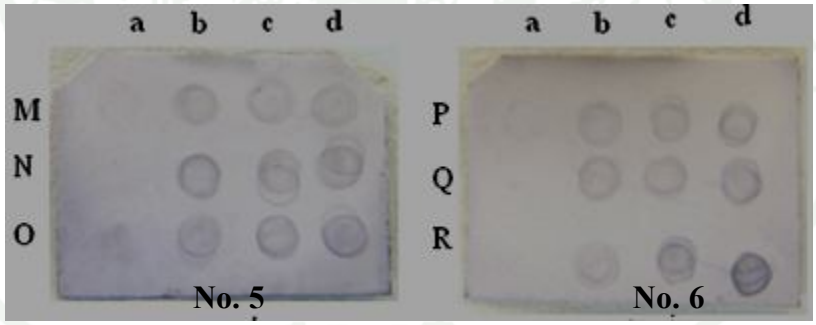


Figure 12 Dot-blot hybridization assay of *Salmonella* DNA on Nitrocellulose membrane no. 5 and no. 6

No. 5:- M a: *Staphyrococcus aureus*., **N a:** no DNA., **O a:** no DNA**., M b:** *S. aberdeen.,* **N b:** *S .cerro***., O b:** *S. cholearasuis*., **M c**: *S. lodon*., **N c:** *S. waycross*.,m **O c:** *S. tennessee.,* **M d:** *S. ohio.,***H d:** *S. emek* **.,O d:** *S. Amsterdam*

No. 6:- P a: *Streptococcus agalactiae*., **Q a:** no DNA., **R a:** no DNA**., P b:** *S. braenderup***., Qb:***S. minisota-;* **R b:** *S. bredeney*., **P c**: *S .djakata*., **Q c:** *S. dublin*., **R c:** *S, heidelbreg.,* **P d:** *S. krefeld*., **Q d:** *S. alachua*., **R d:** ***S.*** *bergen*

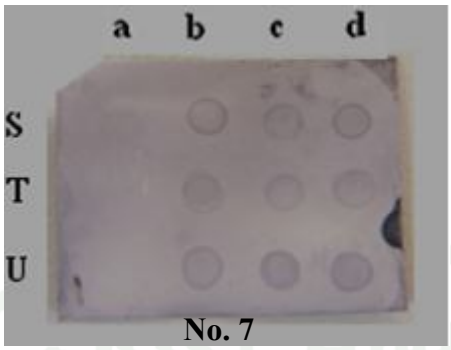


Figure 13 Dot-blot hybridization assay of *Salmonella* DNA on Nitrocellulose membrane no. 7

No. 7:- S a: *Streptococcus suis*., **T a:** no DNA., **U a:** no DNA**., S b:** *S. chicago.,* **T b:** *S .derby***., U b:** *S. fresno*., **S c**: *S. gallinarum*., **T c:** *S. hvittingfoss*., **U c:** *S. onderstepoort.,* **S d:** *S. pomona.,***T d:** *S. urbana* **.,U d:** *S. pullurum*

Table 7 The specificity of Sal3 probe for Salmonella detection with Dot-blot hybridization assay

Sample	Examine	Detected Dig signal[a]	Not detected Dig signal[a]	Rate of detected Dig signal[a] (%)
Salmonella spp. (+)	60	60	0	100
Actinomyces spp(-)	1	0	1	-
Campyrobacter jejuni (-)	1	0	1	-
Corynebacterium spp (-)	1	0	1	-
Escherichia coli (-)	1	0	1	-
Klebsiella spp (-)	1	0	1	-
Pseudomonas spp (-)	1	0	1	-
Staphyrococcus aureus (-)	1	0	1	-
Streptococcus agalactiae (-)	1	0	1	-
Streptococcus suis (-)	1	0	1	-

[a.]Digoxigenin

From table 8 shown Sal3 probe labeled at 3′-end with terminal transferase was specific to *Salmonella* DNA 60 serovars (100%) and No cross hybridization was observed to any of 9 other representatives of negative bacteria.

3. Development fluorescence *in situ* hybridization *Salmonella* detection in pork product

The results of this, FISH experiment using an indirect oligonucleotide (Sal3 probe) was enzymatically labeled at the 3'- end with terminal transferase. The preliminary study, the result of FISH experiments using Sal3 probe labeled at 3'end with terminal transferase, since *Salmonella* cells gave a weak FISH signal (Figure 13C). *Salmonella* is a gram-negative bacterium that is usually permeated by fluorescence-labeled at 5'-end of oligonucleotide probes (single fluorescent per ribosome) without permeabilization step (Nordentoft *et al.,* 1997; Vieira-Pinto *et al.,* 2008). But, Sal3 probe labeled at 3'end with terminal transferase that have large antibody-enzyme complexes and require the diffusion of this probe access to the target molecule. So, the permeabilization step of fixed bacterial cell walls is essentially important for when large probes labeled with high-molecular-weight molecules such as enzymes, antibodies, or (strept)avidin in to fixed whole cells (Zarda *et al.,* 1991, Bidnenko *et al.,* 1998), while minimizing the loss of the target molecule and cell morphology (Furukawa *et al.,* 2006). Labeling probe with multiple molecules of fluorochrome greatly enhances the intensity of positive signal and may be helpful to separate microorganisms from samples (Örmeci & Karl, 2008).

Optimization of permeabilization conditions

Developments of whole-cell detection for *Salmonella* spp., the optimum condition of cell pretreatment for FISH were investigated shown in figure 14. The standard cell permeabilization protocol uses lysozyme. This enzyme may not be effective for microorganisms with unusual cell wall structure (Ishii *et al.,* 2004). The enzyme concentration was varied different reagents composition; different temperature and time of enzymatic incubation were examined in order to allow the permeation of oligonucleotide probe into the cells.

The highest fluorescence signal was observed when cell walls were made permeable with lysozyme concentration. The optimal conditions, first condition; To open the cell walls made lysozyme permeable in buffer containing 100 mM Tris-HCl, 50 mM EDTA pH 8.0 at 25° C for 5 minutes showed that the concentration of 0.1 mg / ml of lysozyme concentration that has a average of the fluorescence signal was 0.73 (18.25%), classified fluorescence signal were emitted at level 2 (Figure 14D). While, in the open cell walls at 25° C for 20 minutes at all concentration of lysozyme were not signals or weak signals (Figure 14I).

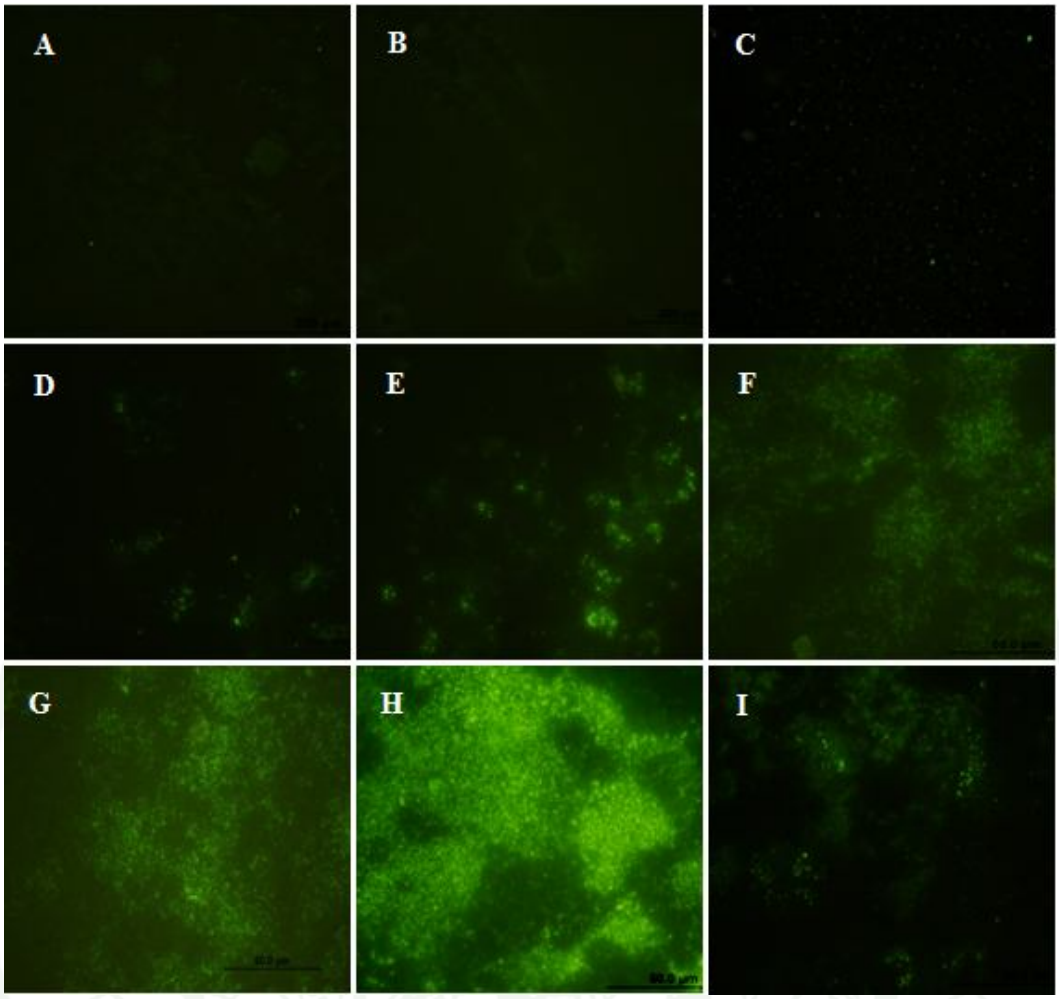


Figure 14 The result of opening the cell walls, permeable with lysozyme. Image of all at magnification ×400.

A: *Escherichia coli*, B: *Staphylococcus aureus*. The ranges of fluorescence signal were emitted as the average percentages that classified into 5 levels. The signal intensities was obtained from *Salmonella enterica* that below, C: no fluorescence signal emitted, D: level 0, fluorescence signals was less than 5%, E: level 1, fluorescence signals was ranged from 6 to 25%, F: Level 2 fluorescence signals was ranged from 26 to 50%, G: Level 3, fluorescence signals was ranged from 51 to 75%, H: Level 4, fluorescent cells signal was ranged from 76 to 100% and I: image of fluorescence signal of *Salmonella* cells that extended lysozyme treatment. Until, the cells can often display a diffuse appearance, suggesting the loss of cell structure and leakage of cell content including rRNA.

Second condition, to open the cell walls to make lysozyme permeable put in buffer containing 10 mM Tris-HCl, 5 mM EDTA pH 8.0 at 37 °C for 1, 3 and 5 minutes. this showed stronger signal intensity than cells treated with first condition. *Salmonella enterica,* cell walls required 1 mg/ml for 3 minutes, showed an average of the fluorescence signal was 3.67 (91.75%). *Salmonella typhimurium*, cell walls required 0.5 mg/ml for 3 minutes, showed an average of the fluorescence signal was 3.30 (82.5%). While, *Salmonella paratyphimurium*, cell walls was required 1 mg/ml for 3 minutes showed an average of the fluorescence signal 3.30 (83.25%). Figure 15D, 15E and 15F respectively. Incubation times depended on the temperature. The best results were achieved with 1 mg/ml of lysozyme permeable in buffer containing 10 mM Tris-HCl, 5 mM EDTA pH 8.0 at 37° C for 3 minutes, this condition is suitable for three serotype of *Salmonella*.

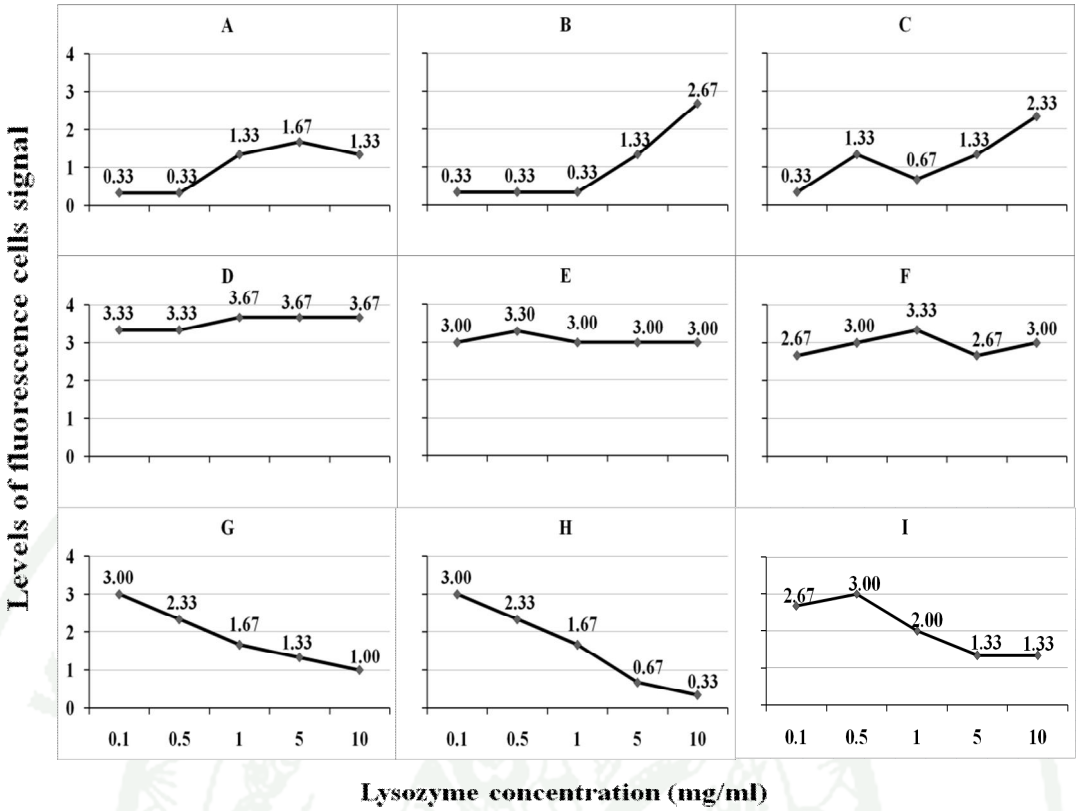


Figure 15 Optimal Lysozyme and temperature (37° C) to obtain the levels of fluorescence cells signal.

[A], [D] and [G] that mean *Salmonella enterica,* temperature difference for lysozyme permeable at 1, 3 and 5 minutes.

[B], [E] and [H] that mean *Salmonella typhimurium*, temperature difference for lysozyme permeable at 1, 3 and 5 minutes.

[C], [F] and [I] that mean *Salmonella paratyphimurium*, temperature difference for lysozyme permeable at 1, 3 and 5 minutes.

Detection of *Salmonella* by FISH in spiked sterile pork samples

The expected Sal3 probe that produces labeled oligonucleotide is to do 3′-tailing. After, *Salmonella enterica* was spiked onto pork meat at levels of 3×10^8 cfu/ml. By application the Sal3 probe with a FISH system in pork samples that were able to reliably detect *Salmonella* spp. In spiked samples at low levels contamination 3×10^7 cfu/ml was possible without pre-enrichment (Table 9). While, the low number

of *S.* enterica presented in pork samples artificially inoculated as well as the presence of debris in the suspension was responsible for occurrence of weak signals or false negative results. Thus, this FISH-experiment should not be considered a "culture-independent" procedure, with detection of the lower number of bacteria samples than at 3×10^6 cells/ml.

Table 8 Results of fluorescence *in situ* hybridization on artificially contaminated pork samples as a function of contamination dose

Inoculums(cfu/ml)	**Artificial contaminated pork sample**						
	3×10^8	3×10^7	3×10^6	3×10^5	3×10^4	3×10^3	3×10^2
observation	(+)	(+)	(+/-)[a]	(-)	(-)	(-)	(-)

[a]Unambiguous (+/-) when microscopic fields (magnification: 400×) with "*Salmonella*-positive" cells were rarely found and contained less than 10 cells.

***In situ* detection of *Salmonella* in pork samples of slaughterhouse by FISH in comparison with culture method**

To evaluate the practicability of FISH for detection of *Salmonella* from naturally contaminated pork samples, 35 samples were sampled and investigated by culture method and by FISH (Table 10). FISH was performed with a set of Sal3 probe specific for *Salmonella* commonly isolated pork samples from one local slaughterhouse of Nakorn Pathom province in Thailand. The FISH allowed the detection of *Salmonella*, appearing as fluorescence signals within the cells in approximately 8 hr. Results of the data showed that 29 of 35 tested samples (82.85%) that were culture positive, and this study found 30 samples (85.71%) with *Salmonella* using the FISH. The results were summarized in Table 2. In this study, moderate levels of sensitivity (93.5%) and specificity (66.6%) were achieved by FISH compared with culture.

Table 9 *Salmonella* spp. Detection on fresh pork samples by FISH and by the ISO6579:2002.

Results of standard culture	**Results of FISH**		
	FISH(+)	**FISH(-)**	**Total**
Culture(+)	27	2 [FN]	29
Culture(-)	3 [FP]	3	6
Total	30	5	35
kappa value of FISH compared with ISO6579:2002			0.46

[FN] false negative
[FP] false positive

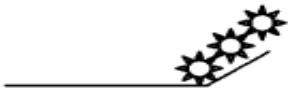
4. Signal amplification technology based on catalyzed reporter deposition (CARD) and its application in Dot-blot hybridization assay with biotinylated sal3 probe for the detection of Salmonella DNA in samples: a potent method to increased sensitivity of DNA hybridization.

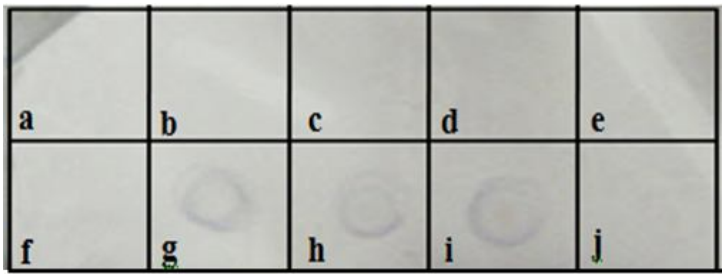
Dot-blot hybridization methods in this study, these were using 3 amplification. Method A, conventional dot hybridization with labeled DIG at 3′- tail labeling DIG hybrids/ anti DIG-AP, detection with NBT/BCIP. Method B, conventional dot hybridization with probe labeled with biotin at 3′- tail labeling biotin hybrids / streptavidin -HRP, detection with DAB. Method C, CARD-dot hybridization: probe labeled with biotin at 3′-tail labeling biotin hybrids / 1°streptavidin-HRP /2°streptavidin-HRP, system tyramide signal amplification (TSA), detection with DAB. All methods were developed to detect *Salmonella* DNA. Serial dilutions from Initial concentration~ 3×10^8 cfu/ml up to -8 of dilution factor and each dilution were extracting DNA and measured concentration (Table 11) and then, DNA were dotted 3 µl on nitrocellulose membrane and hybridized with Sal3 probe.

Table 10 The correlation of *Salmonella*-cell dilution factors and *Salmonella*-DNA concentration.

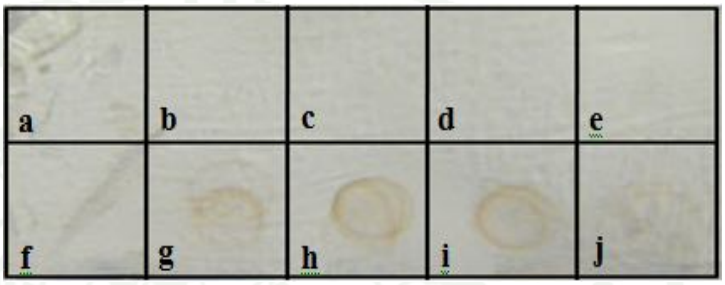
Dilution factor	Number of Salmonella cell	20 µl of DNA extraction, concentration (µg/ml)	3 µl of DNA concentration were dotted on membrane (µg/ml)	Expected cell concentration (cfu/ml)
	3×10^{8}	199.38	29.9	$\sim3\times10^{3}$
-1	3×10^{7}	192.45	28. 86	$\sim3\times10^{2}$
-2	3×10^{6}	176.8	26.52	$\sim3\times10^{2}$
-3	3×10^{5}	49.86	7.47	< 3
-4	3×10^{4}	42.5	6.37	< 3
-5	3×10^{3}	29.24	4.38	< 3
-6	3×10^{2}	18.1	2.71	< 3
-7	3×10^{1}	15.5	2.32	< 3
-8	3×10^{0}	14.85	2.22	< 3

A). System probe labeled DIG at 3′- tail labeling DIG hybrids/ anti DIG-AP, detection with NBT/BCIP.

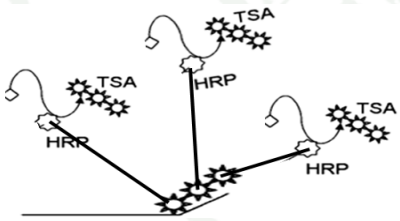




B). System probe labeled with biotin at 3′-tail labeling biotin hybrids / streptavidin-HRP, detection with DAB.



C). System probe labeled with biotin at 3′-tail labeling biotin hybrids /1°streptavidin-HRP /2°streptavidin-HRP, + system tyramide signal amplification (TSA), detection with DAB.



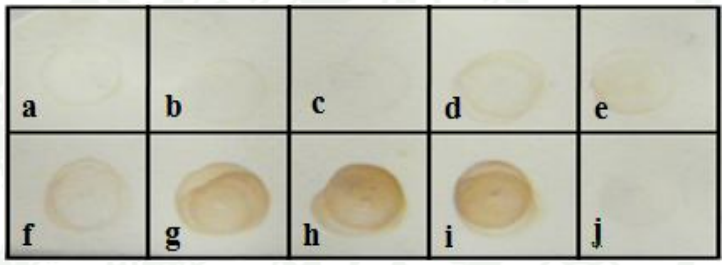


Figure 16 Dot-blot hybridization of *Salmonella*-DNA to Sal3 probe. Salmonella DNA concentration (µg/ml): a= 2.22, b= 2.32, c= 2.71, d= 4.38, e= 6.37, f= 7.47, g= 26.52, h= 28.86, i= Initial concentration~29.9, j= *Escherichia coli* (negative)

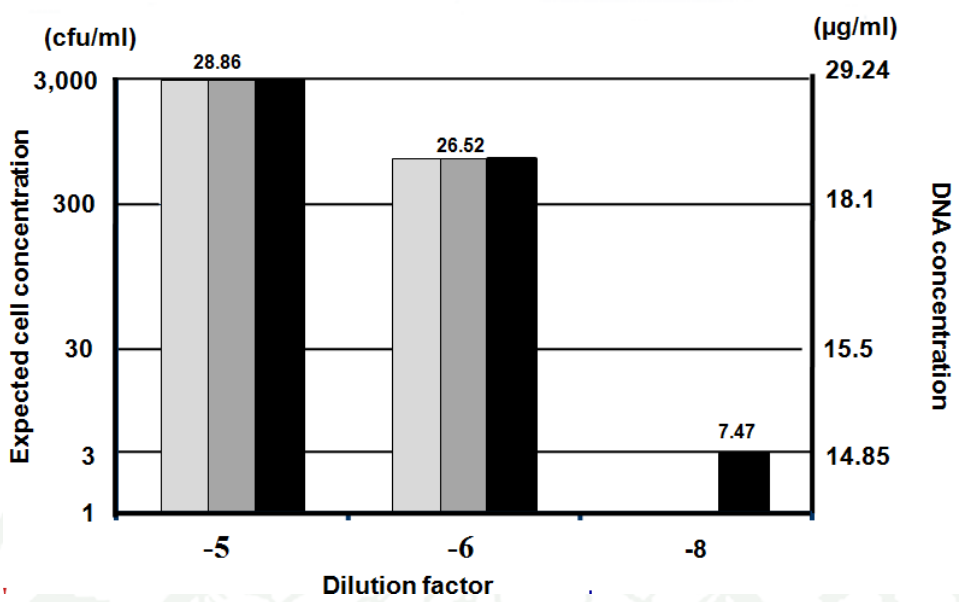


Figure 17 Correlation of the number of epected cell concentration with corresponding recovery rate from dilution factor and the DNA concentration extracted from the initial concentration of the dilution series

The analytical sensitivity of dot-blot hybridization method was determined using serial dilutions of *Salmonella* cells, although positive signals were detectable at each location of *Samonella* DNA. The figure 16 showns 3 methods of probes labeled. Since, method C) or TSA detection method of biotinylated hybrids had a highest sensitivity of the hybridization assay for a well-defined dark-brown positive reaction with no background color, the positive signal that was obtained when a dilution factor of -8 (Figure 16C and Figure 17) or corresponding 7.47 µg/ml of *Salmonella* DNA, it was even possible to detect < 3 cfu/ml in sample (expected cell concentration, table 11) and the number of bacterial cells to detect less than method A and B. While a dilution factor of -7 of *Salmonella* DNA were visualized when the indirect detection of digoxigenin probe by anti-digoxigenin–alkaline phosphatase (AP) conjugate was used (Figure 16A and Figure 17), and indirect detection of biotinylated probe by streptavidin – horseradish peroxidase (HRP) conjugate was used (Figure 16B and Figure 17) that both conventional dot bot hybridization that were able to detect 26.52 µg/ml of *Salmonella* DNA, it was even possible to detect at 300 cfu/ml in sample.

Discussion

The presence of viable Salmonella bacteria in any foods is considered significant and hence, legislation often requires the absence of Salmonella in 25 g of processed food (Roberts *et al.*, 1995). A shortcoming of the conventional culture technique is the total test time required to detect these pathogenic bacteria (72±96 h). This delay has prompted a considerable effort to develop alternative methods to reduce the time of detection of presumptive *Salmonella* bacteria.

1. Sal3 probe

The present study using the Sal3 probe, a specific oligonucleotide probe was developed for hybridization methods (Nordentoft *et al.,* 1997). Section of probe sequence was based upon alignment of a sequence in the 23S rRNA gene from several *Salmonella* serotypes and other bacterial strains. Sal3 probe is located at helix 63 of the 23S rRNA gene (Nordentoft *et al.,* 1997). This segment contains two regions which have been shown to exhibit high conservation of sequence variability in bacteria (Christensen *et al.,* 1994, Van Camp *et al.,* 1993). From the the study of Nordentoft *et al.* (1997) refer, the separation of *Salmonella* serovars into seven homology groups or subspecies has been found when comparing 23S rRNA gene sequences with Sal3 probe (DNA-DNA hybridization studies); the variations were low within genes from serovars of the same subspecies, but they were three times higher when serovars from different subspecies were compared. Which, the 23S rRNA sequence is highly conserved within each subspecies.

Results of the dot blot hybridization experiments are summarized in Table 8. Sal3 probe was specifically hybridized with strains of *Salmonella enterica* subspecies I because it would not discriminate between strains and this allows for the specific detection of the *S. enterica* 60 serovars but does not allow for cross hybridization to any of the negative bacteria. These results corresponds the study FISH experiment of Nordentoft *et al.* (1997), who found the specificity of the Sal3 probe was tested against 55 serovars representing all seven subspecies that showed a high degree of

conservation in the probe target area, because the probe hybridized only to the subspecies I, IIIb, VI and including subspecies II and IV that had a single mismatch. However, Sal3 probe failed to hybridize some strains that had contained two mismatches instead of one. Shows that, a Sal3 probe was specific for all serovars from *S.* enterica species, especially subspecies I constituted the most commonly encountered and the most pathogenic serovars. This was consistent with the results of this experiment which showed Sal3 probe, with included 100% complementary with 23S rRNA of *S.* enteritidis*, S.* typhimurium *and S.* paratyphimurium and all *Salmonella* subspecies were selected (Table 6). Meanwhile, 18 base pairs of Sal3 probe were matching the sequences of all *Salmonella* subspecies that were selected. With the Sal3 probe there were failed to hybridize negative bacteria (at least five mismatches). So, this experiment was selected Sal3 probe was enzymatically labeled at the 3'- end with terminal transferase for hybridization technique. In addition, the use of short oligonucleotide probes reduces the time and temperature required for hybridization. By increasing the probe concentration the velocity of the hybridization is further increased (Nordentoft *et al.,* 1997).

2. Development fluorescence *in situ* hybridization *Salmonella* detection in pork product

FISH has already been successfully implemented for the rapid detection and differentiation of various food pathogens (Vieira-Pinto *et al.,* 2008, Schmid *et al.,* 2005, Rathnayaka, 2011). This study performed a FISH experiment using an indirect oligonucleotide (Sal3 probe) was enzymatically labeled at the 3'- end with terminal transferase, as a result of this oligonucleotide labeled on average, the tail of probe is 40-50 nucleotides in length with several DIG-molecules incorporated that is a more increased signal intensity as compared to a directly conjugated probe (Deere *et al.,* 1998). The preliminary study, the result of FISH experiments using Sal3 probe labeled at 3′end with terminal transferase without permeabilization step, since *Salmonella* cells gave a weak FISH signal (Figure 14C). As 3′- tail labeling DIG has more molecules-number than 5′- labeled with DIG, because large probes can be labeled with a much larger number of chemically tagged bases than small

oligonucleotide probes. This implies that significantly fewer labeled bases are added than with larger probes, thus reducing the relative sensitivity of in situ hybridization, while, the small size of oligonucleotide probes have the advantage of better penetration into fixed tissue and cell matrices than larger RNA probes, making the sensitivity of detection less dependent on penetration problems caused by probe fragment size (Trembleau *et al.,* 1990). Therefore, *Salmonella* cells must be permeabilized by incubating the sample with enzymes such as lysozyme prior to FISH. But, this permeabilization step might cause leakage of ribosomes and rRNA precursor molecules from the cells. To check this, by optimization of permeabilization conditions that is suitable for *Salmonella* cells.

First condition, to open the cell walls covered with different lysozyme solution (0.1, 0.5, 1, 5 and 10 mg/ml) in buffer containing 100 mM Tris-HCl, 50 mM EDTA pH 8.0). The temperature and times tested during lysozyme incubation were 25°C for 5 and 20 minutes (Blasco *et al.,* 2003), shown were not signals or weak signals (Figure 14D and Figure 14I). The two reasons for why a fluorescence signal cannot emit in first condition. Because, appears these could occur from a low penetrating efficiency of antibody or enzyme such as anti-DIG antibody (Furukawa *et al.,* 2006) or an extended lysozyme treatment can result in the hybridization of all of the cells but, as a drawback, the cells can often display a diffuse appearance, suggesting the loss of cell structure and leakage of cell content including rRNA (Bottari *et al.,* 2009). Therefore, this condition was not suitable for *Salmonella* cell walls, because the cell walls had been damaged or rRNA targets of *Salmonella* released from the cell walls. In contrast, the second condition, to open the cell walls, make 1 mg/ml of lysozyme permeable in buffer containing 10 mM Tris-HCl, 5 mM EDTA pH 8.0 at 37 °C for 3 minutes (Hogardt *et al.,* 2000) that is suitable for *S.* enterica*, S.* typhimurium and *S.* paratyphimurium, was validated by comparing both consensus and optimal fluorescence *in situ* hybridization conditions with a specific Sal3 probe to negative controls. Results showed that *Salmonella* specific identifications were possible without losing a significant signal, and no cross-reactions were observed for negative controls (Figure 14A and 14B). Three *Salmonella* strains for this tested showed no

autofluorescence and yielded strong hybridization signals as shown on Figure 14H. So, this last condition was chosen in order to reduce the experimental time.

Detection of *Salmonella* by FISH in spiked sterile pork samples

The expected Sal3 probe that produced labeled oligonucleotide is to do 3′-tailing. After, *S. enteritidis* was spiked onto pork. The results were able to reliably detect *Salmonella* spp. in spiked samples at low levels contamination 10^7 cfu/ml that was possible without pre-enrichment (Table 9). In addition, Stender *et al.* (2001) refers to the existence of low numbers of bacteria in the sample, might limit the detection by microscopic evaluation, since only a small part of specimen is viewed, but became evident after additional enrichment. These explanations might justify the existence of the *S.* enteritidis showing contamination levels lower than 3×10^6 cfu/ml onto pork samples could not be by FISH analysis without prior cultivation. This corresponds with the study FISH experiment of Schmid *et al.* (2005) who found a pre-enrichment step prior for *Campyrobacter* spp. during 3 hr. that should be included to improve detection limit method at levels of 10^6 cfu/g chicken feces. While, low level contamination (10^2 cfu/g chicken feces) was reliably identified after 24 hr of enrichment. So, the low number of *S.* enteritidis presented in the pork sample artificially inoculated as well as the presence of debris in the suspension was responsible for occurrence of a weak signal or false negative results. Thus, this FISH-experiment should not be considered a "culture-independent" procedure, when detection of the lower number of bacteria samples than at 3×10^6 cells/ml.

***In situ* detection of *Salmonella* in pork samples of slaughterhouse by FISH in comparison with culture method**

Preliminary experiments on pork samples obtained from pig from a slaughterhouse at Nakhon Pathom province with the cultural and FISH method, *Salmonella* spp. could even be detected without pre-enrichment, indicating high level shedding of *Salmonella* in pig ($\geq10^7$ cfu/ml). From previous studies, Sal3 probe has been in development and application for *Salmonella* detection in food samples, waste

water and animals live in the water ecosystem by *in situ* hybridization method, for example FISH method (Nordentoft *et al.,* 1997; Fang *et al.,* 2003; Hahn *et al.,* 2006; Vieira-Pinto *et al.,* 2005, 2008; Örmeci *et al.,* 2008, Rathnayaka, 2011) and dot-blot hybridization method (Chunchom *et al.,* 2009). The authours found Sal3 probe has specificity to the serotypes of *S.* enterica species. While, this serotype restriction include that serovars stick are regulated for food safety in Thailand such as *S.* Enteritidis*, S.* Paratyphimurium and *S.* Typhimurium (Chunchom *et al.,* 2009). However, in comparison to the FISH method, the culture method allowed for the recovery of *Salmonella*. Three samples of false positive results from fluorescence *in situ* hybridization were explained by existence of injured cells that may have occurred by different physical and chemical properties of bacteriological culture process ie. temperatures, nutrient concentration, salinity, osmotic pressure, freezing and low pH (pH 3.3). Bacteria pathogens enter into viable but non-culturable state during exposure to a new environment, environmental changes such as culture media (Colwell, 2000; Fang *et al.,* 2003), which FISH experiment in this study that *Salmonella* cells contaminated in pork, they may be stressed and dormant but not dead, and may still be pathogenic, but not be growing on cultivable media. Corresponding to previous FISH experiments, efficiency of FISH experiment could be detected by rRNA of *Salmonella* in viable but non culturable state that still a metabolic activity with a sufficient number of ribosomes detectable by hybridization with Sal3 probe (Vieira-Pinto *et al.,* 2008). Therefore, it is difficult to distinguish viable but non cultural state cells by culture method (Prescott and Fricker, 1999), but FISH targets rRNA could be detection and enumeration of viable bacteria cells is important for the food industry (Ootsubo *et al.,* 2003), which is in fact one of the main advantages of FISH method when compared with the culture method (Blasco *et al.,* 2003, Fang *et al.,* 2003). These characteristics contribute to the success of the implementation of FISH to food microbiology (Vieira-Pinto *et al.,* 2008). In addition, the studies of Fang *et al.* (2003) and Moreno *et al.* (2001) who refers to false positive results may be due to the detection of rRNA from bacteria are just recently dead cells. Which, in this case can prevent false positive results by increasing the pre enrichment step at 37^{o}C after collected samples. This above step will help speed up of the rapid RNA degradation of dead cells that are in the state of inactivated cells for FISH

method. As all reasons above, this FISH experiment has resulted in the low specificity, when compared with the culture method. So, the detection of *Salmonella* spp. cells among the high level competitive microorganisms is possible through this FISH experiment.

Two samples of false negative results from FISH experiment, but identified by the culture method may be associated with influence of the targets visualization of FISH experiment or a small number or insufficient accessibility of target molecules is present (Amann *et al.,* 1995), especially when the microbial bacteria has a low number of bacterial organism in the samples that do not have enough rRNA targets for FISH detection (Amann *et al.,* 1995; Fang *et al.,* 2003). Since, this is only a small part of rRNA in a specimen that might limit FISH detection by view under microscopic evaluation (Stender *et al.,* 2001). The need to detect very low numbers currently by a pre-enrichment step that should be included to improve method sensitivity and reduce the number of false negative results for FISH detection to confirm the presence of *Salmonella* in the enrichment cultures (Mereno *et al.,* 2001; Fang *et al.,* 2003; Blasco *et al.,* 2003; Hahn *et al.,* 2006).

Interestingly, this study os a FISH experiment that showed FISH sensitivity (93.5%) result was higher than results of a previous study (84.41%) (Vieira-Pinto *et al.,* 2012) which, the results differring in this study may be related to Sal3 probe labeled at 3′-end with terminal transferase. A more sensitivity technique of producing labeled oligonucleotide is to do 3′-tailing. In this reaction, terminal transferase is used to add a mixture on unlabeled nucleotide and DIG-1-dUTP, producing a tail containing multiple digoxigenin residues. However, this technique maybe produces a probe with a high background owing to presence of the long tail. But, these problems can be overcome by using proper hybridization conditions, which accordance with this work showed negative controls were absent by FISH experiment. When we added the anti-digoxigenin-FITC the targets showed a high intensity signal. So, the results of high sensitivity of the FISH experiment should be considered as an important rapid screening tool for *Salmonella* spp. detection in pork samples. But, the FISH specificity result was lower than culture method (66.6%), indicating the need

for more to studies of presumption to influence of enrichment media prior to this FISH detection that could be decrease the number of false positive and false negative results, with results that are similar to standard methods.

3. Signal amplification technology based on catalyzed reporter deposition (CARD) and its application in Dot-blot hybridization assay with biotinylated sal3 probe for the detection of Salmonella DNA in samples.

Fluorescence *in situ* hybridization is a powerful molecular tool used to visualize nucleic acids in tissues and cells. However, it has limited sensitivity when applied to low copy numbers of nucleic acids. Rapid detection methods for low cells counts are important for many aspects of quality assurance in food and pharmaceutical processing. Several strategies have been developed to improve the threshold levels of nucleic acid detection by amplification of either target nucleic acid sequences before hybridization (such as *in situ* polymerase chain reaction) or after the hybridization procedure (such as tyramide signal amplification). Catalyzed reporter deposition (CARD) was developed as an *in situ* amplification method (Bobrow *et al.,* 1992; Schönhuber *et al.,* 1997). The method, also called tyramide signal amplification (TSA), has been adopted as a means of enhancing signal strength for immunoblotting and immunocytochemistry (Bobrow *et al.* 1991,1992; Chao *et al.,* 1996; van Gijlswijk *et al.,* 1997) and fluorescent *in situ* hybridization (FISH) on chromosomes (Kerstens *et al.,* 1995; Raap et al. 1995; Speel *et al.,* 1997), bacteria and archaea in marine sediment (Ishii *et al.,* 2004), bacteria in seawater (Pernthaler *et al.,* 2002), bacteria in soil (Ferrari *et al.,* 2006), epiphytic bacteria on marine algea (Tujula *et al.,* 2006), bacteria in biofilms associated with organic matter (Fazi *et al.,* 2005) and bacterial biofilms on seaweed (Tujula *et al.,* 2005) and prokaryotes inside phagotrophic protists (Medina-Sánchez *et al.,* 2005) and within benthic organic matter (Fazi *et al.,* 2005), is used here as an alternative to rRNA enrichment. Therefore, CARD dot blot hybridization that might potentially address the problem of detecting low-abundance RNA targets of bacteria. The method uses horseradish peroxidase to catalyze the deposition of tyramide molecules, preconjugated with either biotin or fluorescent reporters.

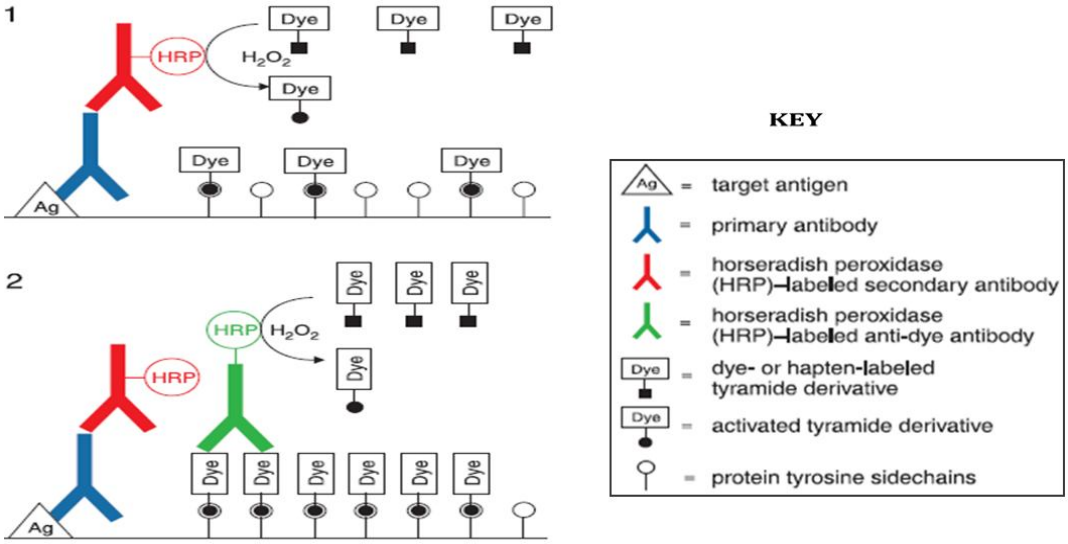


Figure 17 schematic representations of TSA detection methods applied to immunolabeling of an antigen. Stage 1). The antigen is detected by a primary antibody, followed by a horseradish peroxidase (HRP)-labeled secondary antibody in conjunction with a dye-labeled tyramide, resulting in localized deposition of the activated tyramide derivative. Stage 2). Dye deposition, and therefore higher levels of signal amplification, can be generated by detecting dye deposited in Stage 1 with a HRP-labeled anti-dye antibody in conjunction with a dye-labeled tyramide.

A benefit could be expected from the use of CARD-dot blot hybridization. In the present study, a comparative evaluation of the detection efficiency of *Salmonella* DNA by two conventional methods of dot blot hybridization and CARD- dot blot hybridization was carried out on *Salmonella* DNA detection from a dilution factor of *Salmonella* concentration, which exhibited an approximate dilution factor up to -8, it was possible to detect <3 cfu/ml (within 22 hours) that lower dilution factor than -7 of dilution factor of both conventional-dot blot hybridization, it was possible to detect >300 cfu/ml (within 22 hours) and less the low number than FISH analysis was previously applied successfully (3×10^7 cfu/ml onto pork samples within 8 hours). These results corresponds the study CARD-dot blot hybridization experiment of Eschenhagen *et al.* (2008), who found the CARD *in situ* hybridization used SAL998

probe labeled HRP with fluorescent tyramide, 3,3′, 3,5′- tetrammethlbenzidine (TMB) that can be detected the low numbers of salmonella cell (1-2 cells) in a drinking water sample of 10 ml within 9-10 hours.

The TSA has been reported to increase detection sensitivity up to 100-fold, as compared with conventional avidin–biotinylated enzyme complex (ABC) procedures, when compared with standard procedures using monolabelled oligonucleotide probes (Merz *et al.,* 1995; Van Heusden *et al.,* 1997; Speel *et al.,* 1999; Uchihara *et al.,*2000; Haugland, 2005). But, in this study the TSA has been increased the detection sensitivity up to 100-fold when compared with both conventional dot blot hybridization techniques. This is a main reason for why CARD-dot blot assay is increase in the more sensitivity than other detection methods (figure 17). Because, CARD is an enzyme-mediated detection method that is combined with nucleotide probes to localize specific nucleic acid sequences (DNA and RNA) in a membrane or in microscopic preparations of tissues, cells, and chromosomes and allows the detection of rare and even single-copy-number targets. CARD is increased in the sensitivity of detection methods, in the presence of low concentrations of H_2O_2, to convert labeled tyramine-containing substrate into an oxidized, highly reactive free radical that can covalently bind to tyrosine residues at or near the HRP (Bobrow et al., 1992; Adams, 1992; Shindler and Roth, 1996). Tyramines are phenolic compounds, and HRP can catalyze dimerization of such compounds when they are present in high concentrations, probably by the generation of free radicals. If applied at lower concentrations, such as in the signal amplification reaction, the probability of dimerization is reduced, whereas the binding of the highly reactive intermediates to electron-rich moieties of proteins, such as tyrosine, at or near the site of the peroxidase binding site is favored.

Moreover, CARD-dot blot hybridization in this study using Sal3 probe that was enzymatically labeled at the 3'- end with terminal transferase, as a result of this oligonucleotide labeled on average, the tail of probe is 40-50 nucleotides in length with several DIG-molecules incorporated that is an increased signal intensity when compared to a monolabelled oligonucleotide probe (Deere *et al.,* 1998). All of the

above reasons can result, CARD-dot blot hybridization in greatly enhanced signal sensitivity compared to probes with a multiple signal and allow us to detect as low number of *Salmonella* cells as <3 cfu/ ml.

CONCLUSSION

In summary, a novel FISH protocol with Sal3 probe labeled at 3′-end with terminal transferase and developed signal amplification technology based on catalyzed reporter deposition (CARD) for detecting low numbers of *Salmonella* cells and its application in dot-blot hybridization assay *Salmonella* DNA with biotinylated Sal3 probe. Due to disease severity has made it a motivation for appropriated preventive measures. At least a sampling of 25 grams of meat should not be carrying salmonella in the epidemic situated salmonellosis. Although, bacterial culture method is the gold standard with accurate results even though the limitation of this method is time consuming (at least 4-6 days). In this research the fluorescence *in situ* hybridization technique (FISH) is developed for *Salmonella spp*. detection in pork industry of Thailand for food safety and good quality exported meat purpose. The Sal3 probe (5'-ATCACTTCACCTACGTG-3') for *Salmonella spp.* had the specificity achievement to the *Salmonella* nucleotide sequences. The nucleotides sequence alignment with the GenBank database of 23S rRNA of *Salmonella enterica* including *S.* Enteritidis, *S.* Paratyphimurium and *S.* Typhimurium were found with 100 % homology. Those serovar are reference of the major sallmonellosis pathogen in this research. And the Sal3 probe specificity with dot-blot hybridization found a positive results of 60 salmonella serovar (pure salmonella culture) while there were negative result from 9 samples of the negative control group.

The rapid detection of *Salmonella* in pork samples using optimization of cell wall permeabilizing conditions for Sal3 probe labeled at 3′-end with terminal transferase by FISH can be an alternative time saving, reliable and cultivation-independent technique for detection and identification of *Salmonella* in pork production. The results in this study indicate that the optimum condition for cell permiation is using lysozyme at the concentration of 1 mg/ml dissolved in 10mM Tris-HCl, 5mM EDTA, pH 8.0, while incubation proceeded at 37^{o} C for 3 minutes. FISH experiment showed positive results relative to culture method (accuracy, 85.71%). It gave the average score for Fluorescence *in situ* hybridization at level 4 and comparison of the methodological result between the standard method ISO 6579

(2002) and Fluorescence *in situ* hybridization demonstrated by the Cohen's *Kappa* statistics which is equal to 0.46. The statistical measure was corresponding and ranging in the moderate value. The expected result of the Fluorescence *in situ* hybridization in *Salmonella* detection in this study is less time consuming (only 8 hours) and more convenient for testing many samples compared to the bacterial culture method. Nevertheless, our results will contribute to the optimization of permeabilizing conditions, which is one of the most important factors for successful application of routine laboratory detection. It is anticipated that in future studies, a FISH method that is highly sensitivity and specificity of *Salmonella enterica* serotype will be developed, and consequently, this method will enable us to safely consume pork free from *Salmonella* spp.

Catalyze reporter deposition in dot blot hybridization with DAB was used for detecting low numbers of *Salmonella* cells, which its application in dot blot hybridization assay *Salmonella* DNA. Biotinylated Sal3 probe with TSA detection method showed an increased sensitivity with respect to the indirect detection of biotinylated probe and specificity and sensitivity which make the assay suitable in the routine use of a pathogenic detection laboratory. This result has increased the signal intensity and increased detection sensitivity to the minimum number of *Salmonella* cells as <3 cfu/ ml or corresponding 7.47 µg/ml of *Salmonella* DNA, as compared with conventional avidin–biotinylated enzyme complex (ABC) procedures and conventional alkaline-phosphatase (AP), both conventional dot bot hybridization that were able to detect 26.52 µg/ml of *Salmonella* DNA and less than the low number that FISH analysis was previously applied successfully (3×10^7 cfu/ml onto pork samples).

In addition, situation of pork slaughterhouse in Thailand is an open process with opportunities for carcass contamination with *Salmonella* spp. To promote food safety regulation under control by department of livestock that has programmes to reduce pathogenic bacteria like *Salmonella* spp. transmission to meat in slaughterhouse should be implemented. Our study recommenced Sal3 probe labeled at 3′-end with terminal transferase by fluorescence *in situ* hybridization that has a

moderate potential for rapid detection of *Salmonella* in pork meat. But, the results of FISH experiment were applied to *Salmonella* detection that contaminated levels higher than 3×10^6 cfu/ml onto pork. In this case, thirty-five random samplies of *Salmonella* were taken from naturally contaminated pork. Comparisons of experiment on screening pork samples obtained from a pig slaughterhouse with cultural and FISH method, *Salmonella* sp. could be detected without pre-enrichment, indicating high level shedding of *Salmonella* sp. in the pig slaughterhouse. Especially, as long as animal carriers enter the slaughterhouses, *Salmonella* spp. may be transmitted to the consumers. So, FISH experiment can be a promising screening tool for rapid detection of *Salmonella* in pork in Thai slaughterhouse without pre-enrichment.

For CARD-dot blot hybridization, assay a biotinylated probe was constructed by Sal3 probe labeled with biotin at 3′- tail labeling showed an increased sensitivity with respect to the indirect detection of biotinylated probe and a specificity and sensitivity which make the assay suitable in the routine use of diagnostic laboratory, The dot-blot hybridization assay described here can therefore be considered an additional, sensitive and reliable method for the search of low number of *Salmonella* cells both in routine screening and diagnostic work.

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APPENDICES

Appendix A

Chemical reagents and substances

Chemical reagents and substances

1. Bacterial Media and Solution

Mac conkey agar

peptone 190 (pancreatic digest of gelatin)	17.0 g
peptone 180 (Animal tissue - casein polypeptone)	3.0 g
Lactose	10.0 g
Bile salt # 3	1.5 g
Sodium choride	5.0 g
Agar	5.0 g
Nuetral red	0.03 g
Crystral violet	1.001 g
Adjusted to pH	7.1 ± 0.2

Buffered Peptone Water (BPW)

Peptone	10 g
NaCl	5 g
Na_2HPO_4. $12H_2O$	3.5 g
KH_2PO_4	1.5 g
Distilled water	1 L
Adjusted to pH	7.1 ± 0.2
Autoclave at 121°C for 15 minutes.	

Xylose Lysine Desoxycholate (XLD)

Xylose lysine desoxycholate (Oxoid)	53 g
Distilled water	1 L.
Do not autocave. Pour into sterile plates.	

Soyabean Casein Digest Agar; Tryptone Soya Agar (TSA)

TSA (Himedia Laboratories)	40 g
Distilled water	1 L.

Do not autocave.

Pour into sterile plates.

Triple Sugar Iron Agar (TSI)

TSI agar powder (Oxoid)	59 g
Distilled water	1 L.

Aliquots 3 ml were made into 13x100 mm screw capped tubes.

Autocave 121°C for 15 minutes.

After making slants, all tubes were kept at 4°C until used.

TETRATHIONATE BROTH BASE (TT)

TT medium	46 g
Distilled water	1 L.

Heat with frequent agitation to boiling.

Cool down to	45°C
Add Iodine-Potassium solution	20 ml
- Iodine	6 g
- Potassium iodide	5 g
- Disolved with purified water	20 ml

Do not reheat after adding iodine solution.

Rapport-Vassilia Broth (RV)

RV powder (MERK)	42.5 g
Distilled water	1L.

Autocave 121°C for 15 minutes

Kept at 4°C until used.

Brilliant-green Phenol-red Lactose Sucrose Agar (BPLS)

BPLS	57 g
Distilled water	1L.

Autoclave 121°C for 15 minutes

Pour into sterile plates.

2. Dot blot hybridization and Fluorescence *in situ* hybridization

Solution:

10X PBS (pH 7.4) 1L

Nacl	40 g
Na_2 HPO_4	5.75 g
KCl	1 g
KH_2PO_4	1 g

Adjust pH with glacial acetic acid to pH 7.4 ± 0.2 and bring to 1 litre with distilled water.

20X SSC Stock (pH 7.0) 1 L

NaCl	175.32 g
Na3C6H5O7•2H2O [= sodium citrate]	88.3 g

Adjust pH with glacial acetic acid to pH 7.4 ± 0.2 and bring to 1 litre with distilled water.

10X TBS (pH 7.4) 500 ml.

NaCl	80 g
KCl	2 g
Tris- base	30 g

Adjust pH with glacial acetic acid to pH 7.4 ± 0.2 and bring to 500 ml with distilled water.

PreHybridization and Hybridization solution

Deionized formamide	5 ml
20X SSC	1.5 ml

50X Denhardt's solution	200 µl
Yeast tRNA (10 mg/ ml)	200 µl
NaH_2PO_4, pH 7.4	500 µl
Dextrans sulfate	1 g
Distill water	2.5 ml

Store in aliquots at -4 ^{o} C

Wash solution: All this solution should be prepared fresh from the stock immediately before use.

2X SSC, 500 ml

20X SSC Stock	50 ml
dH_2O	450ml

Store at room teperature

1X SSC, 500 ml

20X SSC Stock	25 ml
dH2O	475 ml

Store at room teperature

0.5X SSC, 500 ml

20X Stock	12.5 ml
dH2O	485.5 ml

Store at room temperature

1X TBS, 500 ml

10X Stock	50 ml
dH2O	450 ml

Store at room temperature

TBS-Tween buffer

Add to final concentration of 0.05% Tween 20 (Sigma®)

Blocking agent

5% skim milk in TBS-Tween buffer

Antifade solution

DAPCO	0.23 g
Autocave glycerol	9 ml

Appendix B

The standard methods and Kappa statistics measurement

1.The standard methods

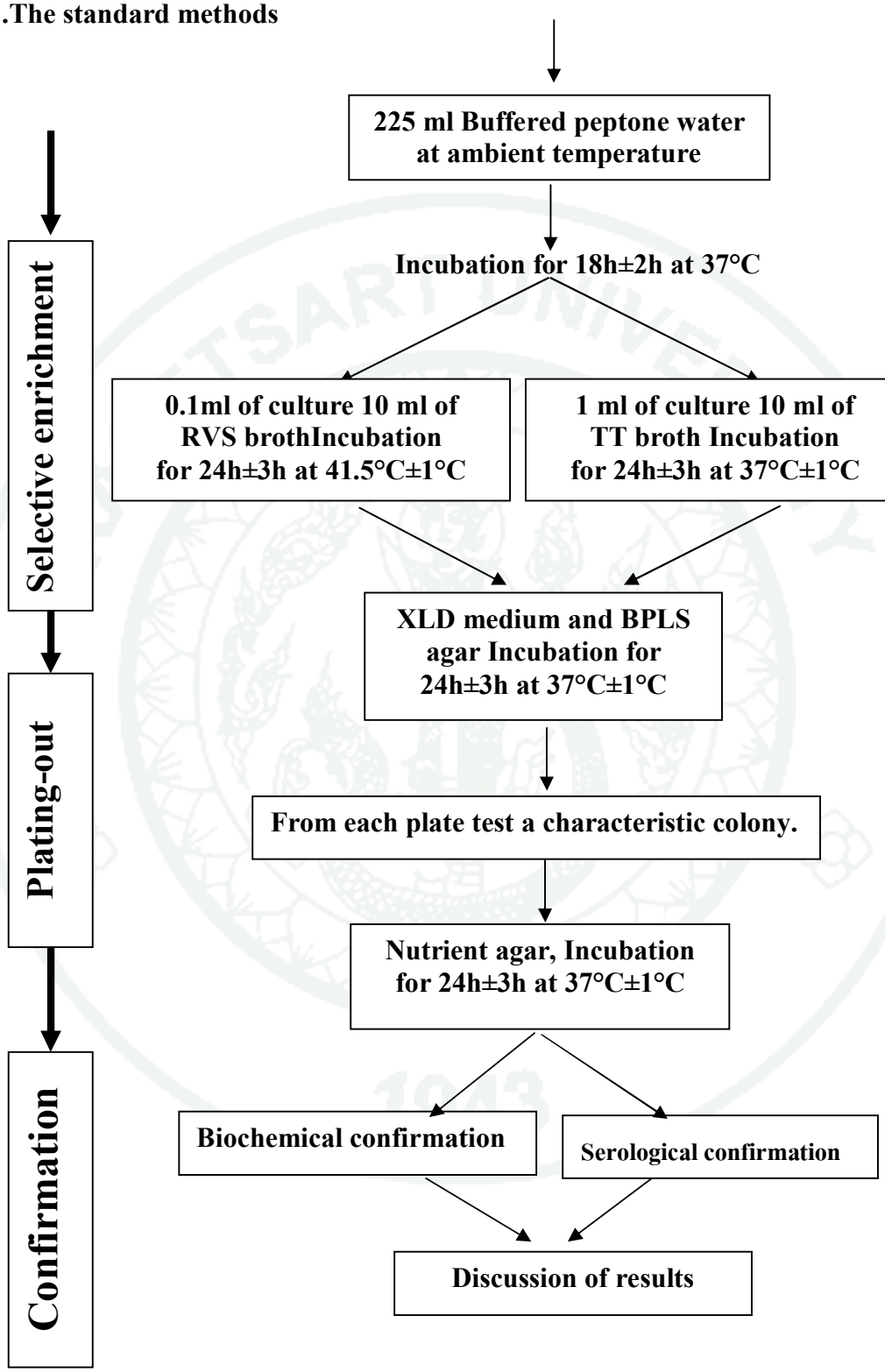

Diagram of ISO 6597: 2002 for *Salmonella* spp. detection.

2. Phenol-Chloroform extraction of DNA and ethanol precipitation

1. DNA was extracted from 100 µl of *Salmonella*-broth sample that mixed with 500 µl of D-solution (4 M guanidiumthiocyanate, 50 mM Tris-HCl, 20 mM EDTA, pH 8.0), was shaken for 5-10 minutes and incubated at room temperature for 5 minute.
2. Add DNA phenol 150 µl and chloroform 150 µl was shaken for 5 minutes.
3. Centrifuge the sample at 13,000 rpm for 5 minutes to separate the phases.
4. Remove about 90% of the upper, aqueous layer to a clean tube, carefully avoiding proteins at the aqueous-phenol interface. At this stage the aqueous phase can be extracted a second time with same procedure.
5. Repeat 2-4 again.
6. Remove about 90% of the upper, aqueous layer to a clean tube, add isopropanol 550 µl and 0.5 µl of glycogen (20ng/ml), invert gently up side down and keep in – 80°C for 40 minutes
7. Centrifuge at 13,000 rpm for 10 - 15 minutes. Carefully decant the supernatant.
8. To wash the DNA pellet with 75% ethanol.
9. Centrifuge at 13.000 rpm for 5 minutes. Decant the supernatant, and dry the pellet by air.

3. Calculation of sensitivity and specificity of method

$$\% \text{ Sensitivity} = \frac{\text{true positive}}{\text{True positive + false negative}} \times 100$$

$$\% \text{ Specificity} = \frac{\text{true Negative}}{\text{True negative + false positive}} \times 100$$

4. Kappa statistics measurement

Evaluating new technologies or test raises the question of whether differences are due to the technology or the interspreters. Kappa, is widely used to measure interobserver variability, that is, how often 2 or more observers agree in their interpretations. Simple agreement, the proportion of agreements between yes and no is a poor measure of agreement because it does not correct for chance. Kappa is the preferred statistic because it accounts for chance. Widely, but inappropriately used in many radiologic studies is the correlation coefficient as a measure of agreement. Two observers may have good (even perfect) correlation, but never agree. One may describe hearts as mildly enlarged, the other severely enlarged.

Agreements between Categorical Measurements: Kappa Statistics

Kappa	Strength of agreement
0.00	Poor
0.01-0.20	Slight
0.21-0.40	Fair
0.41-0.60	Moderate
0.61-0.80	Substantial
0.81-1.00	Almost perfect

The 95% confidence interval of kappa value is (0.279, 0.805). So in terms of the statements in the above guide, agreement here is somewhere between fair and almost perfect. Standard error of the above kappa is 0.134.

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