Original Article

Optimization of Cell Permeabilization for Rapid Detection of *Salmonella* in Pork by FISH

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Abstract

Salmonella spp. are one of the major causes of infectious gastroenteritis. The route of infection is usually ingestion of contaminated food. Due to the severity of the disease, it is worthwhile to identify appropriate preventive measures. Bacterial culture method is the gold standard method, but it is time-consuming (at least 4-6 days). Therefore, in this research, the fluorescence *in situ* hybridization technique (FISH) was optimized for rapid and accurate detection of *Salmonella* spp. in contaminated pork using a 3'-end tailing oligonucleotide probe. The optimum digestion condition for cell permeation with lysozyme was 1 mg/ml at 37^{o}C for 3 min. The lowest concentration of *Salmonella* detected in spiked-pork samples without a pre-enrichment step was 10^7 cfu/ml. Using the FISH method, 30 out of 35 market pork samples were positive, compared to 29 out of 35 positive results produced by the culture method. FISH gave 3 false positive and 2 false negative results. According to Kappa Statistics, agreement between the standard culture method and the fluorescence *in situ* hybridization technique was 0.46, which can be accepted as being in the moderate range of standard value. Thus, FISH should be considered as an important rapid screening tool for detection of *Salmonella* spp. contamination.

Keywords: FISH, oligonucleotide probe, rapid detection of *Salmonella* spp., pork

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Introduction

Salmonellosis is a disease caused by *Salmonella* spp. It is a major problem in terms of food borne diseases all over the world and remains high on the list of pathogens that most concern international food standards agencies, food retailers, and consumers (WHO, 2005; EFSA, 2010). Human salmonellosis causes high morbidity, mortality and economic losses worldwide each year (Ball et al., 2011). The incidence of salmonellosis has increased in conjunction with the dynamics of livestock product consumption (WHO, 2007). *Salmonella* was detected in 55.5% of freshly cut pork, 70.5% of transported pork, and 34.5% of retail products in Thailand (Sanguankiat et al., 2010). This represents a potential risk for consumers that should be correctly identified by monitoring programs to estimate the level of *Salmonella* contamination in slaughterhouses and reduce the presence of *Salmonella* in pork production in order to improve the safety of pork products bought by consumers (Krank et al., 2003).

Detection of *Salmonella* in food is generally performed by ISO 6579 cultural method (FAO/WHO, 2009). This process is laborious and time-consuming (4-6 d), and is rarely carried out in practice. Over past years, many rapid methods for the detection of *Salmonella* have been developed and these methods are generally less time-consuming and labor-intensive than conventional microbiological methods (Swaminathan and Feng, 1994; Burtscher et al., 1999; Baylis et al., 2000). These methods are immunomagnetic methods, colony hybridization, dot blot hybridization, commercial tests and PCR (Datta et al., 1988; Jaton et al., 1992; Hill and Olsvik, 1994; Lantz et al., 1994; Olsen et al., 1995; ICMSF, 1996; Lin and Tsen, 1996; Soumet et al., 1999; Wang et al., 1997). FISH is one of the most rapid methods for monitoring the real risk of alteration in bacterial numbers, and the possibility of pathogenic bacteria, with respect to food contamination (Vieira-Pinto et al., 2008; Bledar, 2009). However, optimization of cell permeation without destroying structural integrity of cell or tissue, and allowing penetration of a probe, is crucial (Yokouchi et al., 2003; Furukawa et al., 2006). In addition, the size and type of probe labeling have an important role concerning the sensitivity of detection (Amann et al., 1992; Bidneko et al., 1998). Thus, it is usually necessary to include a very carefully controlled permeabilization step prior to hybridization. According to previous studies, the FISH protocols using 5'-end oligonucleotide probes without permeabilization treatment gave very weak signals (Vieira-Pinto et al., 2008; Bledar, 2009). There is no report on the permeabilizing conditions of *Salmonella* cell wall and the use of 3'-end labeling oligonucleotide probe when employing FISH. The aims of the present study were to define the optimum condition for permeabilization of the cell wall of *Salmonella* using different concentrations of lysozyme and times for digestion, and the use of 3'-end labeling oligonucleotide probe to increase FISH signal intensity. The optimized FISH and standard culture methods were used to compare the sensitivity for the detection of *Salmonella*-contaminated pork samples purchased from the market.

Materials and Methods

Reference strains and culture: Pure culture of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) and *Salmonella enterica* serovar Paratyphi (*S.* Paratyphi) from the WHO international *Salmonella* and *Shigella* Center, Department of Medical Science in Bangkok, Thailand, were used as positive controls. *Actinomyces* spp., *Campylobacter jejuni*, *Corynebacterium* spp., *Escherichia coli*, *Klebsiella* spp., *Pseudomonas* spp., *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus suis* were kindly provided by the Bacteriology Laboratory, Department of Veterinary Public Health, Faculty of Veterinary Medicine, Kasetsart University. All bacteria were grown aerobically or anaerobically in Tryptic Soy Broth (TSB) at 37°C, and each bacterium was harvested at logarithmic phase in order to obtain cells with high ribosome content.

Oligonucleotide probe: An oligonucleotide probe (5'-AATCACTTCACCTACGTG-3') specific to the 23S specific to the 23S rRNA of *Salmonella enterica* strain was used according to previous studies (Vieira-Pinto et al., 2008; Nordentoft et al., 1997; Örmerci and Karl, 2008). This oligonucleotide probe was tailed with digoxigenin-dUTP at the 3'-end, using Oligonucleotide Tailing Kit, 2nd Generation (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Cell fixation: Cells were grown at exponential growth phase (OD600 = 0.5) and harvested by centrifugation at 13,000 rpm for 3 min. Cell pellets were washed twice using 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). The cells were fixed using 4% paraformaldehyde at 4°C for 4 h, and washed 3 times with PBS. They were then resuspended in 50% ethanol in PBS and stored at -20°C until used.

Enzymatic permeabilization of fixed cells: Ten well Teflon slides (Heinz Herenz, Hamburg, Germany) were precleaned using ethanol, coated with 2% 3-triethoxysilyl propylamine (Merck, Darmstadt, Germany) in acetone for 1 min, incubated twice in acetone for 1 min each, and washed in distilled water (Vieira-Pinto et al., 2008). Then, the slides were air dried and dehydrated using ethanol at concentrations of 50, 80, 96 % (v/v) for 3 min each. Ten microliters of the fixed cells were spotted on the well and air dried. Then, the slides were dehydrated with 50, 80 and 96% of ethanol for 3 min each. Subsequently, the bacterial cell wall was subjected to permeabilization using 2 different enzymatic conditions. For the first protocol, the cells were treated with 10 µl of different lysozyme (Sigma Chemical Co, St. Louis, MO, USA) concentrations (0.1, 0.5, 1, 5 and 10 mg/ml containing 100 mM Tris-HCl, 50 mM EDTA pH 8.0) at 25°C for 5 and 20 min, respectively (Blasco et al., 2003). For the second protocol, the cells were treated with 10 µl of different lysozyme concentrations (0.1, 0.5, 1, 5 and 10 mg/ml in buffer containing 10 mM Tris-HCl, 5 mM EDTA pH 8.0) at 37°C for 1, 3 and 5 min, respectively

(Hogardt et al., 1999). Enzymatic reactions were stopped by rinsing the slides thoroughly with the Milli-Q water. The slides were dried and dehydrated by immersion in 50, 80, and 96% ethanol for 3 min each.

Hybridization of Cells and Counting: For whole-cell hybridization, probes were diluted at a concentration of 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). Ten microliters of the diluted probe were added to each well on Teflon-coated slides. The slides were incubated in a humid chamber at 45°C for 3 h, and were immersed for 15 min in 50 ml of washing buffer (0.9 M NaCl, 20mM Tris-HCl pH 7.4, 0.01%SDS). They were subsequently incubated with 1:800 anti-digoxigenin-fluorescein conjugate (Roche Diagnostics, Mannheim, Germany) in a dark room for 1 h. After incubation, the slides were washed in buffer solution (0.9 M NaCl, 20mM Tris-HCl pH 7.4, 0.01% SDS) at 45°C for 15 min in the dark with agitation, and then rinsed with 1X PBS. They were mounted with 2.3% DABCO (Sigma Chemical Co. Aldrich, St. Louis, Missouri, USA) in 90% glycerol and observed under a fluorescent microscope (Olympus BX51 microscope, Tokyo, Japan). Fifteen visual fields were analyzed and scored for each slide, as in Figure 1.

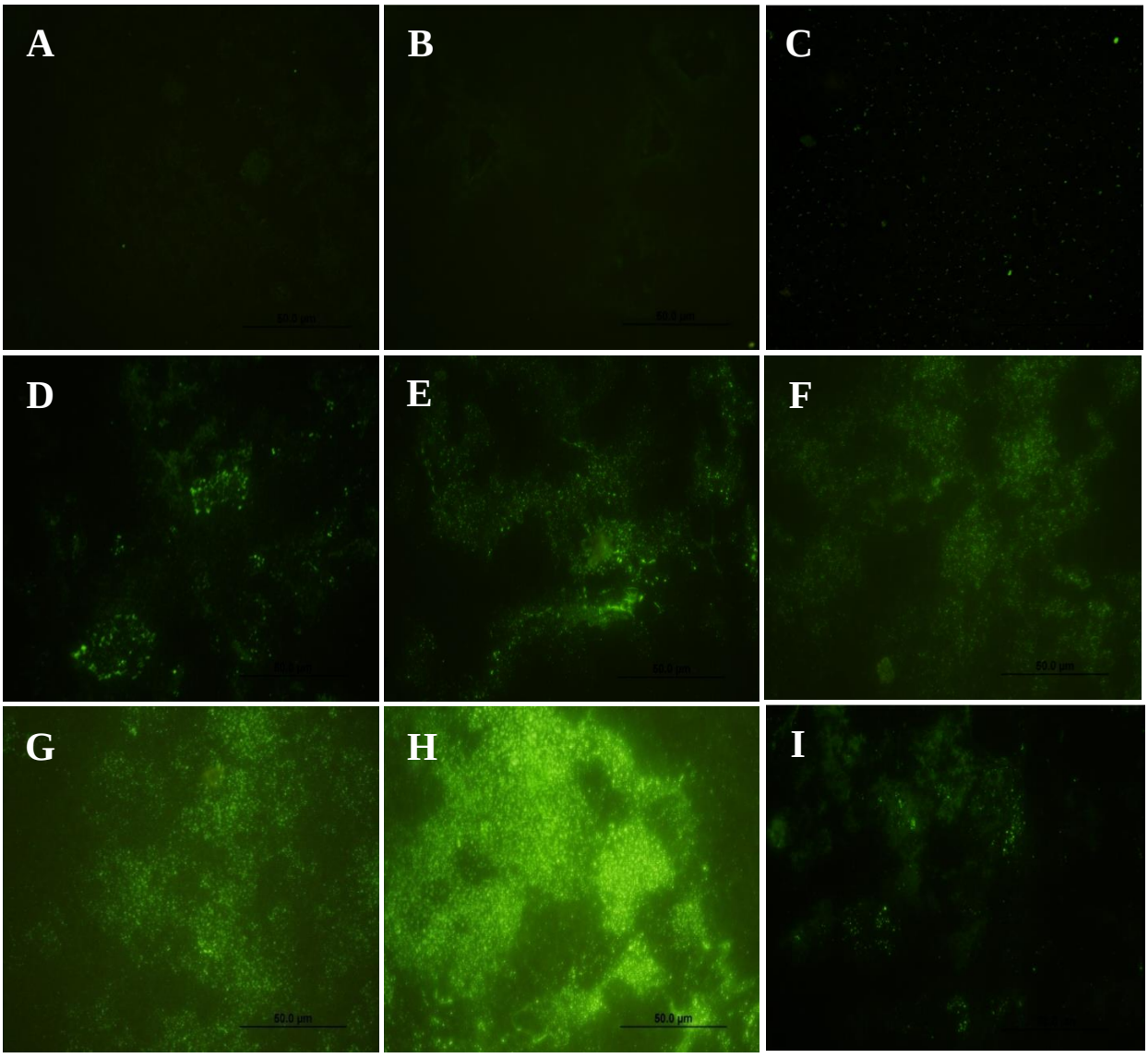

Figure 1 Photograph showing different levels of signal after hybridization of *Salmonella* Enteritidis and other bacteria (magnification ×400). A: *Escherichia coli*, B: *Staphylococcus aureus* and C-I: *Salmonella* Enteritidis. Ranges of fluorescence signals emitted were classified, using average percentages, into 5 levels: A-C, no fluorescence signal emitted; D, Level 0 = Incomplete (fluorescence signals less than 5%); E, Level 1 = Poor (fluorescence signals ranging from 5 to 25%); F, Level 2 = Fair (fluorescence signals ranging from 26 to 50%); G, Level 3 = Good (fluorescence signals ranging from 51 to 75%); H, Level 4 = Very good (fluorescent signals ranging from 76 to 100%); and I, image of fluorescence signal of extended lysozyme treatment of *Salmonella* cells. Cells often displayed a diffuse appearance, suggesting loss of cellular structure and leakage of rRNA. Scale bar: 50 µm

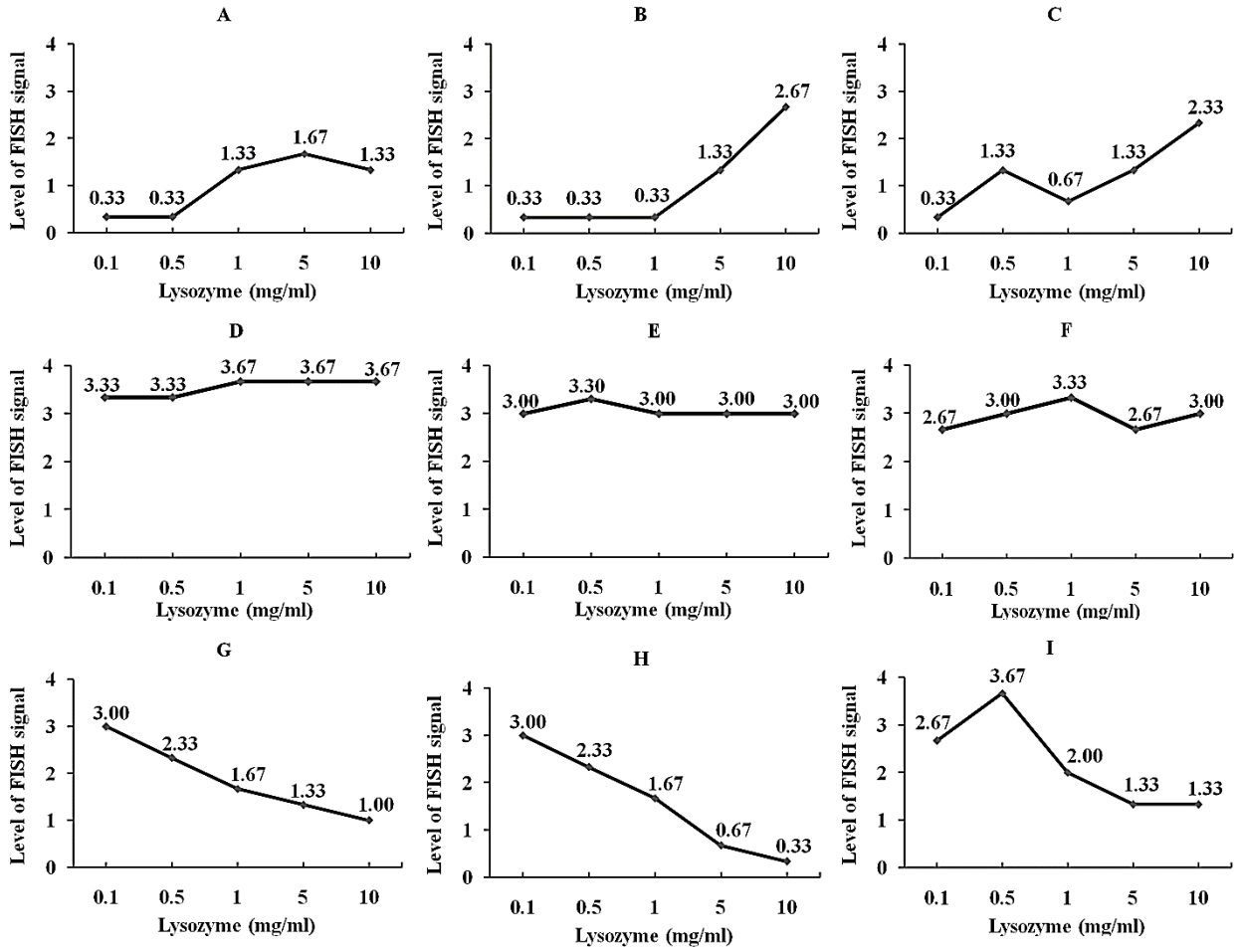

Figure 2 Fluorescence levels of *Salmonella* Enteritidis (A, D and G), *Salmonella* Typhimurium (B, E and H) and *Salmonella* Paratyphimurium (C, F and I) treated with different lysozyme concentrations for 1 min, (A, B and C), 3 min (D, E and F), and 5 min, respectively (G, H and I), at 37°C. The best fluorescence signal was achieved by digestion with 1 mg/ml of lysozyme, at 37°C, for 3 min, for all 3 serotypes of *Salmonella*.

Detection limit for Salmonella detection from pork samples: *S.* Enteritidis were grown in 5 ml of TSB at 37°C for 16 h. Cells were harvested and diluted in distilled water, in order to achieve 1 McFarland standards which was equal to approximately 3×10^8 cfu/ml. Tenfold serial dilution of *S.* Enteritidis was prepared before inoculation. One milliliter of each tenfold dilution of *S.* Enteritidis was spiked into 25 g of *Salmonella*-free pork. Subsequently, each sample was mixed with 25 ml of Bacto peptone water (BPW) containing 0.1% Tween 80 and was homogenized using stomacher (BagMixer® 400W, Interscience, St. Nom, France) at high speed for 90s. The suspension was filtered through 33-µm pore-size nylon screen mesh, and the bacteria were harvested by centrifuging at 7,000 rpm for 20 min. Pellets were resuspended with 2 ml of MilliQ water. One milliliter of the supernatant was used to determine the number of recovered bacteria using plate count agar. Another 1 ml of the supernatant was centrifuged for another 10 min at 13,000 rpm, and cell pellet was resuspened in 1 ml of PBS in order to determine the detection limit of FISH.

Application of FISH method for detection of Salmonella spp. in pork from local market: Thirty-five pork samples were purchased and subjected to FISH. Each sample was cut into 2 pieces of 25 g. A twenty-five-gram piece of pork was put into a sterile bag with 25 ml of BPW, with 0.1% Tween 80, and subsequently, the incubated pork was homogenized using stomacher at high speed for 90 s. The suspension was submitted to bacterial culture, so as to determine the presence of *Salmonella* spp. and the concentration of bacteria, according to ISO 6579:2002. Another piece of pork was incubated and homogenized as the first piece. Subsequently, the 25 ml of suspension was filtered through a 33-µm pore-size nylon screen mesh and the bacteria were harvested by centrifuging supernatant at 7,000 rpm for 20 min. Pellets were washed twice with 1 ml PBS, centrifuged at 13,000 rpm for 5 min, and fixed. The pellets were resuspended with MilliQ water and used to spot on the ten-well Teflon slides used for performing the FISH experiment.

Statistical analysis: Cohen's *Kappa statistic* test was used to determine agreement between the culture method (ISO 6579: 2002) and the FISH method. Good inter-rater reliability was demonstrated using WIN EPISCOPE 2.0 software (Win Episcope 2.0). Differences were considered significant at level of 95% (p-value <0.05).

Results

Optimization of permeabilization conditions: There was no signal detection without lysozyme treatment (data not shown). Regarding the first condition using buffer containing 100 mM Tris-HCl, 50 mM EDTA pH 8.0 at 25°C, the average of fluorescence signal at every concentration of lysozyme for 5 min was very weak (Fig 1C). Although the incubation for 20 min at every concentration of lysozyme showed weak signals, it was better than those incubated for 5 min (Fig 1I). Concerning the second condition using buffer containing 10 mM Tris-HCl, 5 mM EDTA pH 8.0 at 37°C, the optimum permeabilization condition for *S.* Enteritidis (3.67; 91.75%) and *S.* Typhimurium (3.0; 75%) was obtained by the digestion with 1 mg/ml of lysozyme for 3 min (Fig 2). However, the conditions giving the highest fluorescent signal for *S.* Paratyphi (3.67; 91.75%) consisted of the digestion with 0.5 mg/ml of lysozyme for 5 min. There was no cross-reaction with other bacteria (Fig 1A and B). Thus, the optimum permeation condition selected for using in this experiment was the digestion with 1 mg/ml of lysozyme for 3 min at 37°C (Fig 2).

Detection of Salmonella by FISH in spiked sterile pork samples: *S.* Enteritidis of approximately 10^8 cfu/ml was used to spike 25 g of pork. The lowest level of *Salmonella* that could be detected was 10^7 cfu/ml without a pre-enrichment step (Table 1).

Detection of Salmonella in pork samples from a slaughterhouse by FISH in comparison with culture method: To evaluate the practicability of FISH for the detection of *Salmonella* from naturally contaminated pork samples, 35 commercial pork samples were used to detect the contamination, by employing both the culture and FISH methods (Table 2). According to the culture method, 29 out of the 35 samples (82.5%) were positive and 6 samples (17.5%) were negative. With reference to the FISH method, 30 out of the 35 samples (85.71%) were positive, and 5 samples (14.29%) were negative. Twenty-seven positive and 3 negative samples were shared by both methods. The FISH method showed 3 false positive samples and 2 false negative samples. On the other hand, the culture method showed only 1 false negative sample when compared with the FISH method. In this study, moderate levels of sensitivity (93.1%), and specificity (50%), were achieved by the FISH method. According to Cohen's *Kappa statistic* test, the agreement between FISH and ISO6579:2002 was 0.46, which was in the moderately accepted range.

Table 1 Sensitivity of fluorescence *in situ* hybridization using *Salmonella* Enteritidis spiked-pork samples at various concentrations

Inoculums (cfu/ml)	Artificially contaminated pork samples						
	1.5×10^8	1.5×10^7	1.5×10^6	1.5×10^5	1.5×10^4	1.5×10^3	1.5×10^2
Observation	(+)	(+)	(+/-)[a]	(+)	(-)	(-)	(-)

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

Table 2 Number of *Salmonella* spp. detected in 35 commercial pork samples by FISH and culture methods

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 culture method			0.46

Discussion

The conditions of the FISH method for the rapid detection of *Salmonella* spp. contamination in commercial pork was optimized and developed. The permeabilization step using 1 mg/ml of lysozyme in buffer containing 10 mM Tris-HCl, 5 mM EDTA pH 8.0 at 37 °C for 3 min gave the optimum signal for all 3 *Salmonella* spp., and was the best condition for *S.* Enteritidis and *S.* Typhimurium. However, the best condition for *S.* Paratyphi was the digestion with 0.5 mg/ml of lysozyme for 5 min. There was no cross-reaction with the negative controls. The low or non-existent signals obtained from the first condition may be due to the cell wall not being permeabilizated enough to allow efficient penetration by antibodies or enzymes (Furukawa et al., 2006). Another reason might be an issue with too much permeabilization, in the case of longer incubation times, or higher enzyme concentration, which might cause leakage of cellular content including rRNA (Bottari et al., 2009).

In this study, FISH could detect *S.* Enteritidis in the spiked pork at a concentration higher than 10^6 cfu/ml. The total time for detection was 8 hrs, which was significantly less than the standard culture method (4-6 d). The existence of low numbers of bacteria in the sample might have limited the detection sensitivity when using microscopic evaluation (Stender et al., 2001). This explanation might explain the negative result of samples that had *Salmonella* of less than 10^6

cfu/ml in this experiment. Schmid et al. (2005) found that a pre-enrichment step for 3 hrs was necessary to improve the detection limit of the FISH method for the detection of *Campylobacter* spp. at a level of 10^6 cfu/g in chicken feces. Moreover, *Campylobacter* spp. at a level of 10^2 cfu/g in chicken feces can be detected after 24 hrs of enrichment. Therefore, the pre-enrichment step may be crucial for the achievement of higher sensitivity when attempting the detection of *S.* Enteritidis that has a concentration of less than 10^6 cfu/ml.

Regarding the detection of *Salmonella*-contaminated pork samples, FISH gave 30 positive samples out of 35 samples (85.71%), and 5 negative samples (14.29%). The sensitivity of FISH in this study was 93.1%, compared to 84.41% in a previous study (Vieira-Pinto et al., 2012). The increased sensitivity may be due to the difference in the permeabilization step and the method of probe labeling used in this study. The 3′-end tailing probe was used in this study for the detection of *Salmonella* spp., instead of the 5′-end labeling probe used in the previous report (Vieira-Pinto et al., 2012). However, the FISH method in this study gave 3 false positive samples. This might be due to the existence of injured cells resulting from differences in the physical and chemical properties of the bacteriological culture process. Thus, bacteria might enter into a viable but non-culturable state during exposure to a new environment such as culture media (Colwell, 2000; Fang et al., 2003). Therefore, it is difficult to identify viable but non-culturable state cells by employing the culture method (Prescott and Fricker, 1999). Vieira-Pinto et al. (2008) showed that FISH could detect the rRNA of *Salmonella* spp. in a viable but non-culturable state. This is the main advantage of the FISH method, having the ability to target rRNA in order to detect and enumerate viable but non-culturable state bacteria, which is important for the food industry (Blasco et al., 2003; Fang et al., 2003; Ootsubo et al., 2003) and food microbiology (Vieira-Pinto et al., 2008). However, Fang et al. (2003) and Moreno et al. (2001) demonstrated that the false positive results of FISH might be due to the detection of rRNA from freshly dead cells. In this case, the false positive results can be prevented by using the pre-enrichment step at 37°C after sample collection.

Two false negative samples obtained by the FISH method may be associated with the interference of target visualization by debris or insufficient accessibility of target molecules, especially when bacteria are present in low numbers (Fang et al., 2003; Amann et al., 1995). For the detection of low numbers of bacteria, the pre-enrichment step should be included in order to improve the method's sensitivity and reduce the number of false negatives resulting from FISH detection (Blasco et al., 2003; Hahn et al., 2006). Due to the rapidity and sensitivity of FISH, this method should be considered as an important rapid screening tool for *Salmonella* spp. contamination in pork. However, future study needs to increase the specificity and sensitivity of this method by reducing false positive and false negative results, and thereby offer safer pork to consumers.

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บทคัดย่อ

สภาวะการเปิดผนังเซลล์ที่เหมาะสมต่อการเพิ่มความรวดเร็วในการตรวจหาเชื้อซัลโมเนลลาในเนื้อสุกรด้วยวิธี FISH

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ซัลโมเนลลาเป็นหนึ่งในสาเหตุหลักของโรคติดเชื้อในระบบทางเดินอาหาร ซึ่งการติดเชื้อเกิดขึ้นจากการบริโภคอาหารที่ปนเปื้อน มาตรการป้องกันและการตรวจหาเชื้อที่เหมาะสมจึงเป็นสิ่งจำเป็นเพื่อลดความรุนแรงของโรค วิธีการเลี้ยงเชื้อบนอาหารเลี้ยงเชื้อแบคทีเรีย (Culture method) ถือเป็นวิธีการมาตรฐาน แต่เนื่องจากใช้ระยะเวลาตรวจสอบนาน (ไม่น้อยกว่า 4-6 วัน) การศึกษาครั้งนี้จึงได้เพิ่มประสิทธิภาพการตรวจหาเชื้อด้วยวิธีการฟลูออเรสเซ็นต์อินซิตูไฮบริไดเซชั่น (Fluorescence *in situ* hybridization technique; FISH) เพื่อความรวดเร็วและความแม่นยำในการตรวจหาเชื้อซัลโมเนลลาในเนื้อสุกร โดยใช้ตัวติดตามชนิดโอลิโกนิวคลีโอไทด์ที่ติดฉลากด้าน 3′ จากการศึกษา พบว่าสภาพที่เหมาะสมสำหรับการเปิดผนังเซลล์เชื้อซัลโมเนลลา คือ การใช้เอนไซม์ไลโซไซม์ที่ความเข้มข้น 1 mg/ml ที่ 37°C เป็นเวลา 3 นาที วิธีการ FISH สามารถตรวจหาความเข้มข้นของเชื้อซัลโมเนลลาจากตัวอย่างเนื้อสุกรที่ถูกทำให้เกิดการปนเปื้อนเชื้อที่ระดับต่ำสุด คือ ปริมาณเชื้อ 10^7 cfu/ml. และสามารถตรวจพบการปนเปื้อนของเชื้อซัลโมเนลลาในตัวอย่างเนื้อสุกร 30 จาก 35 ตัวอย่าง ส่วนวิธีการ Culture method สามารถตรวจพบการปนเปื้อนของเชื้อซัลโมเนลลาในตัวอย่างเนื้อสุกร 29 จาก 35 ตัวอย่าง โดยวิธีการ FISH ให้ผลบวกลวง 3 ตัวอย่างและผลลบลวง 2 ตัวอย่าง เมื่อเปรียบเทียบการตรวจหาเชื้อซัลโมเนลลาในเนื้อสุกรด้วยวิธีการ Culture method กับวิธีการ FISH พบว่าค่า Kappa Statistics เท่ากับ 0.46 แสดงว่าทั้งสองวิธีสอดคล้องกันและอยู่ในช่วงของค่ามาตรฐานที่ยอมรับได้ (Moderate) ดังนั้นวิธีการฟลูออเรสเซ็นต์อินซิตูไฮบริไดเซชั่นจึงสามารถนำมาใช้เป็นเครื่องมือตรวจหาการปนเปื้อนของเชื้อซัลโมเนลลาได้อย่างรวดเร็ว และมีความเหมาะสมในการคัดแยกเนื้อสุกรปนเปื้อนออกได้ในเบื้องต้น

คำสำคัญ: FISH ตัวติดตามชนิดโอลิโกนิวคลีโอไทด์ วิธีการตรวจหาเชื้อซัลโมเนลลาอย่างรวดเร็ว เนื้อสุกร

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