Original Article

Serotype, Antimicrobial Susceptibility, and Genotype of *Salmonella* Isolates from Swine and Pork in Sa Kaew Province, Thailand

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Abstract

In January 2010, we examined the prevalence of *Salmonella* in the rectal swab of 66 swine on 8 farms and in 25 pork products at 6 meat retailers of Sa Kaew province. *Salmonella* was isolated from 3%(2/66) of swine rectal swab samples and 96%(24/25) of pork samples. In rectal swab samples, both *S.* Weltevreden and *S.* Dumfries were isolated from one sample, and *S.* Stanley from another sample. In pork, a total of 42 isolates (16 serovars) were found ; 8 strains of *S.* Rissen, 5 strains of *S.* Stanley and *S.* Anatum, 4 strains of *S.* Give and *S.* Kedougou, 3 strains of *S.* Welteverden, 2 strains of *S.* Hvittingfoss, *S.* Agona and *S.* Krefeld, and only 1 strain of 7 serovars were isolated. The resistance of *Salmonella* isolates was highest for tetracycline (69%), followed by ampicillin (50%), sulfamethoxazole-trimethoprim (36%), streptomycin (31%), chloramphenicol (14%), cefotaxime (5%), and ciprofloxacin (2%). *S.* Stanley and *S.* Weltevreden were found in both rectal swab and pork samples; however, pulsed-field gel electrophoresis and resistance profiles revealed no relationship between the rectal swab and pork isolates. In our study, *Salmonella* was isolated from only 3% of swine rectal swab samples, however, pork at a meat retailer in Sa Kaew province had a high prevalence of *Salmonella*. The result suggested that cross contamination may occur during slaughter house, transportation, to retail shop.

Keywords: antimicrobial susceptibility, PFGE, pork, *Salmonella* spp., swine.

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บทคัดย่อ

ซีโรทัยป์ การดื้อต่อยาปฏิชีวนะ และจีโนทัยป์ ของ เชื้อ *Salmonella* ที่แยกได้จากสุกรและเนื้อสุกร ในจังหวัดสระแก้วของประเทศไทย

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ผู้วิจัยทำการศึกษาหาความชุกของเชื้อซัลโมเนลลาจากการเก็บตัวอย่างจากก้นสุกรจำนวน 66 ตัวอย่างจากฟาร์มสุกร 8 แห่ง และจากเนื้อสุกร 25 ตัวอย่างจากตลาดขายเนื้อสุกร 6 แห่ง ในจังหวัดสระแก้ว ในปี ค.ศ. 2010 พบว่ามีเชื้อซัลโมเนลลาร้อยละ 3 (2/66) จากตัวอย่างจากก้นสุกร และร้อยละ 96 (24/25) จากเนื้อสุกร โดยตัวอย่างจากก้นสุกรพบเชื้อ *S.* Weltevreden และ *S.* Dumfries จากตัวอย่างหนึ่งตัวอย่าง และเชื้อ *S.* Stanley จากหนึ่งตัวอย่าง ในขณะที่พบเชื้อ 42 ชนิด จาก 16 ซีโรวาร์ในเนื้อสุกร โดยพบ *S.* Rissen มากที่สุดจำนวน 8 สายพันธุ์ตามด้วย *S.* Stanley และ *S.* Anatum เชื้อละ 5 สายพันธุ์ *S.* Give และ *S.* Kedougou เชื้อละ 4 สายพันธุ์ *S.* Weltevreden จำนวน 3 สายพันธุ์ *S.* Hvittingfoss *S.* Agona และ *S.* Krefeld เชื้อละ 2 สายพันธุ์ และพบเพียง 1 สายพันธุ์จาก 7 สายพันธุ์ สำหรับการดื้อต่อยาปฏิชีวนะนั้น พบว่าเชื้อส่วนใหญ่ดื้อต่อยาเตตร้าไซคลิน (ร้อยละ 69) ตามด้วยแอมพิซิลิน (ร้อยละ 50) ซัลฟาเมทรอกซาโซลไตรเมโทรพริม (ร้อยละ 36) สเตรปโตไมซิน (ร้อยละ 31) คลอแรมเฟนนิคอล (ร้อยละ 14) เซฟโฟทาซีม (ร้อยละ 5) และ ไซโปรฟลอกซาซิน (ร้อยละ 2) โดยพบ *S.* Weltevreden และ *S.* Stanley ทั้งจากก้นสุกรและเนื้อสุกร เมื่อนำเชื้อทั้งสองไปศึกษา รูปแบบ PFGE และดูความสัมพันธ์เทียบกับการดื้อต่อยาปฏิชีวนะนั้น ไม่พบว่ามีความสัมพันธ์กัน ในการศึกษาครั้งนี้พบเชื้อเพียงร้อยละ 3 จากก้นสุกร แต่พบการปนเปื้อนของเชื้อจำนวนมากในเนื้อสุกรที่ขายในตลาดสดในจังหวัดสระแก้ว อาจเนื่องมาจากมีการปนเปื้อนตั้งแต่โรงงานฆ่าสัตว์ การขนส่งและร้ายขายเนื้อในตลาดสด

คำสำคัญ: การดื้อต่อยาปฏิชีวนะ จีโนทัยป์ เนื้อสุกร เชื้อซัลโมเนลลา สุกร

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Introduction

Salmonella is one of the most widespread and infectious food-borne bacteria in the world and a major cause of diarrhea in both children and young adults in developing countries (Al-Abris et al, 2005). Animals, particularly chickens and pigs, are considered to be the reservoirs of these organisms, which are easily isolated from feces (Foley and Lynne, 2008). Globally, the main path of infection in humans seems to be consumption of contaminated food, such as contaminated eggs, or cross-contaminated foods and water (Yate, 2005). In Southeast Asia, *Salmonella* spp. are commonly found in chicken egg, chicken meat, and pork sold at local markets (Boonmar et al., 1998; Tran et al., 2004; Padungtod and Kaneene, 2006). There are many reports about *Salmonella* prevalence in Bangkok, Chiang Mai, and Khon Kaen area, where medical and veterinary universities, and national laboratories exist. However, prevalence of *Salmonella* in pork and swine rectal swab in Sa Kaew province has been obscure.

Preliminary studies conducted from 2007–2008 in the provinces of Sa Kaew and Nakorn Phanom found that *S.* Choleraesuis was the most prevalent serotype recovered from the blood of patients with invasive disease, and all isolates were susceptible to norfloxacin (NOR) (100%) but were often resistant to nalidixic acid (NA), ampicillin (AMP), tetracycline (TE), streptomycin (S), and chloramphenicol (C) (75%, 70%, 61%, 54%, and 26%, respectively) (unpublished data). While we are just beginning to understand the human aspect of salmonellosis, only limited information is available on the reservoirs in animals and in meat in such settings.

The objective of this study was to determine the prevalence of *Salmonella* in swine and pork in Sa Kaew province and to investigate the relationship

between *Salmonella* isolates by using serotyping, antimicrobial resistance patterns for 10 antimicrobial drugs, and pulsed-field gel electrophoresis (PFGE).

Materials and Methods

Sample collection: In January 2010, a total of 66 swine rectal swab samples were collected using a commercial swab sample set (Culture swab plus; BD Diagnostics, Brescia, Italy) from 8 local swine farms in Sa Kaew province, Thailand. The piggeries bred about 10-20 heads/farm and collected swine were about 7 months old. We randomly collected 7-12 swab samples/farm. Twenty-five pork samples in 25 meat shops were purchased from 6 local retail markets in the same month and region. All samples were kept at 4^0C in a box and sent directly to the World Health Organization National *Salmonella* and Shigella Center, NIH, Nonthaburi, Thailand for isolation and identification of *Salmonella* by conventional, biochemical, and serological testing. The stored samples were analyzed within 2 days after sampling.

Salmonella isolation and identification: Briefly, a rectal swab sample and a 25-g pork sample were placed in 9 ml and 225 ml of buffered peptone water (Merck, Darmstadt, Germany), respectively, mixed thoroughly and incubated at 37^0C for 18 hours. Next, 1 ml of the pre-enrichment culture was added to 5 ml of Rappaport Vassiliadis (RV) broth (Merck) and incubated at 42^0C for 1 day. After incubation, the RV cultures were dropped onto modified semi-solid Rappaport Vassiliadis (MSRV) agar (Merck) and desoxycholate hydrogen sulfide lactose agar (DHL)(Nissui, Tokyo, Japan) and incubated at 42^0C and 37^0C for 18 hours, respectively. Typical *Salmonella* colonies on MSRV were cloudy area around the colonies due to migration, and the typical colonies on DHL were colorless colonies with black centers. The typical *Salmonella* colonies (1–3) were selected from each specimen for confirmation on the basis of biochemical characteristics (Ewing, 1986) using triple sugar iron agar (Nissui, Japan), lysine indole motility agar (Nissui), catalase and oxidase tests, and other biochemical tests such as MR-VP test, citrate test using Simmons agar, urea hydrolysis test, arginine dihydrolase test, ornithine decarboxylase test, ONPG test, malonate utilization test and carbohydrate fermentation test. Serotyping of *Salmonella* isolates was performed on the basis of somatic and phase 1 and 2 flagellar antigens using agglutination tests with antisera (S&A Reagents Lab, Bangkok, Thailand) according to the Kauffman-White Scheme (Popoff and Minor, 2001).

Antimicrobial susceptibility testing: Antimicrobial susceptibility testing was performed using the disk diffusion method of the Clinical and Laboratory Standards Institute (CLSI, 2010) using BD Sensidiscs (BD Diagnostics, Sparks, MD) with Mueller-Hinton agar plates. Ten antimicrobial agents in the form of disks were employed for susceptibility testing of the 45 *Salmonella* isolates. The concentrations of the antimicrobial agents were as follows: ampicillin (AMP) l0 µg, amoxicillin-clavulanic acid (AMC) 30 µg, chloramphenicol (C) 30 µg, ciprofloxacin (CIP) 5 µg, cefotaxime (CTX) 30 µg, nalidixic acid (NA) 30 µg, norfloxacin (NOR) 10 µg, streptomycin (S) 30 µg, sulfamethoxazole-trimethoprim (SXT) 25 µg, and tetracycline (TE) 30 µg. In this test, *Escherichia coli* ATCC 25922 was used as the quality control strain.

Pulsed-field gel electrophoresis: PFGE macrorestriction analysis was performed in accordance with the PulseNet Protocol (Ribot et al., 2006). Briefly, 6 isolates of *S.* Stanley (5 from pork and 1 from swine rectal swab samples) and 3 isolates of S. Weltevreden (2 from pork and 1 from swine rectal swab samples) were lysed, and their genomic DNA was embedded in agarose plugs. The DNA was digested in the agarose with the restriction enzyme *Xba*I. Restriction fragments were separated using a CHEF-DR III (Bio-Rad, Hercules, CA) with the following reagents and under the following conditions: 1% Seakem Gold agarose (New England Biolabs, MA, USA) and 0.5x Tris-borate-EDTA buffer at 140C and 6 V/cm for 19 hours with switch times of 2.2–63.8 second. *Salmonella* Braenderup H9812 was used as a reference marker. The gel was stained with ethidium bromide for 30 min and destained twice for 20 min with distilled water. The gel image was captured using Gel Doc 2000 (New England Biolabs) and converted to a TIFF file. PFGE profiles were analyzed using Bionumerics software version 3.0 (New England Biolabs). A dendrogram based on the Dice coefficient was generated using the unweighted pair group method with arithmetic averaging (UPGMA) algorithm.

Data analysis: A chi-square test or Yates corrected chi-square test was used to compare the positive rates in different samples and examinations. Differences were considered significant at $p<0.05$.

Definition of Multidrug resistance: Isolates that were resistant to 3 or more classes of antimicrobial agents were multidrug resistance. SXT counted one agent.

Results

Salmonella prevalence and serotypes: Prevalence and serotype in swine rectal swab samples and pork samples are shown in Table 1. Results revealed that only 3% (2/66) of swine harbored *Salmonella* in their rectal contents, while high contamination levels of *Salmonella* were found in pork about 96% (24/25). The prevalence of *Salmonella* in swine rectal swab and pork was statistically different ($p<0.05$). In the rectal swab samples, both *S.* Weltevreden and *S.* Dumfries were isolated from one sample, and *S.* Stanley from another samples. *S.* Stanley isolated from swine rectal swab sample was hydrogen sulfide (H_2S)-producing negative. In pork samples, a total of 42 isolates (17 serovars) were found; 8 strains of *S.* Rissen, 5 strains of *S.* Stanley and *S.* Anatum, 4 strains of *S.* Give and *S.* Kedougou, 3 strains of S. Weltevreden, 2 strains of *S.* Hvittingfoss, *S.* Agona and *S.* Krefeld, and only 1 strain of 7 serovers were isolated.

Table 1 Prevalence and serotype in swine rectal swab and pork samples.

Sample	No. of samples	No. of positive	Serotype	No. of isolates
Swine feces	66	2	*S.* Weltevreden [1,2)]	1
			S. Dumfries [1)]	1
			S. Stanley [3,4)]	1
		Total		**3**
			S. Rissen	8
			S. Stanley [4)]	5
			S. Anatum	5
			S. Give	4
			S. Kedougou	4
			S. Weltevreden [2)]	3
			S. Hvittingfoss	2
Pork	25	24	*S.* Agona	2
			S. Krefeld	2
			S. Derby	1
			S. Schwarzengrund	1
			S. Panama	1
			S. Bovismorbificans	1
			S. Worthington	1
			S. Meleagridis	1
			S. Idikan	1
		Total		**42**

[1] *S.* Weltevreden and *S.* Dumfries were isolated from one rectal swab sample of swine.
[2] One strain from a rectal swab and 2 strains from meat of *S.* Weltevreden were determined PFGE analysis.
[3] *S.* Stanley isolated from a rectal swab was H_2S-negative.
[4] One strain from a rectal swab and 5 strains from pork of S. Stanley were determined PFGE analysis.

Table 2 Antimicrobial resistance of the *Salmonella* isolates.

Source	Number of isolates	Number of resistant to antibiotics[1)] (%)									
		AMP	AMC	C	CIP	CTX	NA	NOR	S	TE	SXT
Rectal swab	3	1 (33)	0	1 (33)	0	0	0	0	1 (33)	1 (33)	0
Pork	42	21 (50)	0	6 (14)	1 (2)	2 (5)	0	0	13 (31)	29 (69)	15 (36)

[1]AMP: ampicillin, AMC: amoxicillinclavulanic acid, C: chloramphenical, CIP: ciprofloxacin, CTX: cefotaxime, NA: nalidixic acid, NOR: norfloxacin, S: streptomycin, TE: tetracycline, SXT: sulfamethoxazole-trimethoprim.

Antimicrobial resistance: Table 2 shows the antimicrobial resistant of *Salmonella* isolates. In swine rectal swab isolates, 2 out of 3 (*S.* Weltevreden and *S.* Dumfries) were susceptible to all drugs, whereas *S.* Stanley was resistant to AMP, C, S, and TE only. Of the pork isolates, all were susceptible to AMC, NA, and NOR. The resistance of *Salmonella* was highest for TE (69%), followed by AMP (50%), SXT (36%), S (31%), C (14%), CTX (5%), and CIP (2%). As shown in Table 3, 45 *Salmonella* isolates were classified with 15 antimicrobial resistance profiles, and 44% (20/45) of isolates were identified as multidrug resistant which were resistant to 3 or more antimicrobial agents. The most resistant profile was AMP-TE-SXT.

Pulsed-field gel electrophoresis (PFGE) profiles and resistance profiles: A diagram of PFGE profiles of the 6 *S.* Stanley isolates restricted with *Xba*-I, constructed using UPGMA algorithm, strain, PFGE profile, source, and resistance profiles are shown in Fig 1. Six isolates of *S.* Stanley were divided into 4 PFGE profiles (S-I to S-IV), and similarity between PFGE profile S-1 and S-IV were within 80%. The PFGE profile S-I consisted of 3 isolates from pork (strain M18, M20, and M22), and 1 isolate from swine rectal swab (strains R6) belonged to the PFGE profile S-II; the other 2 isolates from pork (strain M25 and M4) belonging to the PFGE profile S-III and S-IV, respectively. Resistance profile of strains M18, M20, and M22 belonging to the PFGE profile S-I

Table 3 Resistance Profiles of isolated *Salmonella*.

Profile	Samples	
	Rectal swab	Pork
No resistance demonstrated	2	10
Resistance to 1 agent [1), 2)]		
AMP only		1
S only		1
TE only		4
SXT only		1
Resistance to 2 agents [1),2)]		
S-TE		2
AMP-TE		3
TE-SXT		1
Resistance to 3 agents [1),2)]		
AMP-TE-SXT		6
AMP-S-TE		4
AMP-C-TE		1
Resistance to 4 agents [1),2)]		
AMP-S-TE-SXT		3
AMP-C-TE-SXT		2
AMP-C-S-TE	1	1
Resistance to 5 agents [1),2)]		
C-CTX-S-TE-SXT		1
Resistance to 6 agents [1),2)]		
C-CIP-CTX-S-TE-SXT		1
Total	**3**	**42**

[1]AMP: ampicillin, AMC: amoxicillinclavulanic acid, C: chloramphenical, CIP: ciprofloxacin, CTX: cefotaxime, NA: nalidixic acid, NOR: norfloxacin, S: streptomycin, TE: tetracycline, SXT: sulfamethoxazole-trimethoprim. [2] SXT counted as one agent.

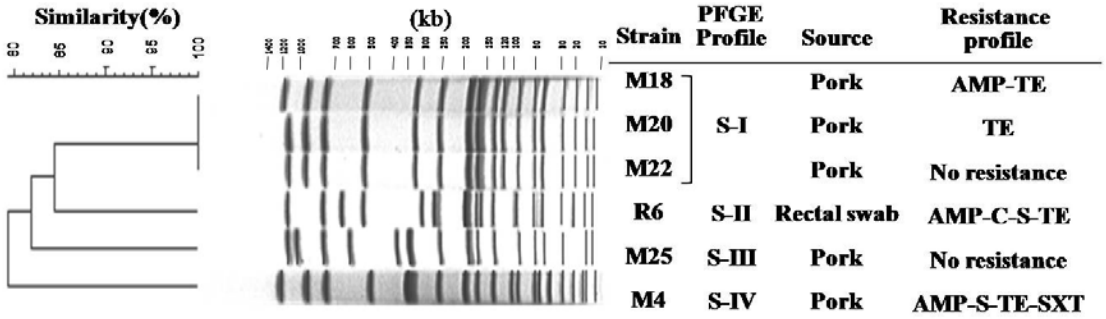

Figure 1 Diagram generated using to the Dice coefficient based on PFGE profiles of the 6 *S.* Stanley isolates restricted with *Xba*-I, constructed using UPGMA algorithms, strain, PFGE profile, source, and resistance profile. Strain R6 isolated from swine rectal swab was H_2S-producing negative.

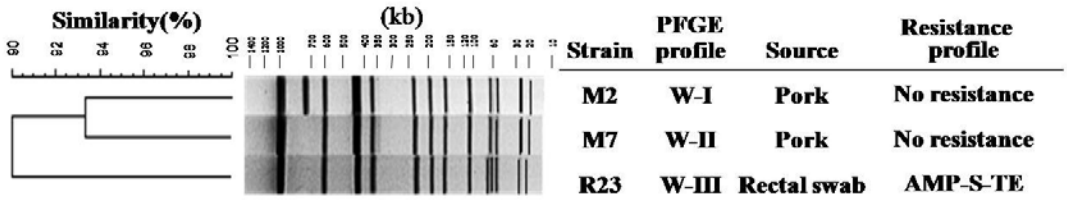

Figure 2 Diagram generated using to the Dice coefficient based on PFGE profiles of the 3 *S.* Weltevreden isolates restricted with *Xba*-I, constructed using UPGMA algorithms, strain, PFGE profile, source, and resistance profile.

was different from each other. Strain R6 isolated from swine rectal swab was H_2S-producing negative, whereas the AMP-C-S-TE resistance profile and the PFGE profile S-II, as well as other strains isolated from pork, were H_2S-producing positive, which indicated a different resistance profile and PFGE profile to those of strain R6.

Figure 2 shows the PFGE diagram of 3 strains of *S.* Weltevreden, strain, PFGE profile, source, and resistance profile. Three isolates of *S.* Weltevreden were 3 PFGE profiles such as W-I to W-3, and similarity between PFGE profile W-1 and W-3 was within 90%. In addition, the resistance profile of strain R23 (AMP-S-TE-SXT) from rectal swab and that of strains M2 and M7 (no resistance) from pork was different.

Discussion

The present study showed that the prevalence of *Salmonella* in swine rectal swab samples in Sa Kaew province was only 3% (2/66) across 8 local swine farms, and the isolated serovar were *S.* Stanley, *S.* Weltevreden, and *S.* Dimfries. This rate is significantly lower than that of a previous study conducted during 2003–2005 in central Thailand, where the overall prevalence of *Salmonella* was 19.5% (146/750, p=0.001) across 250 swine farms and the most prevalent serovar was *S.* Stanley (21.4%), followed by *S.* Rissen (14.3%) and *S.* Bovismorbificans (12.3%) (Pathanasophon et al, 2007). In addition, another study conducted in northern Thailand (Chiangmai) showed a *Salmonella* prevalence of 28% (97/349, p=0.0003) in slaughtered swine (Padungtod and Kaneene, 2006). Our results about *Salmonella* prevalence in swine rectal swab are lower than that of other studies. Our study used pig breeding in small home-based farms in Sa Kaew province. It may be one of the reasons for lower result. This study has limited information on Sa Kaew province, therefore, further details are needed for better understanding the rule of pig in epidemiology of human salmonellosis.

Pathanasophon et al. (2007) reported that the resistance of isolates to AMP was 52.7%, to C was 32%, and to TE was 68.8%, whereas the resistance to gentamicin and CIP was 8.6% and 1.0%, respectively. These findings are close to those of our study, where resistance of swine isolates to C and CIP was 33% and 0%, respectively, however these results were established from only 3 isolates from 2 swine rectal swab samples.

The prevalence and serovars in the swine rectal swab isolates were thought to depend on geographic location. There are several reports concerning *Salmonella* prevalence in Asian countries. For example, in Laos, 76% (37/49) of slaughtered pigs had *Salmonella* in the rectal swab, and the most predominant serovars were *S.* Derby and *S.* Anatum (Boonmar et al., 2008), while in 6 provinces of the Mekong Delta in Vietnam, the prevalence of *Salmonella* in pigs feces was 5.2% (23/439), and *S.* Javiana and *S.* Weltevreden were detected (Tran et al., 2004). In addition, 12% (7/110) of slaughtered pigs in Gunma, Japan harbored *Salmonella*, and the predominant serovars were *S.* Typhimurium and *S.* Derby (Takada et al., 2008). In the present study, 1 swine harbored both *S.* Weltevreden and *S.* Dumfries, while another had *S.* Stanley only. *S.* Weltevreden, *S.* Dumfries, and *S.* Stanley may be enzootic infections in swine in the studied area.

Our study revealed that the high prevalence of *Salmonella* contamination in pork samples at retail markets in Sa Kaew province was 96% (24/25), which

is statistically higher than that of the study by Angkititrakul et al. (2005) who found a prevalence of 65% (26/40) in pork samples from local markets in Khon Kaen, northeast Thailand (p=0.01). In the present study, the most prevalent serovar was *S.* Rissen (17.8%), followed by *S.* Stanley (11.1%) and *S.* Anatum (11.1%), which is the same as the prevalence found by Angkititrakul et al, 2005 (*S.* Risen of 61.5%, *S.* Stanley of 11.5%, and *S.* Lexington of 11.5%). In Khon Kaen, the resistance of pork isolates to TE was 88.5%, whereas our sampling in Sa Kaew province revealed a resistance to TE in only 68.9% of pork isolates. In addition, there was no resistance to norfloxacin in isolates from Khon Kaen and Sa Kaew. These findings suggest that *Salmonella* prevalence in pork at retail meat markets is high in rural areas in Thailand and that the predominant serovars are *S.* Rissen and *S.* Stanley. Furthermore, many isolates were found to be resistant to TE but showed no resistance to new quinolones such as norfloxacin.

Interestingly, our study failed to find any *S.* Choleraesuis in swine or pork samples from Sa Kaew province, Thailand. According to a report of Hendrinksen et al. (2009), there has been an increase in human *S.* Choleraesuis infection and a decrease in swine infection. Between 1988 and 1996, *S.* Choleraesuis was the second most common cause of septicemia globally (Boonmar et al., 1998). Additionally, a study reported the isolation of *S.* Choleraesuis from 54 Thai patients between 2003 and 2005 (Kulwichit et al., 2007). Preliminary studies conducted from 2007-2008 in the provinces of Sa Kaew and Nakorn Phanom, revealed that invasive *S.* Choleraesuis human infections had existed in this area (unpublished data).

Findings regarding the genetic relatedness of *Salmonella* isolates from retail foods of animal origin in the United States (Zhao et al., 2006) and *S.* Mbandaka isolates from a swine finishing farm in Greece (Filioussis et al., 2008) as determined by PFGE were published. In addition, the spread of genetically identical clones of *S.* Typhimurium and *S.* enterica serovar 4,(5),12:i:- in humans and swine in Thailand has been reported (Pornruangwong et al., 2008). A report by Wonderling et al. (2003) showed that PFGE could be used to characterize the heterogenicity and clonality of *Salmonella* isolates obtained from the carcasses and feces of swine at slaughter house. However, our study showed that *S.* Stanley and *S.* Weltevreden isolates obtained from pork and rectal swab samples presented different PFGE profiles and resistance profiles; a finding that implies cross contamination from slaughter houses to meat retailers at local markets. Environmental factors such as reservoir animals, water supply on farms, slaughterhouses, and local markets should be considered with respect to contamination with *Salmonella.* In this study, however, we did not take these factors into account when examining the prevalence of *Salmonella.* To confirm our hypothesis, further studies of the isolates while considering such factors should be performed.

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