MOLECULAR EPIDEMIOLOGY AND ANTIBIOGRAM OF *SALMONELLA* ISOLATES FROM HUMANS, SWINE AND PORK

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Abstract. The objectives of this study were to determine prevalence, antimicrobial resistance pattern and molecular epidemiology by pulsed-field gel electrophoresis (PFGE) of *Salmonella* isolated from humans, swine and pork. Three hundred and thirteen samples (84 humans, 84 swine and 145 pork) were collected from farms, slaughterhouses and retail markets in Nong Bua Lum Phu Province, northeastern Thailand from April 2012 to September 2013. Highest prevalence of *Salmonella* isolated from humans (43%) and swine (52%) were found at markets, followed by farms (28% and 32%, respectively) and slaughterhouses (24% and 26%, respectively). At farms, the most frequently identified serovar from humans was *S*. Stanley but from swine *S*. Rissen, the latter also being the most frequent from humans and pork samples at slaughterhouses and markets. All *S.* Rissen isolates were resistant to ampicillin, sulfamethoxazole/trimethoprim and tetracycline, and showed multidrug resistance patterns. PFGE of *Xba*I-digested chromosomal DNA performed on 36 *S.* Rissen isolates demonstrated five clusters: cluster A and B containing 1 pulsotype each from 1 isolate, cluster C contained 3 pulsotypes from 10 isolates, cluster D contained 2 pulsotypes from 19 isolates, and cluster E contained 1 pulsotype from 5 isolates. Serovars, antimicrobial resistance profiles and PFGE patterns were similar among *Salmonella* isolates from the three sources surveyed suggesting that swine is the major reservoir of salmonellosis and poor hygienic slaughterhouse procedures could promote contamination of pork and infection in humans.

Keywords: *Salmonella*, antimicrobial resistance, humans, pork, swine

INTRODUCTION

Food-borne zoonotic disease caused by *Salmonella* spp is an important public health problem in the world including Thailand. Human outbreaks are associated with consumption of contaminated animal-derived products (Mead *et al*, 1999, Mueller-Doblies *et al*, 2013) and the contamination can occur at any point in the food chain from "farm to table". High incidence of *Salmonella* infection has been found in many animal species and transmitted to humans mainly via contaminated food, especially from animal

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products, such as pork, egg and chicken meat (Chiu *et al*, 2002, Thai *et al*, 2012; Van *et al*, 2012).

Salmonellosis patients show clinical signs of diarrhea, cramp, nausea, vomiting, and may have bacteremia in severe cases (Angkititrakul *et al*, 2005). Isolation rate of *Salmonella* from children with acute dysentery is 18% in Thailand during 1998-2000 (Bodhidatta *et al*, 2002). In 2001 the serovar found in the majority of patients in Thailand was *S*. Weltevreden, followed by *S*. Enteritidis (National Institute of Health, 2001). More recently, Sithigon and Angkititrakul (2011) reported a 27.1%, 36.7%, 19.5% and 10.7% prevalence of *Salmonella* in swine, swine carcass, water and workers, respectively at slaughterhouses.

Sulfamethoxazole-trimethoprim was the drug of choice for treatment of diarrhea in Thailand; however, *Salmonella* isolated from salmonellosis patients recently was found to have increased resistance to this drug (Angkititrakul *et al*, 2005; Van *et al*, 2012). Norfloxacin now is used instead (Bodhidatta *et al*, 2002; Chiu *et al*, 2002; Angkititrakul *et al*, 2005). Patients infected with antimicrobial resistant strains usually require hospitalization (Martin *et al*, 2004). The increase in antibiotic resistance is attributed to misuse or excessive use of antibiotics in human and veterinary practice (Pappaioanou, 2004, Duong *et al*, 2006) as well as in animal husbandry, the latter for several purposes, *eg*, therapeutic, prophylaxis and growth promotion (Chiu *et al*, 2002; Thai *et al*, 2012; Van *et al*, 2012). The use of antibiotics in livestock not only selects for antibiotic-resistant bacteria but may also increase the antibiotic-resistant bacteria in humans via the food chain (White *et al*, 2004; Aarestrup *et al*, 2007). The emergence of multidrug resistance in *Salmonella enterica* from food producing animals has been reported (Van *et al*, 2012; Arguello *et al*, 2013).

Epidemiological studies of bacterial pathogens of medical, veterinary and zoonotic significance commonly focus on their presence and discrimination within and among populations of animals and humans (Terletski *et al*, 2004). There is often an interest in identifying sources of infection and pathways of transmission. Identification of transmission patterns on swine farms is an important step towards the reduction of *Salmonella* contamination in pork. *Salmonella* control is therefore necessary at all the key steps from farm to market to ensure safe products for consumers. This control measure requires rapid and reliable methods in detection, isolation, characterization, and typing of *Salmonella* isolates (del Cerro *et al*, 2002). Many of the traditional techniques used for typing *Salmonella* spp, such as biochemical profiling, serotyping and antimicrobial susceptibility profile (antibiogram) routinely used have an overall low discriminative power, making these methods of limited use in epidemiological studies (Tenover *et al*, 1997; Tadee *et al*, 2015). Molecular methods such as pulsed-field gel-electrophoresis (PFGE) have proved to provide strain specific characteristics, which allow differentiation of outbreak strains from other strains of the same species or subspecies, and are invaluable tools for reconstruction of the pathways of infection and for identification of sources of infection (Tenover *et al*, 1997). PFGE is often considered the gold standard of molecular typing method.

Thus, the application of molecular techniques can be used as an important tool to unravel epidemic patterns, trace source of infection and aid in the development of appropriate intervention strategies to reduce the presence and spread of *Salmonella* infection (Heir *et al*, 2002).

Hence, the purpose of this study was to determine the prevalence and identify serovars of *Salmonella* isolates from humans, swine and pork samples in Nong Bua Lum Phu Province, northeastern Thailand, investigate the molecular epidemiology of *Salmonella* Rissen (the most common serovar) by PFGE, and evaluate antimicrobial susceptibility.

MATERIALS AND METHODS

Sample collection

A total of 313 samples (84 rectal swabs of swine, 84 rectal swabs of humans and 145 of pork samples) were randomly collected from farms, slaughterhouses and retail markets in Nong Bua Lum Phu Province of Northeast Thailand from April 2012 to September 2013. Each sample [300 g of pork or cotton swab in Carry-Blair media (Oxoid, Cheshire, England)] was placed in a sterile plastic bag and kept chilled in an ice box during transport to the laboratory at the Faculty of Veterinary Medicine, Khon Kaen University for isolation and identification.

The study was approved by the Office of Khon Kaen University Ethics Committee for human research, no. HE 572200 and from the Office of Animal Ethics Committee of Khon Kaen University, no. 30/2555.

Bacteria isolation and identification

Samples were examined for the presence of *Salmonella* by using ISO 6579:2002 (ISO, 2002). In brief, cotton swabs from swine and humans were placed in 9 ml aliquots or 25 g of pork samples in 225 ml aliquots of buffered peptone water (BPW; Merck, Darmstadt, Germany) and incubated at 37°Cfor 24 hours. Suspensions were placed on modified semisolid Rappaport medium (MSRV; Merck) and incubated at 42°C for 24hours, streaked on xylose-lysine-desocholate agar (XLD; Merck) and on Hektoen enteric agar (HE; Merck), and incubated at 37°C for 24 hours. Plates were inspected after 24 hours and positive colonies were transferred onto triple sugar-iron-agar (TSI; Merck) and on motility indole-lysine agar (MIL; Merck). Colonies, either from XLD or HE, which tested positive on both TSI and MIL were identified as *Salmonella*. Slide agglutination test with O-antigen (S&A Reagents Lab, Bangkok, Thailand) according to Kauffman-White Scheme (Popoff and LeMinor, 2001) were used to group *Salmonella* isolates, which then were submitted to the laboratory at the Department of Medical Science, Ministry of Public Health, Thailand for further identification of serovars using slide agglutination based on Kaufman-White scheme using commercially available antiserum (S&A Reagents Lab) (Popoff and LeMinor, 2001).

Antimicrobial susceptibility testing

Antimicrobial susceptibility test was performed using a disk diffusion method of the Clinical and Laboratory Standards Institute (CLSI, 2010) employing BD Sensidiscs (BD Diagnostics, Sparks, MD) on Mueller-Hinton agar (MHA, Oxoid) plates. The concentrations of the antimicrobial agents were as follows: ampicillin (AMP) 10 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) 10 μg, sulfamethoxazole/trimethoprim (SXT) 25 μg and tetracycline (TE) 30 μg.

PFGE assay

PFGE was performed according to the "One day (24-28 hours) Standardized Laboratory Protocol for Molecular Subtyping of Non-typhoidal *Salmonella* by

PFGE" (Pulse-Net; Atlanta: CDC, 2006). In short, *S*. Rissen isolates were grown on Trypticase Soy Agar (TSA; Oxoid) at 37°C for 14-18 hours and TE buffer (100 mM Tris pH 8.0 containing 100 mM EDTA) was used to adjust the bacterial concentration to an optical density (OD) of 1.35. Bacterial cells were lysed using 10% sarcosine and 20 mg/ml proteinase K, and DNA was digested with 20 lU *Xba*I by incubating at 37°C for 4 hours. Pulsenet universal strain *Salmonella* Braenderup H9812 was used as a molecular standard marker. Digested DNA fragments were separated by CHEF-DRIII Pulsed-Field Electrophoresis System (Bio-Rad, Hercules, CA) at 6 volts for 18 hours. Gel was stained with ethidium bromide and documented by ChemiDoc™ XRS+ (Bio-Rad) equipped with Bionumerics software (Applies Maths, Kortrijik, Belgium). Dendrogram was produced using band clustering and a Ward tree building approach (CDC, 2000) with an optimization of 1% and a position tolerance of 0.75%.

Data analysis

The prevalence of serovars and resistance to antimicrobials were analyzed using descriptive statistics. BIONUMERICS software V. 3 (Applied Maths NV, Kortrijk, Belgium) was used to analyze PFGE gels using a Dice coefficient similarity index and unweighted pair group average (UPGMA) cluster analysis.

RESULTS

Prevalence and serovars of *Salmonella*

On farms, 28% ($n = 39$) of human and 32% ($n = 84$) of swine rectal swabs were *Salmonella*-positive and 16 *Salmonella* serovars were identified, the majority of serovars in humans being *S.* Muenchen, *S.* Panama, *S.* Stanley, and *S.* Weltevreden, and in swine *S.* Panama, *S.* Rissen, and *S.* Stanley (Table 1). At the slaughterhouses, 24% ($n = 38$) of human rectal swabs and 26% ($n = 120$) of pork samples were Salmonella-positive comprising 12 *Salmonella* serovars, the predominant from humans being *S.* Rissen, followed by *S.* Weltevreden and *S.* Stanley. At the markets, 43% ($n = 7$) from humans and 52% ($n = 25$) from pork samples were *Salmonella*-positive, with 5 *Salmonella* serovars identified, namely, *S.* Bardo, *S.* Give and *S.* Rissen in humans, and *S.* Rissen and *S.* Singapore from pork.

Antibiogram profiles

Salmonella serovars isolated mainly were resistance to AMP, C, SXT and T, and no isolates from any of the sources was resistant to AMC, CIP, CTX or NOR (Table 2). On farms, isolates from both humans and swine were resistant to AMP, C, NA, S, SXT and TE; and the percent antibiotic resistance in swine isolates is significantly higher than from human isolates. Interestingly, there was chloramphenicol resistance in pork isolates, which was not seen in human isolates at the slaughterhouses. At the markets, the percent antibiotic resistance in human isolates was slightly higher than in pork isolates. In addition, a total of 78 (83%) multidrug resistant (MDR) *Salmonella* isolates were identified, comprising14 different MDR patterns, with 72 (92%) isolates resistant to 2-6 antimicrobial agents. Six (8%) isolates were resistant only to 1 antimicrobial agent and 16 (17%) were susceptible to all 10 antimicrobial agents tested.

PFGE analysis of *S.* Rissen isolates

Using PFGE to assess genetic relatedness among *S.*Rissen isolates from 31 swine and 5 human isolates, 8 pulsotypes and 5 clusters (A-E) with 95% pattern similarity were discerned (Fig 1). Cluster A and B contained 1 isolate each from farm swine; cluster C had 3

Table 1
Prevalence of *Salmonella* isolates from farms, slaughterhouses and markets in northeastern Thailand, 2012-2013.

Source	Sample type (*n*)	Number positive (%)	Group	Serovar (number, %)
Farm	Human (39)	11 (28)	B	Derby (1, 9)
				Stanley (2, 18)
			C	Muenchen (2,18)
			D	Panama (2, 18)
			E	Give (1, 9)
				Weltevreden (2, 18)
			I	Vagesak (1, 9)
	Swine (84)	27 (32)	B	Stanley (3, 11)
				Typhimurium(2, 7)
				Gloucester (1, 4)
				Gaminara (1, 4)
				i.4,5,12:i:- (1, 4)
			C	Hindmarsh (1, 4)
				Bareilly (1, 4)
				Rissen (9, 33)
			D	Panama (5, 18)
			E	Eastbourne (1, 4)
			G	Kedougou (2, 7)
Slaughterhouse	Human (38)	9 (24)	B	Stanley (1, 11)
				i.4,5,12:i:- (1, 11)
			C	Bareilly (1, 11)
				Rissen (4, 44)
				Hindmarsh (1, 11)
			G	iv.43:z4z23:-(1, 11)
	Pork (120)	31 (26)	B	Stanley (3, 10)
				Gaminara (1, 3)
				i.4,5,12:i:- (2, 6)
			C	Rissen (13, 42)
				Virchow (1, 3)
			D	Panama (3, 10)
			E	Weltevreden (5, 16)
				Anatumn (1, 3)
			G	Kedougou (2, 6)
Market	Human (7)	3 (43)	C	Rissen (1, 33)
				Bardo (1, 33)
			E	Give(1, 33)
	Pork (25)	13 (52)	B	i.4,5,12:d:- (1, 8)
			C	Rissen (9, 69)
				Singapore (3, 23)

pulsotypes from 8 swine and 2 human isolates; cluster D contained 2 pulsotypes from16 swine and 3 human isolates; and cluster E had 1 pulsotype from 2 swine isolates at slaughterhouses and 4 swine isolates at markets. No relationship between isolates from swine and humans at farms were observed and only isolates from farms were present in every cluster. At slaughterhouses and markets there

Table 2
Antibiogram of *Salmonella* isolated from farms, slaughterhouses and markets in northeastern Thailand, 2012-2013.

Source	Sample type (*n*)	Percent resistance									
		AMP	AMC	C	CIP	CTX	NA	NOR	S	TE	SXT
Farm	Humans (11)	64	-	27	-	-	9	-	45	36	27
	Swine (27)	81	-	33	-	-	-	-	63	63	44
Slaughterhouse	Humans (9)	67	-	-	-	-	-	-	11	56	44
	Pork (31)	77	-	16	-	-	-	-	26	61	55
Market	Humans (3)	67	-	-	-	-	33	-	33	33	67
	Pork (13)	61	-	-	-	-	23	-	8	54	54

AMP, ampicillin 10 µg; AMC, amoxicillin /clavulanic acid 30 µg; C, chloramphenicol 30 µg; CIP, ciprofloxacin 5 µg; CTX, cefotaxime 30 µg; NA, nalidixic acid 30 µg; NOR, norfloxacin 10 µg; S, streptomycin 10 µg; SXT, sulfamethoxazole/trimethoprim 25 µg, TE, tetracycline 30 µg.

was a correlation between isolates from swine and humans, especially in cluster D with 12 isolates from swine and 4 from humans and in cluster C with 9 isolates from swine and 1 isolate from humans. Although clusters C and D had the highest number of isolates, there were no isolates from humans on the farms. There was no swine isolate from slaughterhouses in cluster C isolates from humans working at the markets.

DISCUSSION

Swine is regarded as a major source of salmonellosis (Swanenburg *et al*, 2001; Xia *et al*, 2009**;** Thai *et al*, 2012). In this study, the prevalence of *Salmonella* spp in swine from farms in Nong Bua Lum Phu Province, northeastern Thailand was similar to previous studies (25-27.1%) in Khon Kaen Province, located in the same northeastern region of the country (Angkititrakul *et al*, 2003; Sithigon and Angkititrakul, 2011). This value is lower than those reported in previous studies in 2002 (69.5%) and 2005 (62.9%) in swine in Chiang Mai, northern Thailand (Patchanee *et al*, 2002; Dorn-In *et al*, 2009).

Farm is the starting point of the swine production line and prevention of *Salmonella* infection at this point will block the spread of this infection in pork sold in the markets. Similarly, the slaughterhouse is another important step in the pork production line and inadequacies in hygienic routine can lead to colonization and spread of *Salmonella* to pork via contaminated carcasses, slaughtering equipment and/or workers. In our study, at slaughterhouses the prevalence of *Salmonella* in humans was higher than in previous studies (10.7%) in Khon Kaen Province (Sithigon and Angkititrakul, 2011). However, the prevalence of *Salmonella* in pork

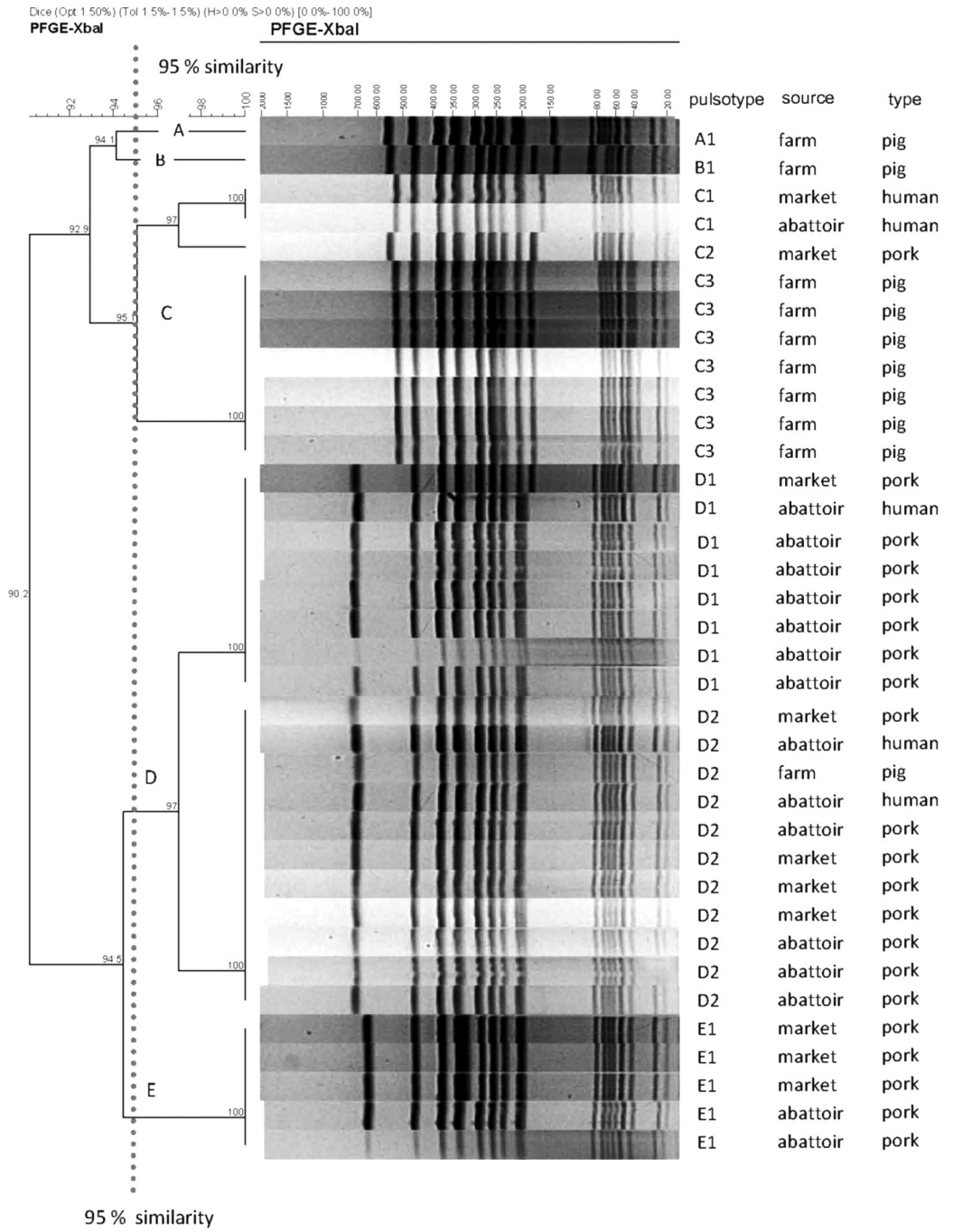

Fig 1–Dendrogram of thirty-six pulsed field gel-electrophoresis-XbaI profiles of *Salmonella* Rissen isolates from farms, slaughterhouses and markets in Nong Bua Lum Phu Province, northeastern Thailand, 2012-2013. Similarity was determined using Dice co-efficient and UPGMA clustering. Percent similarity is indicated at branch site.

in our survey was lower than those reported in lower northern part of Thailand (52.8%) and in Khon Kaen Province (85%) (Angkititrakul *et al*, 2003; Moolsawat *et al*, 2005). There are many factors associated with the *Salmonella* infection in humans including personal hygiene practices and types of tasks undertaken by a worker. Contamination in pork could be from substandard slaughterhouses with unsanitary utensils and water. Therefore, quality pork depends on personal hygiene and proper management in the slaughterhouses.

We found the prevalence of *Salmonella* in pork was highest at the markets, a rate similar to previous studies (58.2%) in minced pork in Khon Kaen Province in which (Angkititrakul *et al*, 2014). This finding is also consistent with that of Butsi *et al* (2009) on *Salmonella* contamination in pork carcasses (33.3%), which increases after slaughtering and processing (65%), reaching highest value (74.3%) at retail markets. Poor sanitary procedures, such as on-floor slaughtering and cutting, absence of separation between dirty and clean zones and on-floor placing of pork in dirty transportation vehicles, are probable sources of contamination. At markets, in the current study the prevalence of *Salmonella* in humans was higher than the previous study (31.2%) in Khon Kaen Province (Jamjane *et al*, 2007). This is probably due to unsanitary conditions in the market and poor hygiene of butchers.

S. Rissen was the most prevalent serovar in swine at farms, in humans and in pork samples at slaughterhouses and at markets, in agreement with previous studies conducted in Khon Kaen and Chiang Mai (Angkititrakul *et al*, 2005; Sithigon and Angkititrakul, 2011; Kumpapong *et al*, 2013). On the other hand, the most frequently identified serovars in humans on swine farms were (on descending order of frequency) *S*. Weltevreden, *S*. Stanley, *S*. Panama and *S*. Muenchen. This might be due that human contract salmonellosis from other sources, such as birds or insects. There were many reports of animals other than swine being reservoirs of this disease (Devi and Murray, 1991, Angkititrakul *et al*, 2008; Kumpapong *et al*, 2013). *S*. Rissen is one of the most common serovars found in swineherds, pork and in gastrointestinal patients in different parts of the world. Vico *et al* (2011) reported MDR *S*. Rissen in several swine farms in Spain. In South Korea, *S*. Rissen is the predominant serotype found in healthy and diarrheal swine with a high frequency of resistance to tetracycline, streptomycin and sulfamethoxazole (Lim *et al*, 2009). Similarly, in Vietnam, *S*. Rissen was isolated from retail pork, with the most frequent antibiotic resistance being to ampicillin, chloramphenicol, streptomycin, sulphonamides/trimethoprim and tetracycline (Thai *et al*, 2012). In addition, an outbreak of *S*. Rissen in the USA resulted in one death in 2009 (Higa, 2011). In Thailand, *S*. Rissen has long been reported to be among the most frequent serovars in swineherds (Dorn-in *et al*, 2009). This serovar is also the most prevalent serovar isolated from pork carcasses and ready-to-eat products in Thailand and has been shown to be efficiently transmitted from swine to humans during pork production and processing (Sanguankiat *et al*, 2010).

PFGE profiling revealed a relationship among the *S*. Rissen isolates from swine farms, slaughterhouses and markets indicating that swine from farm was probably the source of *Salmonella* contamination in the pork samples. *S*. Rissen isolates from humans were found only at slaughterhouses and markets, in particular isolate 4 and 1, respectively.

Identical PFGE pulsotypes obtained

from various production steps in a single area during one sampling day might indicate cross-contamination within that area (Tadee *et al*, 2015). This could be seen clearly in D2 pulsotype in our study, in which *Salmonella* isolates were from swine at farms, from pork and humans at slaughterhouses and from pork at markets. In addition, in D1 pulsotype consisted of *Salmonella* isolates from pork and humans at slaughterhouses collected on the same day. *Salmonella* in swine carriers might have shed bacteria that were then transferred to *Salmonella*-free swine directly or via the environment. In addition, inadequate hygienic precautions in the routine production practices might promote colonization and spread of *Salmonella* to pork via contaminated carcasses, slaughtering equipment and/or workers' hands at any of the slaughtering steps (Swanenburg *et al*, 2001). Furthermore, that the same groups of strains were isolated from the same location on different days provide evidence for the persistence of those strains (Prendergast *et al*, 2009). This notion is given credence by the detection of certain isolates in the market and their C3 pulsotype at the slaughterhouse. This indicates improper cleaning or inadequate hygienic practices in the market and in large areas of the slaughterhouse.

Resistance to antimicrobial agents was similar among the three sources investigated. The highest antimicrobial resistance observed was to AMP followed by TE and SXT, which are the most widely used antibiotics for humans and animals. No *Salmonella* isolates were resistant to amoxicillin/clavulanic acid, cefotaxime, ciprofloxacin and norfloxacin, similar to the previous reports in northern and central Thailand (Sirichote *et al*, 2010; Kumpapong *et al*, 2013; Tadee *et al*, 2015), except for ciprofloxacin, to which 12.5% of live swine and livestock in northern Thailand are resistant (Hanson *et al*, 2002). Similar observations were reported in China, Taiwan and USA (Olsen *et al*, 2001; Chiu *et al*, 2002; Xia *et al*, 2009). Almost *Salmonella* isolates showed multidrug resistance consistent with the previous studies in Korea, Vietnam, Malaysia and Thailand (Lim *et al*, 2009; Thai *et al*, 2012; Van *et al*, 2012; Kumpapong *et al*, 2013; Pornsukarom *et al*, 2015). The high resistance rates in this study might be due to the widespread application of antibiotics to animals in Thailand (Angkititrakul *et al*, 2005). In addition, antibiotics can be readily purchased in veterinary drug stores, and farmers use antibiotics intensively as prophylactics for their animals (Thai *et al*, 2012; Tadee *et al*, 2015). As fluoroquinolones are the drugs of choice for treatment of complicated gastrointestinal infection (Aarestrup *et al*, 2007), it is imperative that this class of antimicrobials not be misused in the swine industry.

In conclusion, this study supports previous studies indicating *S.* Rissen as being common in swine and pork products. *S.* Rissen isolates showed multidrug resistance. Antibiogram profiles and PFGE patterns support notion that swine was the main source of *Salmonella* infection in the region of Thailand surveyed. Introduction of infected swine animals into slaughterhouses, lack of proper hygienic procedures in these premises and poor hygienic practices of employees are factors contributing to the spread of *Salmonella* from farm to slaughterhouse and ultimately to pork in the market.

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