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Salmonella in pork retail outlets and dissemination of its pulsotypes through pig production chain in Chiang Mai and surrounding areas, Thailand

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ABSTRACT

Salmonella spp. is acknowledged as a significant zoonotic foodborne pathogen throughout the world. Contaminated pork consumption is considered as a main cause of human salmonellosis. In the later stage of the pig production chain, poor hygiene and unsuitable storage conditions in retail outlets are considered to be key factors linked to the risk of *Salmonella* infection. The purpose of current study, which was conducted throughout April 2014 to September 2014, was to determine the prevalence and characteristics of *Salmonella* spp. in pork sold at the retail stage in wet markets and supermarkets in the Chiang Mai urban area of Thailand. Additionally, clonal relations between *Salmonella* strains described in this study and those identified in earlier study from the same geographical area were considered. It is provided as a means of contributing to current knowledge regarding *Salmonella* epidemiology with an ultimate aim of improved food security and consumer protection in this region. From a total of 82 pork samples analyzed in this study, 41% were positive for *Salmonella*, with prevalence of 73.2% from wet markets ($n = 30/41$) and 9.8% from supermarkets ($n = 4/41$). Twelve *Salmonella* serovars were identified, *S.* Rissen being the most commonly encountered. Antibiotic resistance of the isolates was highest for ampicillin and tetracycline (53%), followed by streptomycin (44%). Pulsed-field gel electrophoresis (PFGE) and subsequent geographical distribution analysis indicated that the clonal *Salmonella* strains originated from multiple sources had been spread over a wide area. The existence of a common pig supply chain "farm-slaughterhouse-retail" transmission route is inferred. Continuous monitoring of *Salmonella* along the entire production chain is needed to reduce contamination loads and to ensure the safety of pork products for end consumers.

1. Introduction

Salmonella spp. is considered to be one of the most significant zoonotic foodborne pathogens and is a major public health concern throughout the world (Visscher et al., 2011; Magwedere et al., 2015; Gibert, 2015). Approximately 95% of salmonellosis in humans is related to animal-origin food consumption (Lynne et al., 2015); contaminated, undercooked pork are implicated in several human cases (Giovannini et al., 2004; Mürmann et al., 2009; Pires et al., 2014).

Salmonella can be introduced into humans at any point along the pig production chain (White et al., 2001; Dorn-In et al., 2009; Wang et al., 2015). At the farm level, *Salmonella*-infected pigs carrying the organisms in their intestinal tracts can spread *Salmonella* to other pigs directly via the fecal-oral route or indirectly through fecal contamination of the environment (Farzan et al., 2006; Hauser et al., 2011). High bacterial loads in pig intestinal tracts can be expected to cause high contamination rates at every level of the production chain (Tadee et al., 2014). The slaughtering procedure itself can be

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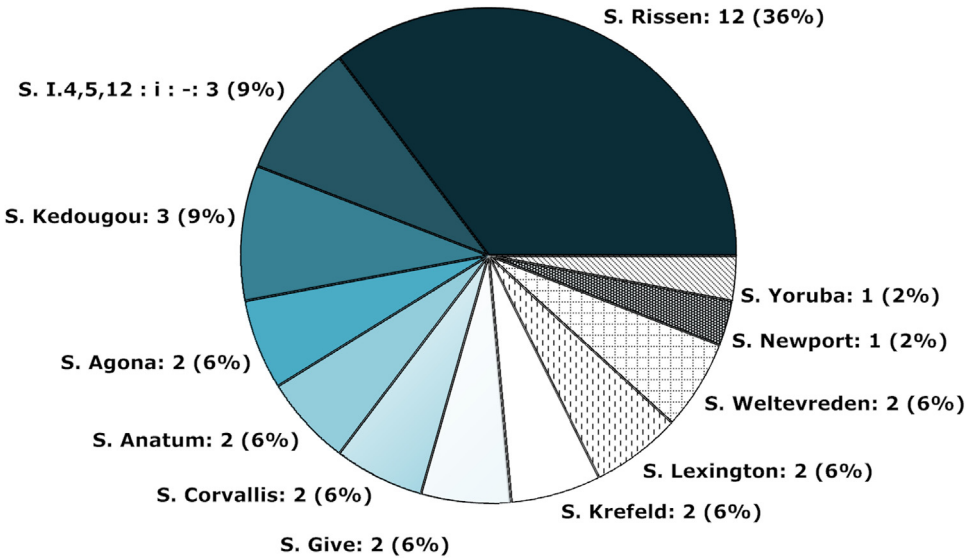

Fig. 1. Distribution of *Salmonella* serovars isolated from pork products from wet markets and supermarkets in the Chiang Mai urban area from April 2013 through September 2014.

a major source of spreading the organism from the pig's intestinal contents to slaughtered carcasses if the processing is not accomplished properly (Swanenburg et al., 2001; Gomes-Neves et al., 2012).

At the later stages of the production chain, the appearance of *Salmonella* on pork at retail markets or butchers results in a high chance of exposure in consumers and is correlated with a large number of human salmonellosis cases (Prendergast et al., 2009; Bollaerts et al., 2010). Appropriate handling and processing techniques, good general hygiene and suitable storage conditions have been suggested as effective means of reducing or stopping colonization activity of *Salmonella* (Berends et al., 1997; Mürmann et al., 2009). Concern about processing procedures is the reason that most health-conscious consumers prefer pork products purchased from a supermarket rather than those obtained from an open marketplace with stalls of fresh food and other items for sales, "wet market" (Hansen et al., 2010). The vacuum sealed packages with origin tracking labels in supermarkets greatly increase the confidence of consumers when they are making their selection (Heather, 2014). Despite these retail practices, there is no guarantee that the pork purchased from supermarkets are free of bacterial contamination (Whittaker et al., 2009).

Tadee and his colleagues demonstrated an association among *Salmonella* pulsotypes at pre-harvest and harvest levels of pig supply chain in Chiang Mai, Thailand (Tadee et al., 2015). Spreading of *Salmonella* from the standard pig farm to slaughterhouse over the wide area was extrapolated. The study, however, included only a limited number of *Salmonella* pulsotyping profiles taken from pork products on sale at a number of retail outlets and butchers. Additional data are required to broaden epidemiological knowledge of *Salmonella* throughout the entire pig production chain in order to improve methods of reducing the risk posed by that organism.

The objectives of this study were to investigate the prevalence and characteristics of *Salmonella* on retail's pork in wet markets and supermarkets in the Chiang Mai urban area, and to determine clonal relationships between *Salmonella* strains described in this study with those *Salmonella* strains identified in the Tadee et al. (2015) study. That expansion of knowledge in *Salmonella* epidemiology could enhance domestic consumer protection and help promote *Salmonella*-free pork through appropriate practices for pork handling and consuming.

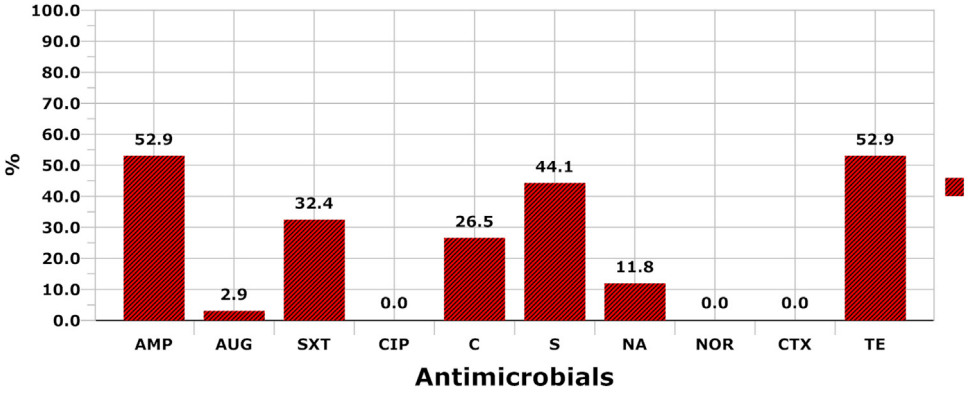

Fig. 2. Antimicrobial resistance prevalence of *Salmonella* isolates originating from pork products at the retail stage from the Chiang Mai urban area between April 2013 and September 2014.
Antibiotic abbreviations: ampicillin (AMP); amoxicillinclavulanic acid (AUG); sulfamethoxazole-Trimethoprim (SXT); ciprofloxacin (CIP); chloramphenicol (C); streptomycin (S); nalidixic acid (NA); norfloxacin (NOR); cefotaxime (CTX); tetracycline (TE).

2. Materials and methods

2.1. Sample sizes calculation

A total of 82 pork samples were designated from "Estimate Percentage" function in http://winepi.net/ (Blas, 2006). While "70%" was an approximate rate of *Salmonella* contamination in pork samples of Thailand (Angkititrakul et al., 2005; Butsi et al., 2009); it was used as expected prevalence. Ten percent and 0.95 were selected as "e" and "Z1-α/2" value, respectively for features parameter needed.

2.2. Sample collection

All whole pork cuts samples of approximately 300 g each, were purposely collected from all 14 wet markets ($n = 41$) and 22 supermarkets ($n = 41$) located in the Chiang Mai urban area (around the Chiang Mai Old City area) between April 2013 and September 2014. At wet markets sampling sites, two or three pork samples were purchased at each target location. Additionally, at least one pork sample was randomly selected from each supermarket. The pork samples were individually packaged in a sterile container and transported in an icebox to the Bacteriology Laboratory, Faculty of Veterinary Medicine, Chiang Mai University, for *Salmonella* isolation and identification within 24 h after sample collection.

2.3. Salmonella *isolation and identification*

Salmonella spp. isolation and identification was implemented following ISO 6579:2002 Amendment 1:2007, Annex D technique, to determine the prevalence of *Salmonella*-positive samples. A 25 g pork sample was weighed using aseptic techniques and stomached for 120 s with 225 mL of buffered peptone water (BPW; Merck, Germany). After incubation at 37 °C for 24 h, an aliquot of 0.1 mL of homogenized mixture was transferred to Modified Semi-solid Rappaport-Vassiliadis agar (MSRV; Oxiod, United Kingdom) and stored at 42 °C for 24 h. The cultures with turbid, grey matter were streaked on Xylose Lysine Deoxycholate agar (XLD; Oxiod, United Kingdom) and Brilliant Green Phenol Red Lactose Saccharose agar (BPLS; Merck, Germany) then incubated at 37 °C for 24 h. Finally, colonies presumptively identified as *Salmonella* by their black color, were definitively identified with biochemical and serum agglutination tests.

2.4. Statistical analysis

Descriptive data regarding the *Salmonella* positive prevalence among pork samples obtained from wet markets and supermarkets were analyzed using PHStat®. Differences in *Salmonella* spp. prevalence among types of market and their odds ratio were analyzed using Fisher's exact test by R® version 3.2.1. If the p-values less than 0.05, the tested values are considered as statistically significant differences.

2.5. Salmonella *serotyping and antimicrobial susceptibility testing*

Salmonella isolates were serovar identified by the WHO National *Salmonella* and *Shigella* Center Laboratory (NSSC), National Institute of Health, Department of Medical Science, Nonthaburi, Thailand. All strains were submitted for susceptibility testing to a panel of ten different antimicrobials using agar disk diffusion, following Clinical and Laboratory Standards Institute guidelines (CLSI, 2011). Antimicrobials tested included ampicillin (AMP) 10 μg, amoxicillin-clavulanic acid (AUG) 20/10 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) 23.75/1.25 μg and Tetracycline (TE) 30 μg. In the sensitivity measurements, *Escherichia coli* ATCC® 25922 was used as a control strain. All strains that evidenced intermediate resistance were combined with the susceptible strains to avoid overestimation of resistance.

2.6. Pulsed-field gel electrophoresis (PFGE)

PFGE genotyping of *Salmonella* was performed following the Centers for Disease Control and Prevention (CDC) standardized PulseNet protocol for *Salmonella* as previously described (Ribot et al., 2006), and also completed by the WHO National *Salmonella* and *Shigella* Center (NSSC), National Institute of Health, Department of Medical Science, Nonthaburi, Thailand. The PulseNet "Universal" standard strain *Salmonella enterica* serovar Braenderup H9812 was used as a reference marker and *Xba*I was used as the digestion enzyme. Dendrograms were developed using BioNumerics software version 7.1 employing the unweighted-pair group method with arithmetic means (UPGMA). The 2.5% optimization values and 2.5% band position tolerances were submitted to comparative analysis. PFGE banding patterns with a similarity index >70% were classified as being in the same genotype cluster.

2.7. Geographical distribution of Salmonella *strains*

The map of the boundaries of Chiang Mai and Lamphun provinces as well as the locations of farms, slaughterhouses and markets was created using QGIS program version 2.10 (QGIS Development Team, 2015). The geographic locations of slaughterhouses and markets were precisely marked with a GPS device; the locations of farms were plotted at the sub-district level due to a lack of exact coordinates. Strains of *Salmonella* were grouped and demonstrated on each location based on 100% similarity threshold by PFGE.

3. Results

Of all the pork samples tested, thirty-four were positive with *Salmonella* (34/82 = 41.5%, 95% CI: 30.8-52.1%). Of the positive samples, thirty had been acquired from wet markets (30/41 = 73.2%, 95% CI: 59.6-86.7%), while only four *Salmonella*-positives samples were obtained from supermarkets (4/41 = 9.8%, 95% CI: 0.1-18.8). There was a statistically significant difference between the *Salmonella* prevalence on Thai retail pork which originated from wet markets and those which originated from supermarkets ($p < 0.01$), with odds ratio, 25.2 (Table 1).

Twelve *Salmonella* serovars were identified. *S.* Rissen was the highest frequency found (36%), followed by *S.* I. 4,5,12: i: – (9%) and *S.* Kedougou (9%). However, two serovars, *S.* Newport and *S.* Yoruba, were detected in only one isolate each (Fig. 1).

Over 80% of all *Salmonella* strains identified in the study were resistant to at least one antimicrobial. The resistance of isolates was highest for ampicillin and tetracycline (53%) followed by streptomycin (44%) (Fig. 2). In contrast, no resistance to ciprofloxacin, cefataxime or norfloxacin was detected. Moreover, only one *Salmonella* isolate identified in this study was resistant to amoxicillin-clavulanic acid.

Dendrograms of PFGE profiles are shown in Fig. 3. Thirty *Salmonella* strains from the present study, comprising *S.* Rissen ($n = 12$), *S.* I. 4,5,12: i: – ($n = 3$), *S.* Kedougou ($n = 3$), *S.* Anatum (n = 2), *S.* Give (n = 2), *S.* Agona (n = 2), *S.* Lexington (n = 2), *S.* Newport ($n = 2$), *S.* Weltevreden ($n = 1$) and *S.* Corvallis ($n = 1$), were compared with 15 other strains previously recovered from pig farms and slaughterhouses in Chiang Mai city and surrounding areas between 2011 and 2013 (Tadee et al., 2015), including *S.* Rissen ($n = 7$), *S.* I. 4,5,12: i: – ($n = 4$), *S.* Typhimurium ($n = 2$) and *S.* Weltevreden ($n = 2$). An

Table 1
Salmonella spp. prevalence (%) and odds ratios in pork products acquired at the retail level from different market types in the Chiang Mai urban area between April 2013 and September 2014.

Type of market	No. of samples	No. of positive samples	% (95% CI)	Prevalence comparison	
				Odds ratio	P-value
Wet market	41	30	73.2 (59.6–86.7)	25.2 (7.3–87.3)	<0.01
Supermarket	41	4	9.8 (0.1–18.8)	References	

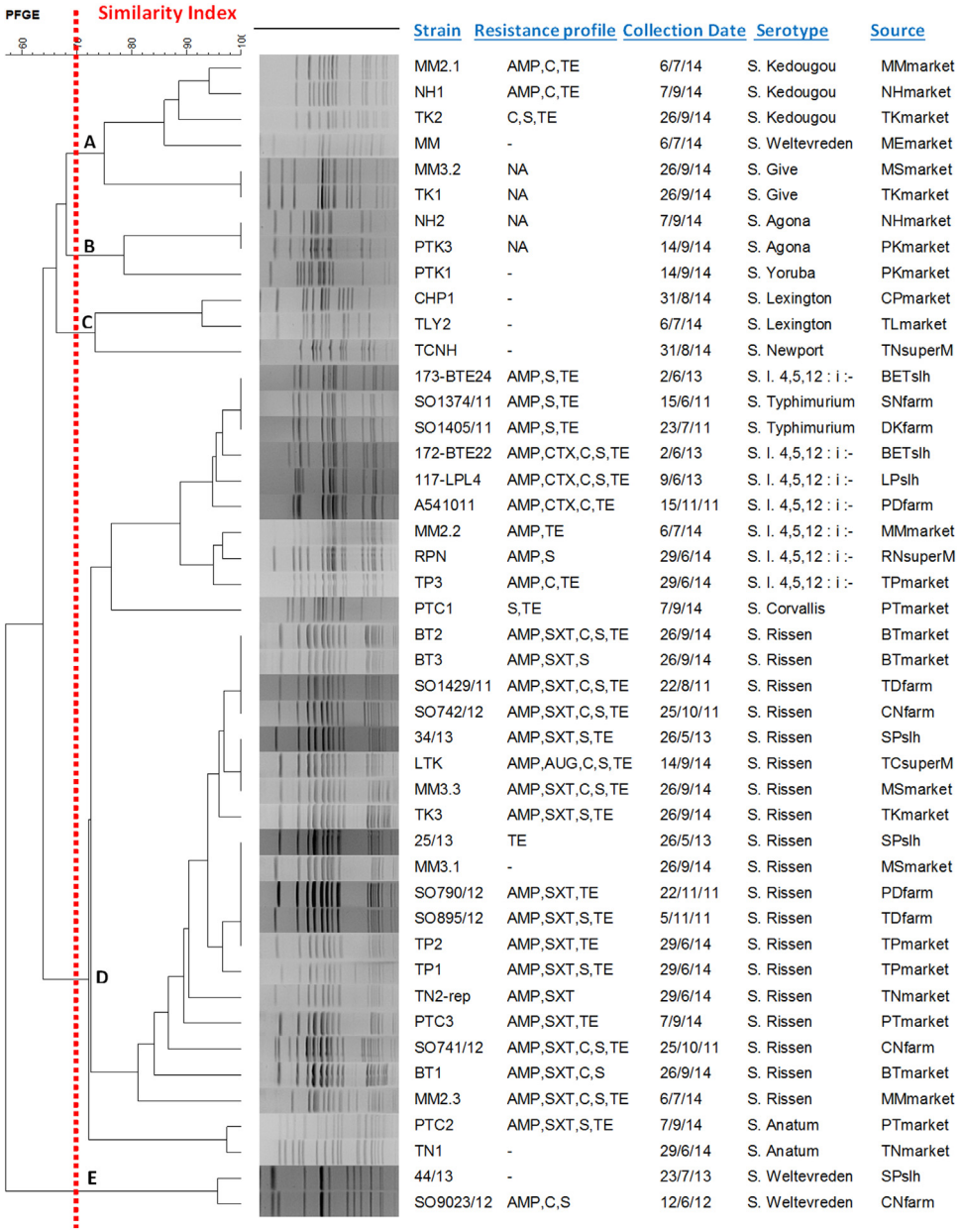

Fig. 3. Dendrograms generated using UPGMA algorithms based on PFGE profiles with their phenotypic characterization and epidemiological data for *Salmonella* strains obtained over a 4-year period in Chiang Mai and surrounding areas.
Antibiotic abbreviations: ampicillin (AMP); amoxicillinclavulanic acid (AUG); sulfamethoxazole-Trimethoprim (SXT); ciprofloxacin (CIP); chloramphenicol (C); streptomycin (S); nalidixic acid (NA); norfloxacin (NOR); cefotaxime (CTX); tetracycline (TE).

appraisal of 45 *Salmonella* strains with a similar index cut-off of 70% using Pulsed-field gel electrophoresis (PFGE) created profiles of five major genotypic clusters (A-E) with 32 different PFGE patterns. Overall, D-Cluster was the major cluster found in this study, involving two-thirds of all strains in the dendrograms. Common PFGE patterns were established of the groups of indistinguishable strains obtained from various production stages and with several spatiotemporal distributions (BT2, BT3, SO1429/11 and SO742/12, as well as 25/13, MM3.1, SO790/12, SO895/12 and TP2). Additionally, shared PFGE patterns were demonstrated in *Salmonella* strains

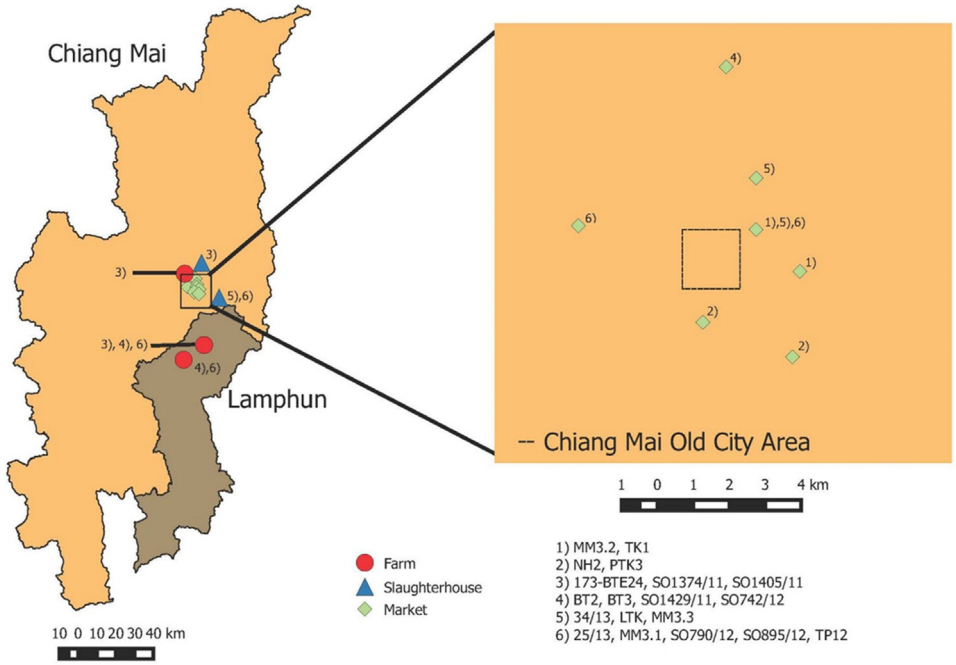

Fig. 4. Geographic location of farms (red cycles), slaughterhouses (blue triangles) and markets (green diamonds) involved in this study. The numbers next to each location in the Old City area refers to the groups of *Salmonella* pulsotypes based on a 100% similarity index. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

recovered from different areas on the same sampling date (MM3.2 and TK1) as well as those recovered during widely separated sampling periods (NH2 and PTK3). However, the two *S.* Rissen strains (TP1 and TP2) derived from TP-market (TP is a local market name) with identical sampling dates were revealed to have distinct PFGE patterns.

In terms of geographic location analysis, six *Salmonella* pulsotypes having more than one strain grouping each are displayed by original location (Fig. 4). Two *S.* Give strains with an identical pulsotype (MM 3.2 and TK1) derived from two markets located approximately 2 km apart were identified. Furthermore, Two *S.* Agona strains which share PFGE patterns (NH2 and PTK3) were also found at nearby sites. Some indistinguishable strains recovered from different production levels were also found. Interestingly, five *S.* Rissen strains with matching pulsotypes (25/13, MM3.1, SO790/12, SO895/12 and TP2) were recovered from farms, slaughterhouses and markets located within a 40 km radius.

4. Discussion

The survey of *Salmonella* contamination in pork at the post-harvest stage in the Chiang Mai urban area between April 2013 and September 2014 was conducted. An overall prevalence was 41% (95% CI: 31–52%). This is not dissimilar to the 29% (95% CI: 18–40%) prevalence determined by Padungtod and Kaneene (2006) who surveyed pork on sale at markets throughout the provinces of Chiang Mai and Lamphun, Thailand, and with the 40% (95% CI: 34–45%) prevalence found on pork being sold in northern-Vietnam (Thai et al., 2012). The high levels of *Salmonella* contamination suggest that is there remains a problem with the quality of the pork being sold in retail outlets within the Chiang Mai study zone and that this important public health situation appears changed from the study conducted a decade ago. Since the desired precision for sample size determination in the study is large (i.e. 10%) leading to a small sample size in the study, the observed prevalence of *Salmonella* contamination in Chiang Mai may not be representative of the infection prevalence on pork being sold in other provinces throughout Thailand.

Interestingly, approximate of 70% of the samples obtained from wet markets in this study were positive for *Salmonella.* Actually, *Salmonella* can grow at a wide range of high temperature. An absence of cover materials, inappropriate storage conditions as well as an inadequate disinfection of retail purchasing areas during the pork cutting and handling processes at wet markets can increase bacterial colonizing activity (Berends et al., 1998a). Moreover, bacterial load is redistributed all over the pork and can proliferate with exposure to contaminated equipment by knives, cutting blocks and working tables (Berends et al., 1997; Tadee et al., 2014). In contrast, only 10% of supermarket-derived pork were found to be contaminated. Sealed packages with appropriate storage conditions including refrigerated shelves (i.e., below 6 °C) can reduce the occurrence of *Salmonella* contamination (Lo Fo Wong et al., 2002; Huang et al., 2015).

Several serovars were identified in this study, with *S.* Rissen being the most commonly encountered serovar at the retail stage. Moreover, Rissen constitutes a dominant serovar found in the pig industry in the northern region of Thailand (Padungtod and Kaneene, 2006; Dorn-In et al., 2009; Boonkhot et al., 2015), and southern-Thailand (Lertworapreecha et al., 2013). It is also one of the most important serovars in humans in this country (Angkititrakul et al., 2005). This situation is a marked contrast to the studies conducted elsewhere which have found Rissen to be an uncommon serovar in pig production, Irish retail pork (Prendergast et al., 2009). Besides, this serovar was not identified at all in pork purchased at the retail level in Germany (Schwaiger et al., 2012) or in food products in the United States (Jackson et al., 2013) as well as in China (Cai et al., 2016). It is likely that geographical area plays a role in the differences in sero-distribution. Unusually, serovars infrequently found in pork production such as Corvallis, Newport and Yoruba were noticed in this study. Most likely, the contamination is not introduced into the pig production process from a previous production stage. Rather, shipping hygiene as well as sanitation and storage conditions at the retail level might be involved

in the cross contamination (Escartín et al., 1995), especially in wet markets. That is supported by our finding of greater *Salmonella* prevalence and diverse serovars at the retail level.

Our findings of high resistance levels of strains to ampicillin, tetracycline and streptomycin and an absence of resistance to ciprofloxacin, cefataxime and norfloxacin are similar to the results of a study by Tadee et al. (2015) which was recently carried out on *Salmonella* strains in the identical geographical area of Thailand. Remarkably, whereas various organisms are often susceptible to broad spectrum antimicrobials widely used in human medicine, only one *Salmonella* strain originating from supermarkets was resistant to amoxicillin-clavulanic acid. That is an indicator that antimicrobial resistance in livestock is becoming an increasingly important concern in human health (Marshall and Levy, 2011).

Following the PFGE banding analysis, some matching patterns of *S.* Rissen originating from pig farms, slaughterhouses and wet markets were demonstrated. Clonal relatedness of these strains has been verified, supporting the idea that pig is the primary origin of *Salmonella* contamination, and can spread this organism to subsequent stages, the slaughtering procedure as well as the retail stage, by cross-contamination (Berends et al., 1998b; Prendergast et al., 2009; Hauser et al., 2011). The farm-slaughterhouse-retail contamination route has been inferred by this study. Additionally, groups of identical PFGE patterns derived from different locations were demonstrated by geographical distribution mapping. The common chain in pork production supplied by producers increases the potential for organisms to spread over a large area (Prendergast et al., 2009). Furthermore, various strains of *Salmonella* were identified from the same location on same day and numerous new *Salmonella* PFGE patterns were discovered at the retail stage. These findings clearly demonstrate at the molecular level that the high risk for infection/reinfection and cross contamination with *Salmonella* in wet markets, butcher shops and supermarkets has been a great concern (Mürmann et al., 2009).

5. Conclusions

The findings from this study indicate *Salmonella* is a hazard to human health throughout the retail stage. Better hygienic practices at supermarkets could reduce the chance of cross-contamination, enhancing domestic consumer protection from the organism. Furthermore, the "farm-slaughterhouse-retail" transmission route in this study is confirmed at the molecular level. Farm control programs should be established with strict biosecurity through appropriate management practices to minimize the *Salmonella* risk at the beginning of production stage. Continuous monitoring and appropriate control measures during the slaughtering process should be also promoted. As a final point, butchers education such as proper products handling, good personal hygienic practices as well as adequate products storage conditions should be underlined at the retail stage, in order to reduce contamination loads and to ensure product quality improvement to downstream consumers.

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