Occurrence and sequence type of antimicrobial resistant
Salmonella spp. circulating in antibiotic-free organic pig farms
of northern-Thailand

Pakpoom Tadee¹ Prapas Patchanee¹ Ben Pascoe² Samuel K. Sheppard²
Dethaloun Meunsene³ Tunyamai Buawiratlert³ Phacharaporn Tadee⁴*

Abstract

Salmonella is one of the major foodborne pathogens, often as the result of pork consumption. Currently, with the situation of antimicrobial resistance, organic farming has been suggested as an alternative for healthier options. However, there is little evidence to support this. In this study, we investigated the occurrence of Salmonella circulating in local organic pig farms in northern Thailand and typed isolated clones to better understand the population structure and transmission dynamics of the underlying Salmonella contamination. In total, 112 samples from 11 organic pig farms were processed from October to December 2018. Salmonella was detected in 9 targeted farms. One-fourth (28/112) of all samples yielded Salmonella. The positives proportion of fecal, feeder swabs, and boot swabs were found to be 32.7% (17/53), 17.7% (6/34), and 20.0% (5/25), respectively. Of the 28 positive strains, Seven Salmonella serotypes were identified, with S. Rissen being the most common (15/28; 53.6%). 89.3% (25/28), 78.6% (22/28) and 71.4% (20/28) of isolated Salmonella resisted against tetracycline, ampicillin, and sulfamethoxazole-trimethoprim, respectively. From multilocus sequence typing (MLST) analysis, the phylogenetic tree hinted that cross contamination within herds, point mutation of the housekeeping genes at period persisted in a herd, and sharing routes of supply chain between farms. A minimum spanning tree (MST) revealed that Salmonella contamination in organic pig farming is possibly linked with conventional farming. Based on the own results, strictly highly organic practices provide a safe alternative enhancing domestic consumer trust and improve public health safety.

Keywords: Salmonella, organic farming, antimicrobial, multilocus sequence typing, Thailand

¹Integrative Research Center for Veterinary Preventive Medicine, Department of Food Animal Clinics, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai, 50100, Thailand
²The Milner Centre for Evolution, Department of Biology and Biochemistry, University of Bath, BA2 7AY, United Kingdom
³Graduate Program in Veterinary Science, Faculty of Veterinary Medicine, Chiang Mai University 50100, Thailand
⁴Faculty of Animal Science and Technology, Maejo University, Chiang Mai, 50290, Thailand
*Correspondence: phacharaporn boonkhot@gmail.com (P. Tadee)
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Introduction

Foodborne illness caused by non-typhoidal Salmonella is an important public health issue and is a significant economic burden worldwide (Crump and Heydeman, 2015; Padungtod and Kaneene, 2006; Pan et al., 2000; Phu et al., 2016; Scallan et al., 2011). Transmission of the pathogen to humans occurs mainly through contaminated food products from livestock (Ivic and Kocic, 2009; Jordan et al., 2006), including consumption of pig or pork products (Campos et al., 2019; Hauser et al., 2011). Clinical infection manifests with the onset of fever, followed by gastrointestinal tract disorders such as nausea, vomiting, and profuse diarrhea within 8–48 hours (Crump et al., 2015). Multidrug-resistant Salmonella can complicate treatment and is a serious rising issue in public health (Dechet et al., 2006), leading to a reduction in first line empirical treatment efficacy, limiting treatment choices, and prolonging illness (Pan et al., 2000).

Salmonella has been identified at all stages of the food production chain (Campos et al., 2019). Initial contamination pressure at farm level is directly related to continued contamination at subsequent levels, through slaughtering, retail, and the final cooking process (Rostagno and Callaway, 2012; Tadee et al., 2015). Pigs are often cited as the primary source of human infection, as Salmonella can multiply in the pig’s gut and spread between animals via the fecal–oral route directly or through the environment. Healthy pigs can also carry Salmonella without showing any signs of infection (Futagawa-Saito, 2008); thus, increasing the opportunity for spread of Salmonella into the human food chain.

Currently, increasing demand has led to pig production being transformed into an intensified industry. Large numbers of antimicrobials have been implemented (Guardabassi et al., 2008) and widespread usage and misuse have contributed to the emergence of drug-resistant bacteria (Yezli and Li, 2012), one of several concerns that has fueled the increase in “organic” farming. Pigs are produced under natural management, where antibiotics and synthetic hormones are prohibited. It has set itself the goal of instituting high-quality yields with environmentally friendly production under animal welfare standards (Barański et al., 2017; Sundrum, 2001). In northern Thailand, local organic pig farms have been established for half a decade. However, data on the Salmonella contamination or any resistance situations have not been clearly determined.

Bacterial typing is essential for effective disease investigation and surveillance (Ferrari et al., 2017). Serotyping is based on immune reaction raised in the host and has limited discriminatory power to distinguish Salmonella clones (Brenner et al., 2000; Liu et al., 2001). Multilocus sequence typing is able to overcome limitations in detection and help distinguish between Salmonella clones and aid outbreak assessment. The method is also more reproducible and by using sequence data that can be compared between laboratories, strains can be characterized nationally or internationally (Jain et al., 2018; Yap et al., 2016). Large global studies use this method for epidemiological studies that clearly and precisely differentiate bacterial genotypic diversity in different hosts and geographical distributions (Foxman, 2011; Liu et al., 2001).

The aims of this study were to determine the occurrence of Salmonella spp. circulating in organic pig farms in northern Thailand, and evaluate diversity of the pathogen. Phenotypic characteristics (serotypes and antimicrobial resistance patterns) and sequence types were measured, to understand the local population structure and dynamic propagation. The results will enhance domestic consumer trust and help improve public health safety in the region.

Materials and Methods

Sample collection: This study was conducted under ethical approval reference number R13/2561 from the Animal Care and Use Committee of the Faculty of Veterinary Medicine, Chiang Mai University (FVM-ACUC). Pig fecal samples, feeder swabs and workers’ boot swabs were collected from local organic pig farms in Chiang Mai, Chiang Rai, and Lamphun provinces, Thailand from October to December 2018. To estimate the sample size in a single proportion, the sample size of this study was determined using the Win Epi online program at (http://www.winepi.net/uk/index.htm) (University of Warwick, 2018). Because of entering 50% for the estimated occurrence will result in the highest sample size. Therefore, a rate of 50% was used as the “expected disease proportion (p)”. Ten percent and 0.95 were selected as the “desired precision (E)” and “confidence level (Z_{a/2})” values, respectively. For the infinite population, at least 97 samples are designated. However, at the end, 112 samples were carefully chosen to provide greater accuracy and reliability of the investigation results.

Eleven open housing system pig farms with a herd size range of 30–60 pigs were randomly selected in the study. The selection criteria of all organic farms were: (1) no antibiotic or hormone usage throughout the pigs’ lifespan; (2) all piglets were born under the organic system; (3) use of natural feed ingredients from an organic agriculture system (either with or without commercial feed); (4) farmed according to animal welfare standards; and (5) either natural mating or artificial insemination for breeding. Approximately ten samples (5 pig feces from digital rectal examination, 3 feeder swabs, and 2 workers’ boot swabs) were collected from each farm. For fecal and feeder swab samples, up to two samples/pen were chosen. All collected samples were kept in separate sterile bags and transferred to the laboratory under chilled conditions.

Salmonella isolation and identification: Isolation and identification of Salmonella spp. were performed following the ISO 6579:2002 Amendment 1:2007, Annex D technique, the detection of Salmonella spp. in animal feces, and environmental samples from the primary production stage (International Standard Organizaion, 2002). 25 g of fresh fecal samples and swab samples were added to 225 ml of and 9 ml of buffered peptone water (BFW) (Merck, Germany) as pre-enrichment media. The mixtures were homogenized for 2 min and incubated at 37 °C for 24
h. An aliquot of 0.1 ml was then dropped on Modified Semi-solid Rappaport-Vassiliadis (MSRV) (Oxoid, United Kingdom) plates and incubated at 42 °C for 24 h. The culture material surrounded by a turbid ring was then streaked on Xylose Lysine Deoxycholate (XLD) agar (Oxoid, United Kingdom), and Brilliant Green Phenol Red Lactose Saccharose (BPLS) agar (Merck, Germany) with incubation at 37 °C for 24 h. Presumptive Salmonella colonies were additionally checked by biochemical measurement, including testing of triple sugar iron (TSI) (Oxoid, United Kingdom), urease, and motile indole lysine (MIL) decarboxylase (Merck, Germany). Salmonella positive samples were recorded. Only one isolate from each sample was chosen for further investigation.

**Serotyping and Antimicrobial susceptibility testing:** All detected Salmonella spp. were serotyped by serum-agglutination according to the White-Kauffmann-Le Minor scheme (Brenner et al., 2000). Antimicrobial susceptibility testing was determined by agar disk diffusion techniques with ten panels of antimicrobial agents, comprising amoxicillin-clavulanic acid 20/10 μg, ampicillin (AMP) 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 (CLSI, 2018). Strains with intermediate resistance profiles were recorded as resistant strains to avoid underestimation.

**Statistical analysis:** Salmonella occurrence with their 95% confidence levels were determined by descriptive statistics. Comparisons between groups of sample types were performed using Fisher’s exact test. All analyses were accomplished using Epi Info™, version 7 (Centers for Disease Control and Prevention, USA). Statistical significance levels were measured at p < 0.05.

**Multilocus sequence typing:** All Salmonella strains detected were genotyped into sequence types (STs) by the MLST technique. Total genomic DNA was extracted according the protocol described by Liu et al. (2011). Seven housekeeping genes, including aroC (chorismate synthase), dnaN (DNA polymerase III beta subunit), hemD (urophorosynigogenIII cosynathase), purE (phosphoribosylaminomimidazole carboxylase), sucA (alpha ketoglutarate dehydrogenase), hisD (histidinol dehydrogenase), and ThrA (aspartokinase I/homoserine dehydrogenase) were taken for MLST profiling. Polymerase Chain Reaction (PCR) amplification of all seven genes was accomplished using the methods previously described by Achtman et al. (2012). Then, the PCR products were sent for sequencing to the Macrogen Service Center, Republic of Korea. All sequences obtained in each gene were transformed into allele number and compiled for sequence type (ST) data from the database of http://mlst.warwick.ac.uk/mlst/ (University of Warwick EnteroBase, 2020).

Using Bionumerics® software, version 7.6 (Applied Maths, Belgium), by the unweighted pair group method with arithmetic mean algorithms (UPGMA) through individual similarity matrices, cluster analyses of the seven genes were executed. Local epidemiological results of strains currently circulating in organic pig farms in the study area were displayed in phylogenetic networks. In addition, to develop understanding of evolution and population structure, minimum spanning tree (MST) analysis was used to analyze the genetic relationship between Salmonella strains obtained from this study together with the geographically and temporally matched strains previously submitted to the MLST database (University of Warwick EnteroBase, 2020). Warwick Medical School, University of Warwick. Those included were Thai strains recovered from pig (n = 218), human (n = 42), plant (n = 37), frozen food (n = 30), aquatic animal (n = 16), dried food (n = 13), poultry (n = 9), soil (n = 6), invertebrate (n = 2), cooked food (n = 1), and marine mammal (n = 1), submitted during 2009–2018. The STs, which are closely associated in loci characteristics, were demonstrated close together.

**Results**

**Salmonella occurrence:** From October to December 2018, 112 fecal and environmental samples were collected from 11 local organic pig farms (farms A–K). Eight to 13 samples were obtained from each. One-fourth (28/112; 95% CI: 17.30–34.07%) of all samples yield Salmonella. The positives proportion of fecal, feeder swabs, and boot swabs were found to be 32.08% (17/53; 95% CI: 19.92–46.32%), 17.65% (6/34; 95% CI: 6.76–34.53%), and 20.00% (5/25; 95% CI: 6.83–40.70%), respectively. There was no association between Salmonella frequencies detected and types of sample taken. Salmonella contamination was detected in nine of the eleven farms tested, with only two farms (farms F and K) negative. Between 10–20% of the samples obtained from six farms (farms B, D, E, G, H, and I) were positive to Salmonella. For the three remaining farms (farms A, C, and J), 50–60% detection rates were distributed.

**Salmonella sero-distribution:** Seven Salmonella serotypes were identified in the study. S. Rissen was the most common (15/28; 53.57%), followed by monophasic S. Typhimurium 1,4,[5],12:i – (7/28; 25%), S. Weltevreden (2/28; 7.14%), and one strain (1/28; 3.57%) of each of S. Hvittingfos, S. Krefeld, S. Stanley, and S. Typhimurium.

**Antimicrobial susceptibility testing:** Of all 28 strains taken from Salmonella positive samples, only one (3.57%) was susceptible to all antimicrobials tested, while 24 strains (85.71%) were resistant to at least three different antimicrobials, classifying them as multidrug-resistant (MDR) strains. Resistance of Salmonella from the current study could be found concerning eight of ten antimicrobials tested. No resistance was observed to ciprofloxacin and norfloxacin. Most strains were resistant to tetracycline (25 strains; 89.29%), followed by ampicillin (22 strains; 78.57%) and sulfamethoxazole-trimethoprim (20 strains; 71.43%) (Figure 1).
Multilocus sequence typing: Sequencing data of seven housekeeping genes of all strains were submitted to the Salmonella spp. definition database to query allelic numbers and corresponding STs. In total, 12 different MLST genotypes were assigned. Six of them were matched with the previously described in MLST database (University of Warwick EnteroBase, 2020), could be assigned in ST number. The majority were of ST469 (n = 14), followed by three strains of ST34 and two strains of ST365. Six others could not be assigned a ST, highlighting the diversity of Salmonella isolates found on these farms. Variation in the sucA and/or hisD or thrA loci enabled assignment to ST complex or clonal complex (CC) level. Five strains were assigned to CC1, and the other one was CC66. The genetic relationship between all 28 Salmonella strains was explored using sequence variation from the seven housekeeping genes with UPGMA algorithms (Figure 2).

Six clusters were determined with two or more MLST gene differences, representing different CCs. The strains grouped in the dominant serovar Rissen ST469 were mostly obtained from three nearby farms (farms A–C, which each has a similar zip-code), and two identical strains were also obtained from farms approximately 30–70 km away from each other (farms E and I). Besides, strains grouped in Weltevrenden ST365 were also obtained from farms spread more than 30 km apart (farms A and I).

Serovar Rissen ST469 from farm A was recovered from feeder swabs and fecal samples. Similar to the ST34 strains from farm J, the strains were recovered from a boot swab and fecal samples. Moreover, in farms A, B, E, H, I, and J, at least two ST characteristics were identified.

In order to better understand the genetic relatedness of the pathogen, a minimum spanning tree (MST) was constructed to analyze the 28 strains alongside 375 Thai strains previously submitted to the MLST database in the ten-year period, 2009–2018 (Figure 3). A total of 403 Thai strains was constructed describing a population from 11 different hosts. Seventy-one STs were distributed. More than two-fifths of the strains were closely related and shared allelic profiles in at least five loci, with similar CCs shaded in gray. ST34 was the most frequently detected, followed by ST465, ST365, and ST29. Members grouped in the top four STs were recovered from more than one host origin, such as organic pig, pig, plant, soil, poultry, food, and human. For the members grouped in ST365, one was obtained from an invertebrate. On the other hand, some STs were unique to a single host origin; ST185, ST410, ST590 were specific to plant sources. Similarly, ST1 and ST292 were specific to human and aquatic animals, respectively.

Figure 1  Antimicrobial resistance rates of Salmonella strains isolated from local organic pig farming in northern Thailand. Antibiotic abbreviations: amoxicillin-clavulanic acid (AMC); ampicillin (AMP); chloramphenicol (C); ciprofloxacin (CIP); cefotaxime (CTX); nalidixic acid (NA); norfloxacin (NOR); sulfamethoxazole-trimethoprim (SXT); streptomycin (S); tetracycline (TE)
This is the first cross-sectional study of *Salmonella* spp. circulating in organic pig farming in the northern region of Thailand. We identify overall positives of 25% (95% CI: 18–35%), which is only slightly lower than rates identified in conventional pig farms from the same region during the same time period (Tadee et al., 2014), that reported an occurrence of 31% (95% CI: 27–33%). This is far higher than rates identified in other similar study on organic farms from other country in Asia, where an occurrence of 8% (95% CI: 6–10%) was reported in a study of Korean organic pig farms by Tamang et al. (2015). These high levels of *Salmonella* contamination suggest that there is a breakdown in organic practices in rural northern Thailand.

All samples acquired from farms F and K were negative. However, only ten samples were collected from each farm, so these farms cannot yet be considered *Salmonella*-free, exactly. Although all the farms followed organic principles, at the present time farm K is the only one in northern Thailand that has received official accreditation from the Thai government, suggesting that standards may be higher on these farms. Organic products may provide an alternative for health-conscious local consumers. Of that farm, one hundred percent use of natural feed ingredients is likely beneficial (Rostagno and Callaway, 2012), with a direct effect on the pigs' gut microbiota (García-Feliz et al., 2009; Rajić et al., 2007). The use of low pH fermented grain and natural organic feed stuff are act as a prebiotic, promoting growth of lactic acid bacteria to the detriment of the preferred conditions for *Salmonella* (Farzan et al., 2006; Hedemann et al., 2005).

As one might expect following comparison among sample types, *Salmonella* positive percentage from fecal samples tended to be higher than the value acquired from boot and feeder swab samples. Expectedly, *Salmonella* usually multiplies in pig's gut (Futagae-Saito et al., 2008). Ingested *Salmonella* can be shed via feces within 2 h, without any clinical symptom and spread to the wider environment, such as stable floors or feeders (Hauser et al., 2011; Rostagno and Callaway, 2012). Consequently, hygienic practices in stable cleaning, boot disinfecting, and feed management are very important in reducing the risk of contamination (Rajić et al., 2007). However, other on-farm stressor factors including handling for mating, transportation as well as high density in the rearing area are also considered as multifactorial effects that increase the chance of *Salmonella* shedding (García-Feliz et al., 2009). For that reason, effective herd management allowing natural behavior expression should be fulfilled. This could also be considered an explanation for why *Salmonella* could not be found on farms F and K, which seemingly had higher hygiene standards than the other farms, even though natural feeds were not 100% implemented on farm F.

All 28 detected *Salmonella* strains were susceptible to norfloxacin and ciprofloxacin, both of which are fluoroquinolones, which are necessary in parenteral treatment of severe gastroenteritis in human cases (Bruzzese et al., 2018; Malik, 2017). Fluoroquinolones remain the most effective antimicrobials for pig related salmonellosis in humans. However, nine-tenths of strains tested in the study were MDR. High resistance rates of antimicrobials recently used in livestock production, such as ampicillin, tetracycline, or sulfamethoxazole-trimethoprim were found. This is

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**Figure 2** Dendrograms generated using UPGMA algorithms based on MLST profiles with the phenotypic characterization and epidemiological data of *Salmonella* circulating on organic pig farms in northern Thailand.
consistent with reports on *Salmonella* isolated from conventional pig farms in northern Thailand that also revealed high levels of resistance to these drugs (Chuanchuen and Padungtod, 2009; Tadee et al., 2014). Generally, antimicrobial usage is currently prohibited in all organic farms selected for this study. Spread of MDR *Salmonella* seems not to be the effect of selective pressure in the antibiotic-free organic pig farming. This finding illustrates the complicated and widespread nature of resistance in the farm community (Rostagno and Callaway, 2012). Breeders reared on conventional farms that enter the organic system may already carry drug-resistant *Salmonella* in their guts, providing an opportunity for transmission and infection. Accordingly, it is important to note that a resistance has also emerged in antimicrobial-free herds (Österberg et al., 2016). In relation to farms F and K, breeders are produced on farm, thus limiting the possibility of pathogen transmission from off-farm sources.

**Figure 3** Minimum spanning tree (MST) analysis of 403 Thai population *Salmonella* strains recovered from various host origins during 2009–2018. Size of circles indicates the number of strains grouping in the same STs. Branch styles coding: thick black solid lines connect ST types with one locus difference; thin black solid lines connect ST types with double and three loci differences; gray dashed lines connect four loci differences; gray dotted lines connect more than four loci differences, respectively. Gray shaded areas indicate at least five shared loci between STs (grouping in common ST complexes).
Shared STs belonging to different sample types were identified, suggesting a common source of contamination. Within herds, cross transmission can occur via carriers or any contaminated materials and the probability of re-infection could occur continuously (Rajić et al., 2007; Rostagno and Callaway, 2012). If we consider the geography of our collection, 12 strains of ST469 were recovered from three different farms (farms A, B, and C) located in nearby areas, with the same zip-code. Farm staff, environmental contamination, and the sharing of routes for pig transportation could play a significant role in dissemination. Nevertheless, associations of spatial relatedness were also noted at farms located far apart (farms E and I). As a previously noted, common supply chains such as breeders, gilts, or feed stuffs might be inferred as an important route. S. Rissen was the most common serotype detected and is a dominant clone Salmonella contamination of pig farms in northern Thailand over the last 15-years (Padungtod and Kaneeue, 2006; Tadee et al., 2014). In view of this, Salmonella contamination of both conventional and organic pig farms is probably linked.

Diversity in MLST genotypes in some farms provides evidence that multiple strains are present within a single herd. Farms A, B, E, H, and I harbored at least two genotypical characteristics of Salmonella. This may represent different sources of infection. For another explanation, variation in one or two alleles can be explained by point mutations in the housekeeping genes during persistent colonization of the herd. This is potentially what we see with the ST34 strains from farm J, where only one to three nucleotide changes can be identified in the sucA gene. Using the allele sequence comparison analysis function of the pubMLST Salmonella spp. MLST database (Jolley et al., 2018), point mutations can be specified: 155A>G of thrA alters thrA_2 to thrA_904, with 99.8% identity. With the same identity, a variation 127G>A of sucA shifts sucA_9 to sucA_862. Additionally, variations 15C>T, 18A>G, and 54C>T of sucA make over sucA_9 to sucA_925 with only 99.4% identity. This allelic profile has not yet been referenced in the database and a new ST profile will need to be added.

As part of expanding knowledge of regional epidemiology, results of the genetic relatedness of Thai Salmonella strains obtained from several host origins from ten collection years were displayed in MST. A diverse set of 403 Salmonella spp. strains were evaluated. Sixty percent of them were pig associated strains, supporting a strong interest in Salmonella contamination of pork products in the region. Strains grouped in the majority STs were obtained from the host origins linked with human food chain. From the finding, pig, chicken and aquatic animals could be important source of salmonellosis. They are denoted as the primary source of infection and can spread the pathogen to environment and subsequent level (Compos et al., 2019; Rostagno and Callaway, 2012). In addition, most strains derived from organic pig farms were clonally related to the pig associated strains from the conventional farming system. Predominant types, especially ST34 and ST469 have been spread into pig farms across the country and have been a source of contamination in the pig population for over a decade.

One of the ST365 strains was recovered from invertebrate, which could be an additional potential reservoir of salmonellosis. Moreover, ST1 was found to be unique in the human host origin. Not surprisingly, all strains belonging to ST1 were derived from Salmonella Typhi, which is renowned as the host specific serotype for humans (Yap et al., 2016). For STs which were unique to other origins, the low numbers of strains submitted does not reflect that these cannot be transmitted or spread to the others. This requires further study to develop a complete understanding of their epidemiology.

Consumers demand choice and healthier options. Organic products aim to provide increased food security, animal wellbeing as well as consideration of the effects on environmental sustainability. However, in this study Salmonella was detected in nine of the eleven farms tested. All farms tested claimed to follow organic principles, but only one had received official Thai government certification – from which no Salmonella contamination was detected. Although Salmonella occurrence was slightly lower in these organic farms, contamination with multidrug resistant clones suggests that domestic consumers should still be wary and that subsequent meat products still pose a public health risk.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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