Genetic Diversity and Antimicrobial Resistance Determinants of Clinical *Salmonella* Enteritidis in Thailand

（タイで患者から分離された *Salmonella* Enteritidis の遺伝学多様性と抗菌薬耐性決定因子）

Fuangfa Utrarachkij

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ABBREVIATIONS

AMC	amoxicillin/ clavulanic acid
AMP	ampicillin
BK	Bangkok
C	central
CHL	chloramphenicol
CIP	ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CTX	cefotaxime
D	Nei's diversity index
E	eastern
GEN	gentamicin
GyrA	gyrase subunit A
LB	Luria Bertani
MDR	multidrug resistance
MICs	minimum inhibitory concentrations
MLVA	multilocus variable number tandem repeat analysis
MST	minimum spanning tree
N	northern
NAL	nalidixic acid
NE	north-eastern
NOR	norfloxacin
PCR	polymerase chain reaction
PMQR	plasmid-mediated quinolone resistance
QRDR	quinolone resistance-determining region
S	southern
SE	*Salmonella enterica* serovar Enteritidis
STR	streptomycin
SXT	sulfamethoxasazole-trimethoprim
TBE	Tris-borate EDTA
TET	tetracycline
VNTR	variable number tandem repeat
W	western

PREFACE

Human pathogenic *Salmonella enterica* are classified by their antigenic formulas into typhoidal serovars (*S. enterica* serovar Typhi and *S. enterica* serovar Paratyphi A) and non-typhoidal serovars, which contains more than 2500 serovars. Non-typhoidal serovars usually caused self-limited diarrhoea; however, extra-gastrointestinal infections are also caused by the predominant serovars as *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis (*S.* Enteritidis, SE).

Gastroenteritis caused by non-typhoid *Salmonella* is a global public health concern, accounting for 4.8 million of disability-adjusted life years and 81,300 deaths in 2010 (43, 48). In a global monitoring, SE was reported as the first ranked *Salmonella* serovar accounting for 43.5% of human salmonellosis (26). SE is one of the zoonotic pathogen that transfers to human through food animal consumption, particularly chicken and chicken egg. The global emerging of SE infection was probably because of its persistent and invasive behaviour in these common food animals (29). Importantly, the use of antimicrobial in poultry farming has been considered as main factor affecting the existence of antimicrobial resistant SE. This make the issue of multidrug resistance in SE strains increase significantly concern (30).

In Thailand, SE was reported as the second most common serovar isolated from clinical samples and predominantly isolated from chicken meat as showed in Fig. 1 and 2 (6). SE was also the major cause of invasive non-typhoidal salmonellosis defined as cases with positive blood salmonella culture (24, 59). Moreover, SE isolates from Thai patients and chicken meat exhibited significant increase in antimicrobial resistance since 1994 (7) and multidrug resistance, including drug of choice as fluoroquinolone were observed in the past decade (25, 59). The study on genetic diversity and antimicrobial resistance profiling of clinical SE isolates in Thailand can provide better epidemiological data for tracing of SE infection. However, the national epidemiological data of SE in Thailand was limited to serovar identification and antimicrobial susceptibility, without any genetic polymorphisms among the isolates. This thesis aimed to elucidate the clonal distribution of clinical SE isolates in throughout Thailand.

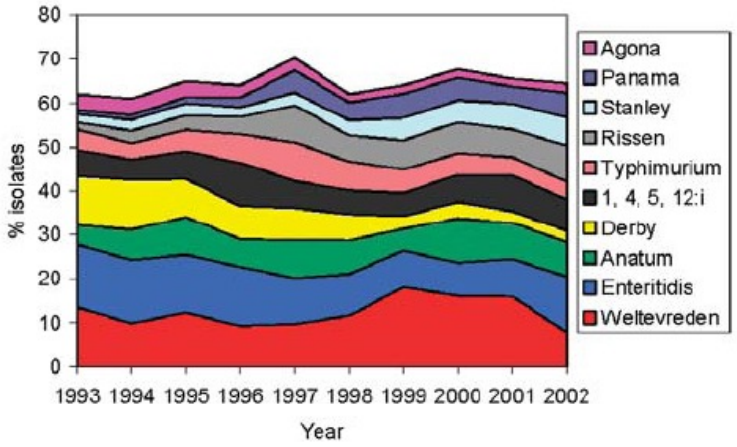

Fig. 1 Trend over time for the 10 most common *Salmonella* serovars causing human infection between 1993 and 2002 in Thailand (Bangtrakulnonth A. *et al*, 2004)

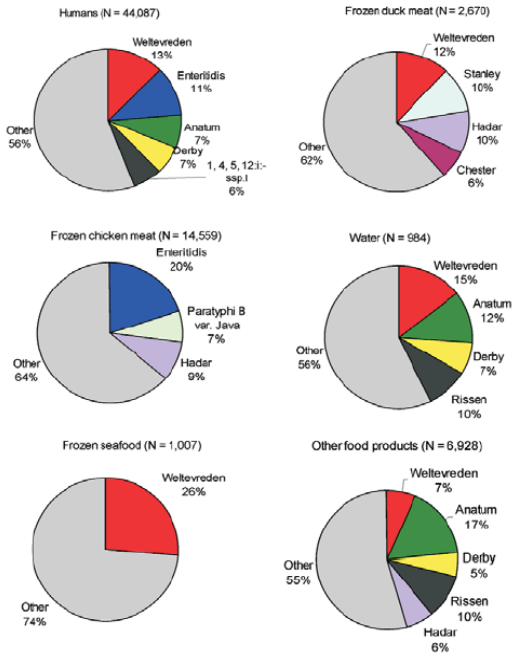

Fig. 2 Distribution of the most common *Salmonella* serovars among the different reservoirs. (Bangtrakulnonth A. *et al*, 2004)

In chapter I, a retrospective analysis of SE isolates collected during 2004-2007 in Thailand was conducted by drug susceptibility profiling and MLVA typing. In chapter II, the quinolone resistance determinants were further investigated in clinical nalidixic acid resistant SE isolates collected nationwide in Thailand from 2004 to 2007. In addition, the relationship between these resistance determinants and MLVA typing results was analysed to elucidate the dissemination of specific clones.

CHAPTER I

Genetic Diversity and Antimicrobial Resistance Pattern of *Salmonella* Enteritidis Clinical Isolates in Thailand

Non typhoid *Salmonella* is a major concerned foodborne pathogen with continuously high incidence over a ten year period (13). Usually, severe cases of salmonellosis can be treated with antibiotics; however, the emergence of drug resistant strains has become a serious problem. In Thailand, clinical SE typically possesses a lower percentage of multidrug resistance. However, a drastic increase of antimicrobial resistance in SE was observed in 1994 (7). Moreover, during the period 2001-2006, higher resistance to ampicillin (AMP), tetracycline (TET), sulfamethoxasazole-trimethoprim (SXT), streptomycin (STR) and nalidixic acid (NAL) were reported in clinical SE (59).

In epidemiological surveillance, drug resistance profiling and genetic typing have been utilised as useful tools for tracking the overseas spread of *Salmonella* by foreign visitors (60) and exported food products (1). Therefore, a survey of antimicrobial resistance patterns and genetic diversity including invasive virulence potential in SE circulating in individual countries could provide important information for control measures. Multilocus variable number tandem repeat analysis (MLVA) was developed as a suitable technique for discriminating various highly clonal pathogenic salmonellae. The first developed MLVA for typing *S*. Typhimurium used a set of eight variable number tandem repeat (VNTR) loci and showed a higher resolution than other molecular typing methods (40). Subsequently, the method was optimised for *S. enterica* serovar Typhi (51) and SE (9, 46) to simplify the interpretation of results, which achieved consistency and comparability across different laboratories (9, 50).

Recently, Hendriksen *et al*. reported that clinical SE isolates collected in 2008 in Thailand were highly resistant to AMP (60%), NAL (83%) and ciprofloxacin (CIP, 90%) (25). In contrast, lower percentages of resistance to AMP (21%), NAL (55%) and CIP (0 %) were reported in the same country during 2001-2006 (59). To assess the real situation at this turning point, a retrospective analysis of SE isolates collected during

2004-2007 in Thailand was conducted by drug susceptibility profiling and MLVA typing in the present study.

Materials and Methods

Salmonella strains

A total of 192 clinical SE isolates were collected from 2004 to 2007 from seven different regions of Thailand: Bangkok (BK), and central (C), eastern (E), northern (N), north-eastern (NE), southern (S) and western (W) provinces (Table 1). The strains consisted of 99 and 93 isolates from blood and stool samples, respectively. All strains were isolated from epidemiologically unlinked patients in private and governmental hospitals and regional medical centres throughout Thailand. The isolates were identified by biochemical tests including triple sugar iron and lysine-indole-motility tests, and serovars typed according to the Kauffmann-White serotyping scheme (52) at the WHO National *Salmonella-Shigella* Center, belonging to the Ministry of Public Health of Thailand.

Antimicrobial susceptibility testing

Antimicrobial susceptibility of the studied SE isolates was determined by the Kirby-Bauer disk diffusion method using 11 antimicrobials, namely, amoxicillin/ clavulanic acid (AMC), ampicillin (AMP), chloramphenicol (CHL), ciprofloxacin (CIP), cefotaxime (CTX), gentamicin (GEN), nalidixic acid (NAL), norfloxacin (NOR), streptomycin (STR), trimethoprim/sulfamethoxazole (SXT) and tetracycline (TET) (Becton–Dickinson, Sparks, MD, USA). The results of inhibition zones were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (18). *Escherichia coli* strain ATCC 25922 was used as the quality control organism in the assays. For statistical analysis purposes, strains with intermediate interpretation were regarded as resistant. All comparisons and associations were analysed using Chi square test with significant level at P value < 0.001.

DNA extraction

SE strains were cultured on Luria Bertani (LB) agar (Becton–Dickinson) at 37°C for 18-24 hr. A single colony of each strain was inoculated in 5 mL of LB broth and incubated with shaking at 37°C for 4-6 hr. A DNeasy® Blood & Tissue kit (Qiagen Inc., Valencia, CA, USA) was used for the extraction and purification of SE genomic DNA from 1 mL of LB broth culture according to the manufacturer's protocol.

Multilocus variable number tandem repeat analysis

Selected VNTR loci and primer sets for polymerase chain reaction (PCR) were synthesised in accordance with previous publications (9, 15, 17), as shown in Table 2. The PCR reaction mixture for SE1, SE3, SE5, SE6 and SE9 loci consisted of 0.4 mM dNTP (New England Biolabs Inc., Ipswich, MA, USA), 0.4 μM of each primer set, 0.025U of GoTaq DNA polymerase (Promega, Madison, WI, USA) and 1 μL of diluted DNA template (1-10 ng/μL) in 1 × Green Go Taq buffer. For the amplification of SE2, SE7, SE8 and SE10 loci, an enhancer mixture consisting of 0.25 mM dNTP, 10% enhancer solution (Invitrogen™, Grand Island, NY, USA), 1.7 mM $MgSO_4$, 0.4 μM of each primer set, 0.025U of GoTaq DNA polymerase and 1 μL of diluted DNA template (1-10 ng/μL) in 1× PCR buffer (Invitrogen™) was used. The amplification was carried out in a thermal cycler (iCycler, BioRad, Hercules, CA, USA) under the following conditions: an initial denaturation at 95°C for 2 min, 35 cycles (SE2, SE6, SE8, SE10) or 40 cycles (SE1, SE3, SE5, SE7, SE9) of 95°C for 10 sec, 50°C for 20 sec and 72°C for 40 sec, and a final elongation at 72°C for 5 min. Amplification products of long repeat loci (SE6, SE7, SE8, SE10) were separated in 2.5% agarose gel by electrophoresis at 50 V for 25 min using Tris-borate EDTA (TBE) as the electrophoresis buffer. The electrophoresed gel was stained with ethidium bromide and visualised under UV light for the presence of expected amplification products. The numbers of repeat units in the long repeat loci were calculated from the estimated size of amplicons. Amplification products of short repeat loci (SE1, SE2, SE3, SE5, SE9) were analysed by capillary electrophoresis using an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster, CA, USA), and the number of repeat units were identified by sequencing. Based on the diversity of alleles, the discriminatory capacity of each locus was evaluated by the Nei's diversity index (D) calculated as $1-\Sigma (\text{allele frequency})^2$.

Four polymorphic short repeat VNTR loci (SE1, SE2, SE5, SE9) and one long repeat locus (SE7) were selected for further analysis of MLVA types of all SE isolates. The SE7 locus of isolates was amplified by enhanced PCR and calculated by the number of repeat units as described above. The short repeat loci were amplified using multiplex PCR, and the product size of each locus was simultaneously determined with multicolour capillary electrophoresis as previously described (41). Each forward primer of four primer sets was labelled with a fluorescent dye, NED, 6FAM, VIC or PET, as shown in Table 2. The reaction mixture (20 μL) consisted of Multiplex PCR mixture 1 (0.1μL) (Takara Bio Ink, Shiga, Japan), Multiplex PCR mixture 2 (10 μL), a primer premix of SE1, SE5, SE9 (0.08 μM each) and SE2 (0.16 μM each) and 1 μL of diluted DNA template (10 ng/μL). The reaction was conducted by an initial denaturation at 96°C for 1 min, 30 cycles of 96°C for 10 sec, 50°C for 20 sec and 72°C for 30 sec and a final elongation at 72°C for 5 min. The amplicons were analysed with an internal size marker (600 LIZ size standards, Applied Biosystems) in an ABI 3130 Genetic Analyzer. The number of repeat units of each VNTR locus was calculated from the amplicon size by Peak Scanner software (Applied Biosystems). The MLVA type was further analysed by designating each isolate with the number of repeat units in all five loci. Minimum spanning trees were drawn by BioNumerics ver. 6.0 (Applied Maths, Sint-Martens-Latem, Belgium).

Detection of *SdfI* and virulence-associated genes

All SE isolates were analysed for the presence of *Salmonella* Enteritidis specific fragment (*SdfI*) and virulence-associated genes *spvA*, *sodC*1 and *sopE* using primer sets synthesized either by methods available on our premises or in accordance with previous publications (3, 22), as shown in Table 3. The 20-μL PCR mixture contained 1 μL of diluted DNA template (1-10 ng/μL), 0.25 mM dNTP mixed (New England Biolabs Inc.), 0.4 μM of each primer set and 0.025U of GoTaq® DNA polymerase (Promega) in 1 × Green GoTaq® buffer. The amplification was carried out in iCycler using the following cycles: an initial denaturation at 95°C for 1 min, 35 cycles of 95°C for 10 sec, 55°C for 10 sec and 72°C for 40 sec and a final elongation at 72°C for 5 min.

Results

Antimicrobial resistance profiles

Antimicrobial susceptibility tests revealed that 190 isolates examined were susceptible to GEN, and that 165 of them (86.8%) were also resistant to at least one other antimicrobial. Resistance to NAL (83.2%, 158/190) was the most common resistance in the studied isolates, followed by resistance to CIP (51.1 %) and AMP (50.5%). Other resistance was detected for TET (16.3%, 31/190), SXT (15.8%, 30/190), STR (13.7%, 26/190), AMC (4.2%, 8/190), CHL (2.1%, 4/190), NOR (0.5%, 1/190) and CTX (0.5%, 1/190) (Fig 3). Resistance to NAL and CIP showed a constantly high frequency during the period 2004-2007, whilst a significant increase ($P < 0.001$) in resistance to AMP was observed in 2006.

Resistant isolates displayed 31 different antimicrobial resistance profiles, with 17 profiles containing a single isolate. Of these, AMP/NAL was the most prevalent resistance profile in both blood (20.7%) and stool (24.1%) isolates. The second most common profile, AMP/CIP/NAL, was observed in blood (19.5%) and stool (9.6%) isolates. Other common resistance profiles observed for at least three years at a lower frequency were NAL (7.9%), CIP/NAL (11.5%), AMP/CIP/NAL/STR (8.5%) and CIP/NAL/SXT/TET (7.9%). Based on the definition of multidrug resistance (MDR), that is, resistance to ≥ 3 antimicrobial categories (45), at least 25.5% of the resistant isolates were MDR (Table 4). However, no significant difference between blood and stool isolates was observed for the overall resistance rate and frequency of multidrug resistance ($P = 0.09$ and 0.5, respectively). Drug resistant SE isolates were spread throughout Thailand with 58% in the central region and 75-95% in other regions. The two most common resistance profiles, AMP/NAL and AMP/CIP/NAL, were also found in all geographical regions with a wide range of prevalence (8-50%). NAL, CIP/NAL, AMP/CIP/NAL/STR and CIP/NAL/SXT/TET were prevalent profiles distributed in all but one or two regions. MDR strains were found in all regions (Table 5). The drug susceptibility of two isolates from stool was not examined because of their extinction during storage.

VNTR variations

The variation of copy numbers in 9 VNTR loci (4 long repeat loci and 5 short repeat loci) was preliminarily analysed in 83 SE isolates. The results showed diversity in a long repeat locus (SE7) and four short repeat loci (SE1, SE2, SE5, SE9) while no diversity was found with three long repeat loci (SE6, SE8 and SE10 with tandem repeat numbers of 16, 1 and 8 respectively) and a short repeat loci (SE3 with the tandem repeat numbers of 3). Polymorphic loci SE1, SE2, SE5, SE7 and SE9 were selected for further analyses in the MLVA typing scheme. In all SE isolates, five polymorphic loci showed different discriminatory capacities with the Nei's index ranging from 0.06 to 0.65, and with SE5 having the highest diversity level (Table 6).

Distribution of MLVA types

The MLVA types of SE isolates were defined with five code numbers designated by the tandem repeat number in loci of SE1, SE2, SE5, SE7 and SE9. For example, MLVA type 5-5-10-7-3 denotes that a strain contained 5, 5, 10, 7 and 3 repeat units in those loci, respectively. Using these five polymorphic VNTR loci, 20 MLVA types were identified in SE strains circulating in Thailand (Fig 4). Various MLVA types were identified in SE isolates from different regions. The numbers of MLVA types identified in isolates from the Thai regions BK, C, N, NE, S, W and E were 10, 8, 8, 8, 7, 7 and 5, respectively. Three common MLVA types having a difference at locus SE5, 5-5-11-7-3, 5-5-9-7-3 and 5-5-10-7-3, were spread in each geographical area, with 5-5-11-7-3 being the most common type. Other MLVA types were found in Thai regions with less than 10% of the isolates, of which 10 unique MLVA types were represented by a single isolate (Fig 4).

A minimum spanning tree (MST) was generated to elucidate the population structure of isolates based on MLVA types. MST displayed a cluster of three adjacent MLVA types, namely, 5-5-11-7-3, 5-5-9-7-3 and 5-5-10-7-3, consisting of 75% of isolates from blood and stools in even numbers (Fig 5). The ratio of each MLVA type in Thailand during the study period is shown in Fig. 6. There was an increasing trend in frequency of MLVA type 5-5-11-7-3 from 2004 to 2007, with a peak in 2006. In contrast, a steadily decreasing trend was observed for MLVA type 5-5-9-7-3.

Association of MLVA types and drug resistance

The association between drug resistance and MLVA types was analysed. Isolates resistant to NAL, CIP and TET were widely observed in 20 MLVA types. MLVA types 5-5-11-7-3 had a significantly higher rate of resistance to NAL ($P < 0.001$) and AMP ($P < 0.001$), whilst MLVA type 5-5-9-7-3 showed higher resistance to SXT ($P < 0.001$) and TET ($P < 0.001$). In addition, MDR isolates were mainly found in MLVA type 5-5-9-7-3 (47%), followed by 5-5-11-7-3 (23%) and 5-5-10-7-3 (19%).

Detection of *sdfI* and virulence-associated genes

All isolates were shown to carry the SE specific DNA sequence *sdfI* and prophage-encoded, virulence-associated genes *sodC*1 and *sopE*, whilst the plasmid virulence gene *spvA* was observed in 97.9% (188/192). Three out of four *spvA* negative isolates were collected from stools and the remaining one from blood.

Discussion

Our study demonstrated a high rate of resistance to NAL, CIP and AMP in clinical SE strains circulating in Thailand during the period 2004 - 2007. It is worth noting that the rate of resistance to AMP in this study significantly increased from 25.0% in 2004 to 71.7% in 2006, whilst those to NAL were persistently high throughout the study period (2004 - 2007) (Fig. 3). AMP resistance rate in each year showed the surge reflected by expansion of strain with MLVA type 5-5-11-7-3. Compared with previous survey data of Thailand taken in the periods 1993-1994 (7), 2001-2006 (59) and 2008 (25), we suggest that there are increasing trends of SE resistance to NAL and AMP. Drastically high rate of CIP-resistance was seen in our study with isolates during 2004-2007 and that with isolates in 2008 by Hendriksen *et al*. comparing with the previous survey data of Thailand taken in the periods 1993-1994 (7) and 2001-2006 (59). This discrepancy might be because of the difference of the breakpoint of CIP by the former CLSI criteria (4 mg/L) used in previous survey and new CLSI criteria (0.06 mg/L) amended in 2012 used in our study and EUCAST criteria (0.064 mg/L) used in the study of Hendriksen *et al*. (25)

The emergence of antimicrobial resistant SE is likely associated with human therapeutic use and misuse of antibiotics in food animals, which serve as a source of resistant SE in human infection (5). In Thailand, AMP is an accepted drug for systemic salmonellosis (53), and both enrofloxacin and amoxicillin are generally used in broiler farming (49). In addition, SE was observed as the predominant serovar isolated in Thailand from 28% of retail chicken meat in 1997 (8) and 20% of frozen chicken during the period 1993-2002 (6). These factors may be associated with the increasing antibiotic resistance rate of SE in Thailand.

In this study, we found the three most common antimicrobial resistance profiles (AMP/NAL, CIP/NAL and AMP/CIP/NAL) in almost half of the studied isolates. In contrast, the other half of the isolates exhibited 28 distinct antibiograms, some of which were isolated from both blood and stools over the study period (Table 4) and shown to be distributed in different regions (Table 5). This result may indicate that SE in Thailand has survived under various antibiotic selective pressures that contribute to diverse antibiograms.

The MLVA types used in this study showed lower discriminatory power for subtyping of SE isolates than those from the United States (9, 16, 17) or European countries (46) possibly because of the high clonality of SE in Thailand (Fig. 5 and 6). Each VNTR locus showed different Nei's indices (Table 6). We found that only five VNTR loci (SE1, SE2, SE5, SE7 and SE9) had polymorphisms in SE isolates from Thailand that were similar to those observed by Hendriksen *et al*. (25). Interestingly, the Nei's index of each polymorphic locus analysed in this study was lower than those in studies from other countries (9, 16, 17). With these five VNTR loci, all isolates were grouped into 20 MLVA types. Amongst these, three closely related MLVA types with a single locus difference at SE5 (5-5-11-7-3, 5-5-9-7-3 and 5-5-10-7-3) accounted for 75% of the SE widely spread (Fig. 4) during the 4-year period (Fig. 6). This finding indicated that endemic SE strains in Thailand were highly clonal by sharing a common ancestor. These SE may have been introduced as a single source relatively recently, but spread and persisted in Thailand for a period of time.

Significant associations were observed between the MLVA types and the resistance to each antimicrobial. MLVA types 5-5-11-7-3 showed significant association with resistance to NAL ($P < 0.001$) and AMP ($P < 0.001$) (Table 7). The trend of AMP resistant SE during 2004-2007 (Fig. 3) was associated with that of MLVA type 5-5-11-7-3 (Fig. 6), for which the AMP resistance ratio was around 90%. This result clearly demonstrated the clonal expansion of AMP resistant SE in Thailand. MLVA type 5-5-9-7-3 was significantly associated with SXT ($P < 0.001$) and TET ($P < 0.001$) resistance (Table 7). SE with this MLVA type showed a decreasing trend from 2004 to 2007 that related to SXT resistance (Fig. 6). The majority of MDR SE (88%) belonged to three dominant MLVA types, 5-5-9-7-3, 5-5-11-7-3 and 5-5-10-7-3. Since locus SE5 is well known to be hypervariable, the origin of these SE might be same. Furthermore, MDR phenotypes in these SE might contribute to spreading under continuing antimicrobial selection pressure.

We confirmed the presence of *Salmonella* difference fragment (*sdfI*), which has been proposed as a specific marker for the identification of SE isolates (3). The presence of *spvA* in most SE isolates from blood (99.0%) and stools (96.8%) provided evidence for carrying the virulence plasmid. Moreover, all SE strains harboured *sodCI* and *sopE*.

Summary

SE is predominant non typhoidal *Salmonella* causing extra-gastrointestinal infections and raising concern about emergence of antimicrobial resistance. To trace the history of antimicrobial resistance in *Salmonella* Enteritidis (SE) circulating in Thailand, we characterised clinical isolates obtained during 2004-2007. Antimicrobial resistance profiles, multilocus variable number tandem repeat analysis (MLVA) types and 3 representative virulence determinants (*spvA*, *sodC*I and *sopE*) were established from SE isolates (n = 192) collected from stool and blood of patients throughout Thailand during the period 2004-2007. Resistance was found in SE against 10 out of 11 antimicrobials studied. The highest resistance ratios were observed for nalidixic acid (83.2%), ciprofloxacin (51.1%) and ampicillin **(**50.5%), and 25.5% were multidrug resistant. Based on five polymorphic tandem repeat loci analysis, MLVA identified 20 distinct types with three closely related predominant types. A significant increase of AMP resistance from 2004 to 2006 was strongly correlated with that of a MLVA type, 5-5-11-7-3.

Table 1 Number of *Salmonella* Enteritidis isolates collected from different regions of Thailand during the period 2004-2007

Regions	Blood samples (n = 99)				Stool samples (n = 93)			
	2004	2005	2006	2007	2004	2005	2006	2007
Bangkok (n=62)	11	13	2	3	5	11	8	9
Central provinces (n=13)	1	4	2	1	-	-	1	4
Eastern provinces (n=9)	3	1	1	3	-	-	-	1
Northern provinces (n=33)	6	2	1	6	2	4	8	4
North-eastern provinces (n=27)	2	2	9	7	2	3	1	1
Southern provinces (n=20)	-	2	6	4	-	2	3	3
Western provinces (n= 28)	3	2	-	2	9	5	4	3
Total (n=192)	26	26	21	26	18	25	25	25

Table 2 Characterisation of VNTR loci selected for MLVA typing and primer sequences for amplification

Locus	Primer sequences[d] (5'—3')	Repeat size (bp)	Position at SE P125109	Reference
SE1-F[a]	**NED-**AGACGTGGCAAGGAACAGTAG	7	2504994-74	(9)
SE1-R	**GTTTCTT**CCAGCCATCCATACCAAGAC		2504728-47	
SE2-F[a]	**6-FAM-**CTTACGATTATACCTGGATTGTTGG	7	4617817-02	(9)
SE2-R	**GTTTCTT**GGACGGAGGCGATAG		4617615-30	
SE3-F[a]	CAACAAAACAACAGCAGCAT	12	2073240-59	(9)
SE3-R	GGGAAACGGTAATCAGAAAGT		2073547-27	
SE5-F[b]	**VIC-**CGGGAAACCACCATCAC	6	3073216-32	(9)
SE5-R	**GTTTCTT**CAGGCCGAACAGCAGGAT		3073427-10	
SE6-F[b]	CGGTGGCGGAGATTCTAATCA	33	3510975-95	(17)
SE6-R	ACGCCGTTGCTGAAGGTAAT		3511412-393	
SE7-F[a]	CCGACCCAATAAGGAG	61	2961466-79	(15)
SE7-R	CTTACCGTTGGTAGTTTGTTA		2961949-29	
SE8-F[c]	TTGCCGCATAGCAGCAGAAGT	87	2812703-23	(9)
SE8-R	GCCTGAACACGCTTTTTAATAGGCT		2813171-49	
SE9-F[c]	**PET-**CGTAGCCAATCAGATTCATCCCGCG	9	533460-36	(9)
SE9-R	**GTTTCTT**TGAAACGGGGTGTGGCGCTG		533132-53	
SE10-F[b]	GCTGAAGAAGCGGCAAAAC	45	774231-49	(17)
SE10-R	GTACCGCTATCTTTCGATGGC		774760-40	

a: Primer and VNTR sequences derived from a partial genome sequence of *Salmonella* Enteritidis LK5 (http://www.sanger.ac.uk/Projects/Salmonella/SEN_genepred.embl).

b: Primer and VNTR sequences derived from a *Salmonella* serotype Typhimurium LT2 genome sequence (reference sequence number NC 003197).

c: Primer and VNTR sequences derived from a *Salmonella* serotype Enteritidis PT4 genome sequence (http://www.salmonella.org/genomics/sen.dbs).

d: Sequence of reverse primers (SE1,SE2,SE5,SE9) that were modified by adding nucleotides at the 5' end for clear peaks by capillary electrophoresis.

Table 3 List of primers and product sizes for detection of *Sdf I* and virulence-associated genes

Primer name	Primer sequence (5'---3')	Product size	Reference
SpvA-F	GTCAGACCCGTAAACAGT	641bp	(22)
SpvA-R	GCACGCAGAGTACCCGCA		
SopE-F	AGCGCATCTGAGGGCCG	565bp	This study*
SopE-R	GTTCATATTAATCAGGAAGAGGCTC		
*SodC*I-F	TCACAGTTTCAGAGACACCTTACGG	327bp	This study*
*SodC*I-R	CTTTATGGATCATCAATGAGTGACC		
Sdf-F	TGTGTTTTATCTGATGCAAGAGG	333bp	(3)
Sdf-R	CGTTCTTCTGGTACTTACGATGAC		

*Originally designed primer based on *sop*E and *sod*CI sequences of *S.* Enteritidis strain P125109

Table 4 Frequency of antimicrobial resistance profiles in blood and stool *Salmonella* Enteritidis isolates in Thailand during 2004-2007

Resistant profile*	Blood (N = 99)				Total blood	Stool (N = 91)				Total stool	Total
	2004	2005	2006	2007	N (%)	2004	2005	2006	2007	N (%)	N (%)
Resistances	**21**	**17**	**21**	**23**	**82(82.8)**	**15**	**22**	**23**	**23**	**83 (91.2)**	**165 (86.8)**
AMP				1	1(1.2)		1		1	2(2.4)	3(1.8)
NAL	3	1	1	1	6(7.3)	1	3		3	7(8.4)	13(7.9)
STR							1			1(1.2)	1(0.6)
TET								1		1(1.2)	1(0.6)
AMP/NAL	2	2	4	9	17(20.7)	5	4	5	6	20(24.1)	37(22.4)
CIP/NAL	5	5	1		11(13.4)	3	2		3	8(9.6)	19(11.5)
NAL/SXT	2				2(2.4)						2(1.2)
AMC/AMP/NAL								1		1(1.2)	1(0.6)
AMC/AMP/STR							1			1(1.2)	1(0.6)
AMP/CHL/NAL									1	1(1.2)	1(0.6)
AMP/CIP/NAL		4	5	7	16(19.5)		2	3	3	8(9.6)	24(14.5)
AMP/NAL/STR							1	1		2(2.4)	2(1.2)
AMP/NAL/SXT		1	1		2(2.4)						2(1.2)
CIP/CTX/NAL							1			1(1.2)	1(0.6)
CIP/NAL/STR						1				1(1.2)	1(0.6)
CIP/NAL/SXT	4				4(4.9)	1				1(1.2)	5(3.0)
CIP/NAL/TET			2	1	3(3.7)		2	2	1	5(6.0)	8(4.8)
NAL/SXT/TET							1			1(1.2)	1(0.6)
AMC/AMP/CIP/NAL	2				2(2.4)	2				2(2.4)	4(2.4)
AMP/CIP/NAL/NOR				1	1(1.2)						1(0.6)
AMP/CIP/NAL/STR		1	3	1	5(6.1)		1	6	2	9(10.8)	14(8.5)
AMP/CIP/NAL/SXT				1	1(1.2)						1(0.6)
AMP/CIP/NAL/TET			1		1(1.2)						1(0.6)
AMP/NAL/SXT/TET			1		1(1.2)						1(0.6)
CHL/STR/SXT/TET									1	1(1.2)	1(0.6)
CIP/NAL/SXT/TET	3	2	1	1	7(8.5)	1	1	3	1	6(7.2)	13(7.9)
AMC/AMP/CIP/NAL/STR		1			1(1.2)						1(0.6)
CIP/NAL/STR/SXT/TET						1			1	2(2.4)	2(1.2)
AMC/AMP/CIP/NAL/STR/TET			1		1(1.2)						1(0.6)
AMP/CHL/NAL/STR/SXT/TET								1		1(1.2)	1(0.6)
CHL/CIP/NAL/STR/SXT/TET							1			1(1.2)	1(0.6)
Susceptible	**5**	**9**		**3**	**17 (17.2)**	**3**	**2**	**2**	**1**	**8 (8.8)**	**25 (13.2)**
Total isolates	**26**	**26**	**21**	**26**	**99 (100)**	**18**	**24**	**25**	**24**	**91 (100)**	**190 (100)**

*Abbreviations of antimicrobial drugs are as follows, AMC, amoxicillin clavulanic acid; AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; CTX, cefotaxime; NAL, nalidixic acid; NOR, norfloxacin; STR, streptomycin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline

Table 5 Frequency of antimicrobial resistance patterns in *Salmonella* Enteritidis isolated from different regions of Thailand

Resistance profile*	Total (n=190)	No. (%) of isolates in different regions[#]						
		BK (n=62)	C (n=12)	E (n=9)	N (n=32)	NE (n=27)	S (n=20)	W (n=28)
Resistances	**165**	**55(89)**	**7(58)**	**8(89)**	**30(94)**	**25(93)**	**19(95)**	**21(75)**
AMP	3		1(14)		1(3)			1(5)
NAL	13	6 (11)			2(6)	3(12)	1(5)	1(5)
STR	1	1(2)						
TET	1				1(3)			
AMP/NAL	37	9(16)	2(29)	4 (50)	5(17)	2(8)	7(37)	8(38)
CIP/NAL	19	8(15)			5(17)	2(8)	2(11)	2(10)
NAL/SXT	2			2(25)				
AMC/AMP/NAL	1						1(5)	
AMC/AMP/STR	1					1(4)		
AMP/CHL/NAL	1				1(3)			
AMP/CIP/NAL	24	9(16)	1(14)	1(13)	4(13)	5(20)	2(11)	2(10)
AMP/NAL/STR	2	2 (4)						
AMP/NAL/SXT	2	2(4)						
CIP/CTX/NAL	1					1(4)		
CIP/NAL/STR	1					1(4)		
CIP/NAL/SXT	5	1(2)			1(3)	2(8)		1(5)
CIP/NAL/TET	8	4(7)				1(4)	3(16)	
NAL/SXT/TET	1				1(3)			
AMC/AMP/CIP/NAL	4	2(4)			1(3)			1(5)
AMP/CIP/NAL/NOR	1	1(2)						
AMP/CIP/NAL/STR	14	3(6)	3 (43)		4(13)	2(8)		2(10)
AMP/CIP/NAL/SXT	1					1(4)		
AMP/CIP/NAL/TET	1					1(4)		
AMP/NAL/SXT/TET	1					1(4)		
CHL/STR/SXT/TET	1				1(3)			
CIP/NAL/SXT/TET	13	4(7)		1(13)	3(10)	1(4)	3(16)	1(5)
AMC/AMP/CIP/NAL/STR	1	1(2)						
CIP/NAL/STR/SXT/TET	2	1(2)						1(5)
AMC/AMP/CIP/NAL/STR/TET	1					1(4)		
AMP/CHL/NAL/STR/SXT/TET	1							1(5)
CHL/CIP/NAL/STR/SXT/TET	1	1(2)						
Susceptible	**25**	**7(11)**	**5(42)**	**1(11)**	**2(6)**	**2(7)**	**1(5)**	**7(25)**

*Abbreviations of antimicrobial drugs are as follows: AMC, amoxicillin clavulanic acid; AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; CTX, cefotaxime; NAL, nalidixic acid; NOR, norfloxacin; STR, streptomycin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline

[#]Abbreviations of the regions are as follows: BK, Bangkok; C, Central; E, East; N, North; NE, North-East; S, South; W, West

Table 6 Nei's index of VNTR loci used to discriminate MLVA types in *Salmonella* Enteritidis isolates from various international geographic locations

Locus	This study (n = 192)	Thailand[a] (n = 40)	USA[b] (n = 245)	Indiana[c] (n = 41)	Minnesota[d] (n = 153)
SE1	0.24	0.23	0.59	0.55	0.62
SE2	0.07	0.05	0.79	0.68	0.73
SE3	0[e]	0	0.51	0.41	0.54
SE5	0.65	0.68	0.73	0.64	0.77
SE6	0[e]	0	0.03	0	0.07
SE7	0.12	nd	0.50	0.48	0.60
SE8	0[e]	0	0.48	0.51	0.45
SE9	0.06	0.09	0.44	0.38	0.53
SE10	0[e]	nd	0.04	0	0.05

nd; not determined

a; Clinical strains isolated in 2008 (Ref.25)

b; Clinical strains isolated in various state in USA during the period 2000-2007 (Ref.16)

c; Clinical strains isolated in the period 1990-1999 (Ref.17)

d; Clinical strains isolated in the period 1998-2003 (Ref. 9)

e; n = 89

Table 7 Distribution of antimicrobial resistance in 20 MLVA types of *Salmonella* Enteritidis isolates from Thailand

Antimicrobial resistance	5-5-11-7-3	5-5-9- 7-3	5-5-10-7-3	4-5-10-7-3	4-5-11-7-3	5-5-10-null-3	5-5-12-7-3	4-7.3-11-7-2	5-5-8-7-3	5-5-13-7-3	4-5-6-7-3	4-5-9-7-3	4-5-13-7-3	5-4-9-7-3	5-5-11-5-3	5-5-11-null-3	5-5-12-null-3	6-9-8-7-2	8-9-8-4-2	8-11-12-8-2
Overall resistance*	86	30	16	6	2	6	5	1	3	3	1	1	0	1	1	1	1	0	1	0
(%)	(97)	(94)	(73)	(60)	(33)	(86)	(100)	(33)	(100)	(100)	(100)	(100)		(100)	(100)	(100)	(100)		(100)	
NAL (n = 158)	83#	30	15	6	2	6	4		3	3	1	1		1	1	1	1			
CIP (n = 97)	43	23	11	2	2	5	1		3	3		1		1		1	1			
NOR (n = 1)	1																			
AMP (n = 96)	80#	2	5				5			3					1					
AMC (n = 8)	5		1				1			1										
TET (n = 31)	5	14#	2		1	4		1	1							1	1		1	
SXT (n = 30)	5	19#	1			1			2					1					1	
STR (n = 26)	17	3	3	1			1												1	
CHL (n = 4)	2	1																	1	
CTX (n = 1)						1														
MDR$^{\eta}$ (n = 42)	20	14	3	0	0	1	1	0	1	1	0	0	0	0	0	0	0	0	1	0
(%)	(23)	(47)	(19)			(14)	(20)		(33)	(33)									(100)	
Total (n = 190)	89	32	22	10	6	7	5	3	3	3	1	1	1	1	1	1	1	1	1	1

*Resistance to at least one antimicrobial

$^{\eta}$Resistance to at least 3 classes of antimicrobials defined by Magiorakos AP *et al*.(45)

Statistical significance ($P < 0.001$)

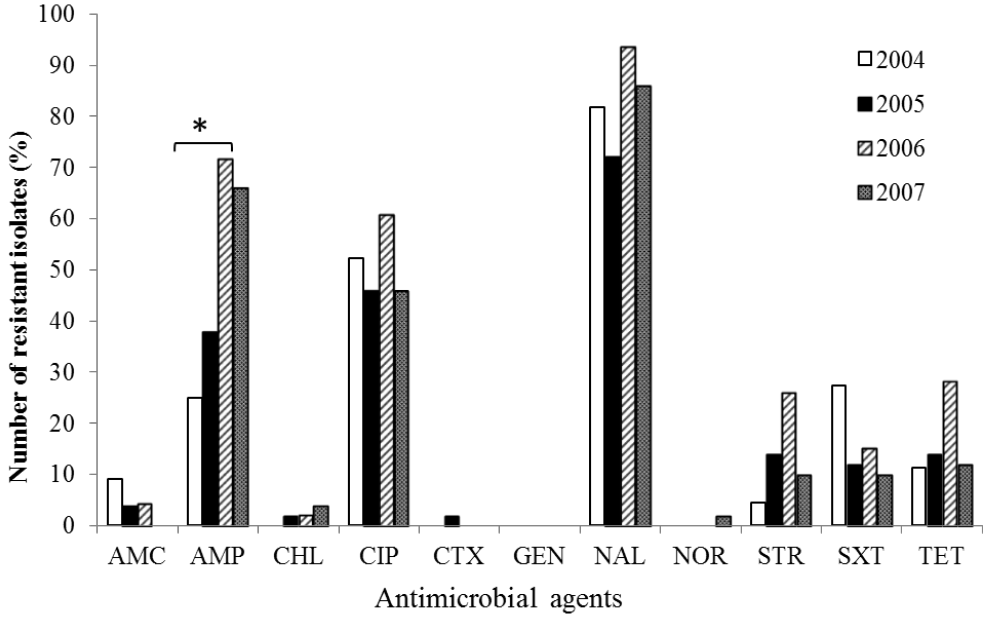

Fig 3 Rate of resistance to antimicrobials in *Salmonella* Enteritidis distributed in Thailand by year (2004-2007). Abbreviations of the antimicrobial agents are as follows: AMC, amoxicillin/clavulanic acid; AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; CTX, cefotaxime; GEN, gentamicin; NAL, nalidixic acid; NOR, norfloxacin; STR, streptomycin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline. The asterisk denotes statistical significance ($P < 0.001$) obtained by the Chi square test.

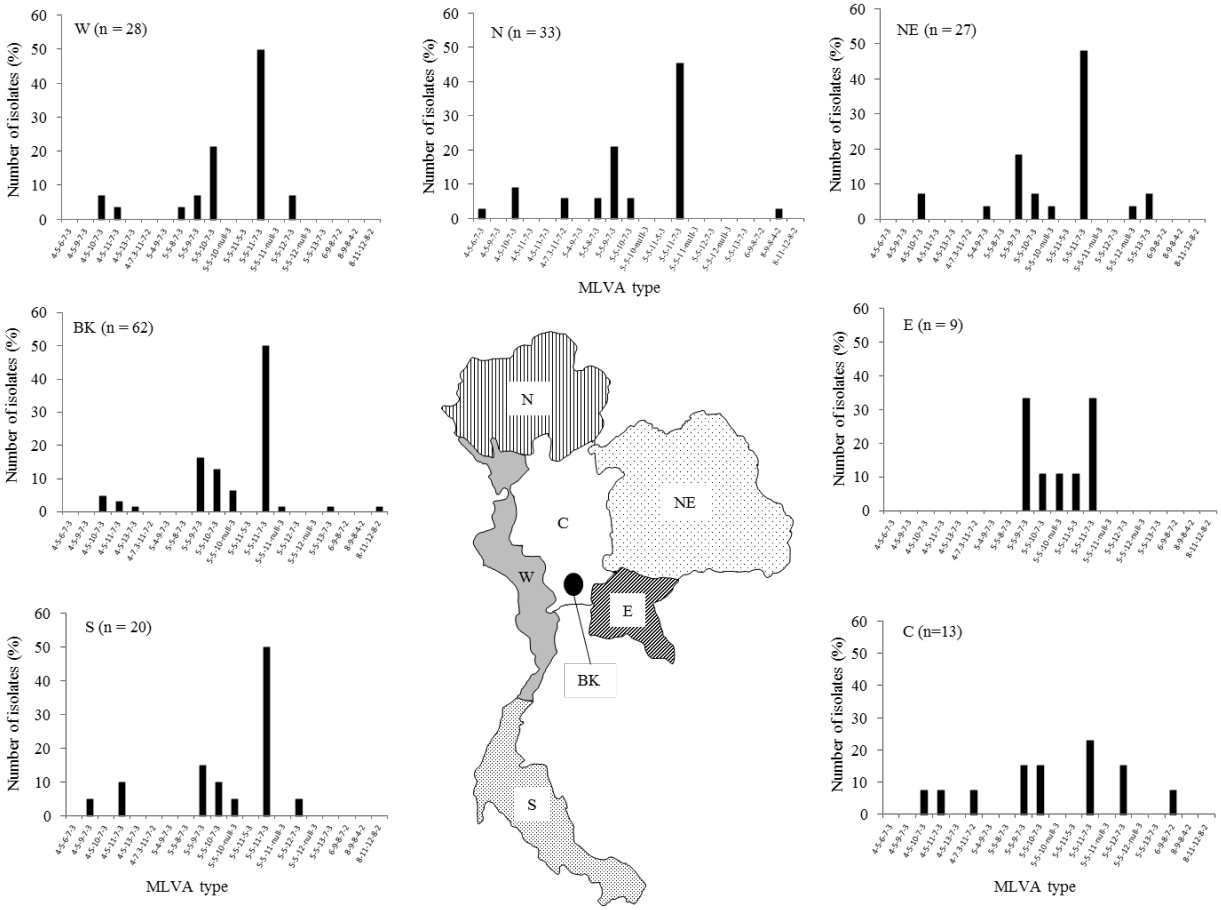

Fig 4 Distribution of MLVA types of *Salmonella* Enteritidis in different Thai regions. Region abbreviations: N, north; NE, north-east; C, central; W, west; E, east; and S, south regions of Thailand; BK, Bangkok. Different MLVA types are shown with the code numbers of tandem repeat in loci SE1, SE2, SE5, SE7 and SE9

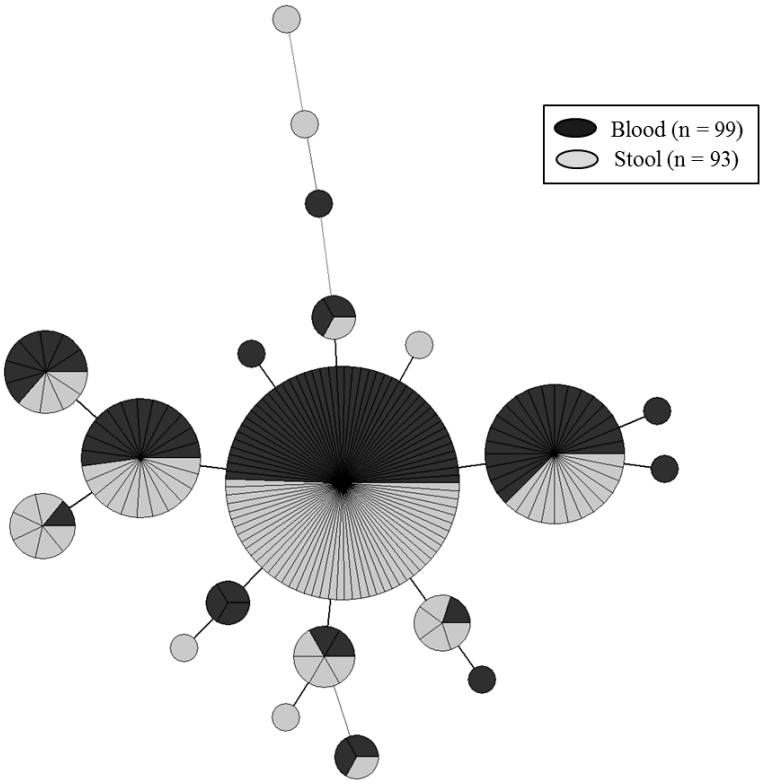

Fig 5 Minimum spanning tree of MLVA types of *Salmonella* Enteritidis in Thailand. Each circle in the tree represents a MLVA type determined by five VNTR loci. The circle sizes depend on the number of isolates, which is indicated by the number of sections in each circle. The length of the connecting lines between circles corresponds to the number of different loci between the two connected MLVA types. The three large circles represent commonly found MLVA types: 5-5-11-7-3 (n = 89), 5-5-9-7-3 (n = 32) and 5-5-10-7-3 (n = 23).

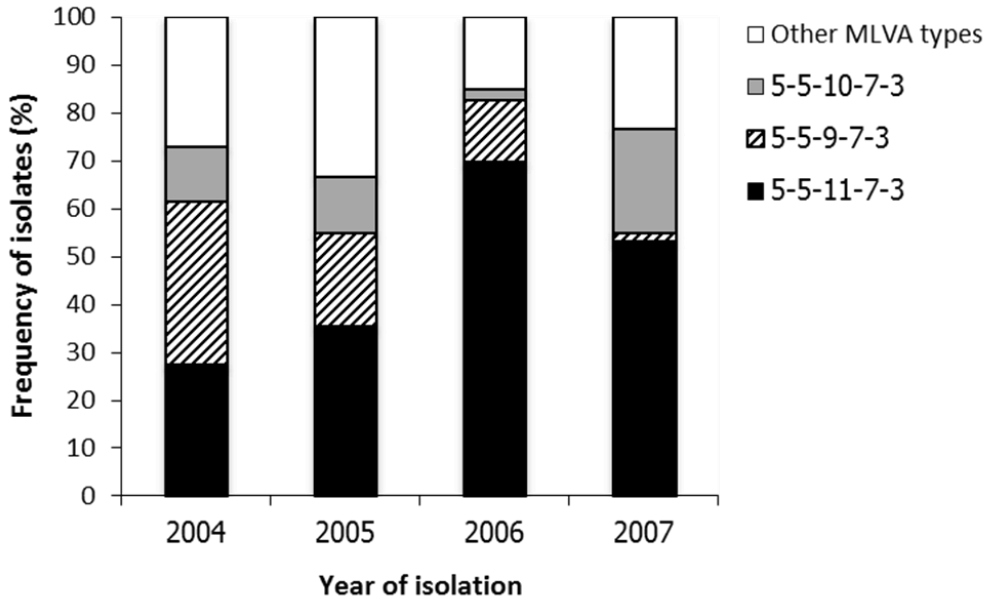

Fig 6 Ratio of MLVA types by year (2004-2007). Each square bar represents a MLVA type as follows: ■, MLVA type 5-5-11-7-3; ■, MLVA type 5-5-9-7-3; ■, MLVA type 5-5-10-7-3; □, other MLVA types.

CHAPTER II

Quinolone Resistance Determinants of Clinical *Salmonella* Enteritidis in Thailand

Nontyphoidal *Salmonella* is a global public health concern, particularly regarding antimicrobial resistance (30, 59). At present, a high nalidixic acid (NAL) resistance rate in *S*. Enteritidis, which is considered potentially invasive, is an emerging problem in many countries including Thailand (10, 24, 47, 59). NAL resistance can be the cause of reduced susceptibility to ciprofloxacin (CIP) (2, 24), which is the most effective therapeutic drug of choice.

In *Salmonella*, quinolone resistance is mainly mediated by point mutations of topoisomerase genes in the quinolone resistance-determining region (QRDR), usually in *gyrA*, occasionally in *gyrB* or *parC* and rarely in *parE* (28). Mutations causing amino acid substitutions in DNA gyrase subunit A (GyrA) of *Salmonella* have been commonly reported at codons 83 and 87 with different frequencies in serovars (19) and geographical areas (39). Quinolone resistance in *Salmonella* can also be acquired by a number of plasmid-mediated quinolone resistance (PMQR) determinants, including the *qnr* genes, *aac(6')-lb-cr*, *qepA* and *oqxAB* (38, 56). Of these, *qnr* genes encode a family of proteins with pentapeptide repeat that can bind to DNA gyrase and protect it by preventing an interaction with quinolones (56). At present, *qnr* gene variants, *qnrA, qnrB, qnrD*, *qnrS* and *oqxAB* have been reported in nontyphoidal *Salmonella* worldwide (11, 12, 27, 32, 33, 37, 54, 61).

In Thailand, an increasing trend of quinolone resistance in *S*. Enteritidis was reported during 2001-2008 (25, 59). However, the QRDR mutational status and the prevalence of *qnr* genes in the serovars have not been elucidated. Therefore, this study aimed to investigate these quinolone resistance determinants in clinical NAL-resistant *S*. Enteritidis isolates collected nationwide in Thailand from 2004 to 2007. In addition, the relationship between these resistance determinants and multilocus variable number tandem repeat analysis (MLVA) typing results was analysed to elucidate the dissemination of specific clones.

Materials and Methods

Salmonella strains

A total of 190 *S.* Enteritidis clinical isolates recovered from randomly selected blood (n = 99) and stool (n = 91) specimens were used in this study. The isolates were collected in regions throughout Thailand, namely, Bangkok (n = 62), Northern (n = 32), Northeastern (n = 27), Eastern (n = 9), Central (n = 12), Western (n = 28) and Southern (n = 20) provinces by the WHO National *Salmonella* and *Shigella* Center, Ministry of Public Health, Thailand, during the period 2004-2007. Isolates were first categorised into susceptible, intermediately or fully resistant to NAL, CIP and NOR (Fig. 7) by the Clinical and Laboratory Standard Institute (CLSI) zone size criteria (18).

Determination of minimum inhibitory concentrations

The minimum inhibitory concentrations (MICs) of NAL, NOR and CIP against intermediately or fully resistant strains by CLSI zone size criteria were determined by E-test (Biomerieux SA, Marcy l'Etoile, France) according to the manufacturer's protocol. The clinical breakpoint used in this study was NAL, resistant (R) $\geq$ 32 mg/L, susceptible (S) $\leq$ 16 mg/L; NOR, R $\geq$ 16 mg/L, S $\leq$ 4 mg/L; and CIP, R $\geq$ 1 mg/L, S $\leq$ 0.06 mg/L, according to the CLSI guideline (18). *Escherichia coli* ATCC 25922 was used as the quality control organism.

Detection of topoisomerase gene mutations

Genomic DNA was extracted using the DNeasy® Blood & Tissue kit (Qiagen Inc., Valencia, CA, USA) in accordance with the manufacturer's protocol. Mutations in the QRDR of topoisomerase genes *gyrA, gyrB* and *parC* were analysed by PCR and sequencing. We didn't analyze *parE* as no previous report showed mutations in *parE* in quinolone resistant *Salmonella*. Three primer sets were designed to amplify these QRDR (Table 8). The PCR mixture (20 µL) contained 1 µL of DNA template (1-10 ng), 0.25 mM dNTP (Biolabs Inc., New England, USA), 0.25 µM of each primer and 1 U of GoTaq® DNA polymerase in 1× Green GoTaq® buffer (Promega Madison, WI, USA). The amplification was conducted in an iCycler (BioRad, Hercules, CA, USA) under the following conditions: predenaturation at 96°C for 1 min; 35 cycles of denaturation at

96°C for 10 sec; annealing at 52°C for 10 sec and extension at 72°C for 30 sec; and a final extension at 72°C for 5 min. DNA sequences of amplified fragments were analysed using a BigDye® terminator version 3.1 cycle sequence reaction kit and an ABI 3130 genetic analyser (Applied Biosystems, Foster, CA, USA) in accordance with the manufacturer's instructions. Obtained sequences were compared with that of the reference strain P125109 with accession number NC_011294 in the GenBank.

PCR amplification and sequence confirmation of *qnr* genes

As *qnrA, qnrB* and *qnrS* have been reported to be widely spreading (56), the presence of these genes in isolates was examined by PCR using the primer sets designed by Robicsek *et al*. (55) under the amplification conditions used in their study. DNA sequences of amplicons were analysed as above.

Characterization of plasmid carrying *qnrS*

Plasmids were purified from *S*. Enteritidis strain by NucleoSpin® plasmid kit (Macherey-Nagel, Duren, Germany) and *Escherichia coli* Top10 (Thermo Fisher Scientific Inc., Waltham, MA, USA) was transformed according to the manufacturer's manual. Colonies grown on the Luria-Bertani broth (Thermo Fisher Scientific Inc.) containing 0.1 μg/mL CIP were selected for further analysis. Plasmids in transformants were identified using nuclease S1 digestion before PFGE as previously described (60). Briefly, DNA embedded in agarose was digested with 0.1 U/mL nuclease S1 (Promega, Medison, WI) and separated by 1% agarose gels in a CHEF DR-III system. Electrophoresis was carried out at 6 V/cm at an angle of 120° with pulses ramping 30 sec for 13 hr in 0.5 x Tris–borate–EDTA buffer at 14°C. Southern blot hybridi-zations were performed by standard methods. To determine the location of *qnrS* on plasmids, specific probes labeled with digoxigenin using the PCR DIG Labeling Mix (Roche Diagnostics GmbH, Mannheim, Germany) were used. Hybridization procedures and conditions were performed according to the manufacturer's manual.

Conjugation experiments were done among *S*. Enteritidis harbouring *qnrS* positive strains and *E. coli* Top10 or DH5α (Thermo Fisher Scientific Inc.) as recipients by mating on Luria Bertani (LB) agar plate. Transconjugants were selected on LB agar containing streptomycin (100 μg/mL) + CIP (0.1 μg/mL) and confirmed by indole positivity as a marker for *E. coli*. Antimicrobial resistance profiles of *S*. Enteritidis isolates and transconjugants were determined by E-test or disk diffusion method in accordance with CLSI interpretation.

Multilocus variable number tandem repeat analysis

MLVA typing was conducted using previously designed primers for PCR amplification of five variable number tandem repeat (VNTR) loci, SE1, SE2, SE5, SE7 and SE9 (9, 15), with some modifications (Table 9). The number of repeat units in locus SE7 was calculated from the amplicon size estimated by electrophoresis. Short repeat loci (SE1, SE2, SE5, SE9) were simultaneously amplified using four primer sets with fluorescence-labelled forward primers, and each product size was determined by capillary electrophoresis as previously described. The product size of each locus was measured and converted into the number of repeat units by Peak Scanner software (Applied Biosystems).

Antimicrobial resistance phenotypes

Antimicrobial resistance of 190 *S*. Enteritidis isolates to eight drugs, amoxicillin clavulanic acid (AMC), ampicillin (AMP), cefotaxime (CTX), chloramphenicol (CHL), gentamicin (GEN), streptomycin (STR), tetracycline (TET) and trimethoprim/ sulfamethoxa-zole (SXT), was determined by the disk diffusion method according to the CLSI guideline (18).

Results

Quinolone resistance phenotypes

The disk diffusion test following the CLSI zone size criteria demonstrated that 158 isolates were intermediately or fully resistant and remaining 32 isolates were susceptible to all of NAL, CIP and NOR. The distribution of MICs in 158 *S*. Enteritidis isolates with intermediate or fully resistance to NAL are shown in Table 10. A high MIC of NAL (≥ 256 mg/L) was observed for 87.3% (138/158) of the isolates, whilst the remaining 20 isolates presented MICs ranging 16-64 mg/L. In addition, 143 isolates (90.5%) were NAL resistant (NAL^R). Except for one, all isolates were susceptible to NOR. As for CIP, 145 isolates (91.8%) had decreased susceptibility (CIP^{DS}, 0.064 < MIC <1 mg/L), whilst only three isolates (1.9%) were resistant (CIP^R). The most common phenotype, NAL^RCIP^{DS} was observed in 82.3% (130/158) of the isolates (Table 10).

Topoisomerase gene mutations and presence of *qnr* genes

The sequencing results of *gyrA*, *gyrB* and *parC* demonstrated that 138 of the studied isolates presented mutations in the QRDR of *gyrA*, while no isolates had mutations in either *gyrB* or *parC*. Mutations in *gyrA* were observed at codons 83 and 87, with three distinct amino acid substitutions in each codon (Table 10). Mutations appeared more frequently at codon 87 (87/138, 63.0%) than at codon 83 (50/138, 36.2%). A mutation causing an amino acid substitution of aspartic acid to tyrosine at position 87 (Asp87Tyr) was most frequently identified (83/138, 60.1%), followed by Ser83Tyr (40/138, 29.0%). Eight isolates had mutations at codon 83, serine (T<u>C</u>C) → isoleucine (<u>AT</u>C), causing amino acid substitution Ser83Ile, and one isolate had a double amino acid substitution in the QRDR (Ser83Phe + Asp87Tyr). In addition, a total of nineteen isolates were identified as positive for *qnrS1* by PCR and sequencing. In contrast, 32 isolates susceptible to all of NAL, CIP and NOR by CLSI zone size criteria were with wild type *gyrA* and negative for *qnr* genes.

Characterization of plasmids in *qnrS1* positive *S.* Enteritidis isolates

A total of nineteen isolates were identified as positive for *qnrS1* by PCR and sequencing (Table 11). Transformation experiments using extracted plasmids from *S.* Enteritidis strains positive for *qnrS* were performed using *E. coli* Top 10 as a recipient and all experiment gave CIP resistant transformants. Then, S1 nuclease analyses using PFGE followed by southern blotting were done to show the size of plasmids to be 104.7 kbp and 52.7 kbp in strain ID134 and others, respectively (Fig. 8). Trans-conjugation experiments using the representative strains, SE-50 and SE-110, demonstrated the increase of MICs. The MICs of CIP and NOR against recipient *E. coli* Top 10 were <0.002 and <0.016 mg/L, respectively, whereas those against transconjugants with SE-50 were 0.125 and 0.5 mg/L, respectively. Similar increase of MICs of CIP and NOR were observed with transconjugant between *E. coli* DH5α and SE-110. Transconjugants also showed the reduction of the zone of inhibition by NAL and AMP (Table 12).

Relationship between quinolone resistance determinants and level of quinolone resistance

The relationship between quinolone resistance determinants and the MICs of NAL, NOR and CIP for each isolate is shown in Table 10. Among the 138 *gyrA* mutants, all but two isolates showed high-level resistance to NAL (MIC ≥ 256 mg/L). The double mutant with Ser83Phe/Asp87Tyr showed a fully resistant phenotype to NAL (≥ 256 mg/L), NOR (16 mg/L) and CIP (4 mg/L). In contrast, single codon mutants were found associated with a wide range of MICs for NOR (0.19 - 4 mg/L) and CIP (0.032 - 2 mg/L).

Isolates harbouring *qnrS1* and wild-type *gyrA* exhibited an atypical quinolone resistance phenotype that could be identified as susceptible or low level resistance to NAL (MIC ≤ 32 mg/L) and decreased susceptibility to CIP (≥ 0.125 mg/L) (23). One *qnrS1*- carrying isolate with a Ser83Tyr substitution in *gyrA* presented high MICs for all quinolones: NAL (≥ 256 mg/L), NOR (3 mg/L) and CIP (2 mg/L). Two isolates did not have *gyrA*, *gyrB* or *parC* QRDR mutations or *qnr* genes, although they showed high resistance to NAL (≥ 256 mg/L) and decreased susceptibility to CIP.

Relationship between quinolone resistance determinants and MLVA types

Asp87Tyr and Ser83Tyr, two dominant mutants observed in the isolates, showed an opposite trend of prevalence. Indeed, the frequency of Asp87Tyr significantly increased from 38.9% in 2004 to 74.4% in 2007 (P = 0.003), while that of Ser83Tyr significantly decreased from 52.8% to 11.6% during the same period (P = 0.001). The mutants were identified at similar frequencies in both blood and stool specimens (Table 13). MLVA types of isolates were analysed and are shown with the number of repeat units in the order of VNTR loci SE1-SE2-SE5-SE7-SE9 (Table 14). Asp87Tyr was strongly associated with type 5-5-11-7-3, while Ser83Tyr was related to another type 5-5-9-7-3. Most *qnrS1*-carrying strains were associated with type 5-5-11-7-3 and resistant to both ampicillin (AMP) and streptomycin (STR) (Table 11). All eight isolates harbouring Ser83Ile were found to have similar MLVA types with no amplification of locus SE7 (Table 15). All the resistance determinant types and MLVA types were widely distributed in Thailand (Table 16).

Discussion

To elucidate the quinolone resistance acquisition mechanism, 190 *S.* Enteritidis clinical isolates investigated, 158 were elucidated to be intermediately or fully quinolone resistant, and 156 were identified to carry quinolone resistance determinants examined in this study. Amongst these, 138 isolates had mutations in *gyrA* and 19 isolates carried *qnrS1*. The MIC_{50}, at which 50% of isolates (i.e. 50% of the population) are inhibited, for NAL was ≥ 256 mg/L, while that for NOR and CIP was 0.75 and 0.125 mg/L, respectively (Table 10). MIC_{50} of NAL, NOR and CIP for isolates with *qnrS1* were 24, 1.5 and 0.38 mg/L, respectively. In contrast, those for isolates with GyrA amino acid substitutions were ≥ 256, 0.75 and 0.125 mg/L, respectively, and MIC of an isolate with both *qnrS1* and a GyrA amino acid substitution (Ser83Tyr) was ≥ 256, 3 and 2 mg/L, respectively. In the context of phenotype distribution among isolates, all 138 isolates with GyrA single amino acid substitution and 3 of 18 isolates with *qnrS1* were resistant to NAL. In contrast, all isolates with *qnrS1* and all isolates with GyrA single amino acid substitutions were susceptible to NOR. Similarly, all isolates with *qnrS1* and 137 isolates with single GyrA single amino acid substitution were susceptible

to CIP. An isolate with both *qnrS1* and a GyrA amino acid substitution (Ser83Tyr) was intermediately resistant to CIP. Only one isolate with GyrA double amino acid substitution (Ser83Phe + Asp87Tyr) was resistant to both NOR and CIP. These results confirmed the higher potency of fluorinated quinolones as DNA gyrase inhibitors against strains with *qnrS1* and GyrA single amino acid substitutions or both. In the current study, *gyrA* mutations causing single amino acid substitutions associated with a higher MIC of NAL and lower susceptibility to CIP were observed, which concurs with data from studies of clinical *S.* Enteritidis isolates in other countries (36, 39, 44). In addition, the highest MICs of CIP and NOR were estimated for an isolate with a double mutation causing two amino acid substitutions. This finding is in agreement with previous reports describing the *Salmonella* requirement of multiple mutations for high-level resistance to fluoroquinolones (28, 42).

In our study, the mutation causing an amino acid substitution at codon 87 was predominantly Asp87Tyr (83/138), which was also found in many other countries including Spain (44), the United Kingdom (19), Norway (39) and Ireland (34). Ser83Tyr was the second predominant mutation (40/138) and was also observed in Norway (39). In our study, we found strong correlations between specific *gyrA* mutations and MLVA types (Table 14). Interestingly, unlike the cases in Norway, which were associated with travel to Southeast Asia, our data suggest a clonal expansion of quinolone resistant strains within Thailand.

We identified Ser83Ile in eight isolates distributed nationwide in Thailand (Table 15). This is the second finding all over the world following that reported from Malaysia (62). All isolates with this substitution presented high MICs of NAL ($\geq$ 256 mg/L), NOR (0.25-1 mg/L) and CIP (0.094-0.38 mg/L). In addition, isolates with Ser83Ile possessed similar MLVA types, of which different alleles were observed only at a single locus in two isolates (Table 15). Interestingly, all eight isolates with Ser83Ile harboured an identical null allele in locus SE7, which is in the clustered regularly interspaced short palindromic repeats, which are known to be highly polymorphic in *Salmonella* (20). This evidence indicates that strains with this new mutation might have originated from a single source that clonally expanded throughout Thailand under certain selective pressures. At codon 83, serine (T<u>C</u>C) to tyrosine (T<u>A</u>C) or

phenylalanine (TTC) substitution requires only a single base change. In contrast, a substitution to isoleucine (ATC) needs a secondary base substitution. The mutated codon of ATC causing Ser83Ile might be derived from a base substitution of serine (TCC) via phenylalanine (TTC) during bacterial survival. It is likely that isoleucine may provide some advantages to the enzymatic activity of DNA gyrase compared with phenylalanine. Low prevalence of Ser83Phe and the expansion of the clone with Ser83Ile seem to support this hypothesis. Further studies seemed to be necessary to clarify this.

In our current study, we found 19 in 190 *S.* Enteritidis isolates (10 %) carried *qnrS1*. In contrast, *S.* Enteritidis strains carrying the *qnrS1* gene are rarely reported. For example, a study in 13 European countries found 125 *Salmonella* isolates carrying *qnrS1*, but only two were *S*. Enteritidis from pigs in Poland (63). Limited number of *S*. Enteritidis isolates from neonatal calf diarrhea in Egypt carried *qnrS* (4). In Asian countries, *qnrS* was identified in several clinical *Salmonella* serovers mainly from serovar other than Enteritidis as reported in Korea (31) and Taiwan (32). The report from China also reported low percentage of *qnrS* in *S*. Enteritidis (0.8%, 1/126) (64). In Thailand, *qnrS1* was previously detected in 16 *S*. Corvallis isolated from human and food animals (12) and recently observed in clinical *S*. Stanley and *S*. Anatum from poultry (58). To our best knowledge, this is the first work reporting clinical *S*. Enteritidis carrying *qnrS1* in Thailand.

As *qnrS1* is a PMQR gene, *qnrS1* in *S.* Enteritidis may have resulted from the acquisition of transferable plasmids from other bacteria. The existence of transferable plasmids carrying *qnrS1* with the size of 104.7 kbp and 52.7 kbp in strain ID134 and others, respectively, was confirmed by the nuclease S1 analysis of CIP resistant *E. coli* transformants (Fig. 8). The transferability of the plasmid carrying *qnrS1* was also confirmed by conjugation (Table 12). The AMP resistant phenotype of transconjugants (Table 12) supported the idea of the coexistence of *qnrS1* and the gene encoding β-lactamase, *bla*, in the same plasmid as has been reported in *Klebsiella pneumoniae* (bla_{SHV-2}) (14) and *S. enterica* serovar Stanley (bla_{LAP-2}) (21). This will be elucidated by our further study.

We found that all *qnrS1*-carrying strains with wild type *gyrA* also showed atypical quinolone resistant phenotype with decreased susceptibility to CIP and susceptible or low level resistance to NAL (CIP^{DS} $NAL^{S/LR}$) (Table 11), which is consistent with the observations reported by Gunell *et al*. (23). In contrast, other studies demonstrated that *qnrS* in *Salmonella* caused other resistance phenotypes, including susceptibility to both CIP and NAL (CIP^{S} NAL^{S}) (44) and decreased susceptibility to CIP and resistance to NAL (CIP^{DS} NAL^{R}) (31). In the present study, we also found an isolate carrying *qnrS1* with a *gyrA* mutation that showed high MICs for NAL (≥ 256 mg/L) and CIP (2 mg/L), which is consistent with observations in Korea (35) and the United Kingdom (27). These findings seemed to suggest that *qnrS* could enhance CIP resistance via the *gyrA* mutation. In fact, phenotype differences in isolates with *qnrS* may be associated with the level of Qnr protein expression. Similar to a single mutation in *gyrA*, carriage of *qnrS* alone does not confer strong fluoroquinolone resistance to *S. enterica*. Thus, reducing the quinolone pressure (e.g. NAL) on resistant strains could be crucial to prevent the emergence and spread of *S*. Enteritidis resistant to fluoroquinolone (e.g. CIP).

Since we found two *S*. Enteritidis isolates that were resistant to NAL and reduced susceptibility to CIP by without *qnrA*, *B* and *S1* genes and target mutations in type IV topoisomerase encoding genes, the other mechanisms such as an alteration of permeability (64), an enzymatic inactivation encoded by *aac(6')Ib cr* and effux pump encoded by *qep* and *oqxAB* (38, 56, 57) might be playing roles in developing the resistance to quinolones. Further investigation of other PMQRs will be conducted by our future study.

Summary

Salmonella Enteritidis has emerged as global concern regarding quinolone resistance and invasive potential. Although quinolone-resistant *S.* Enteritidis has been observed with high frequency in Thailand, information on the mechanism of resistance acquisition is limited. To elucidate the mechanism, a total of 158 clinical isolates of nalidixic acid (NAL)-resistant *S.* Enteritidis were collected throughout Thailand, and the quinolone resistance determinants were investigated in the context of resistance levels to NAL, norfloxacin (NOR) and ciprofloxacin (CIP). The analysis of point mutations in type II topoisomerase genes and the detection of plasmid-mediated quinolone resistance genes showed that all but two harboured a *gyrA* mutantion, the *qnrS1* gene or both. The most commonly affected codon in mutant gyrA was 87, followed by 83. Double codon mutation in *gyrA* was found in an isolate with high-level resistance to NAL, NOR and CIP. A new mutation causing serine to isoleucine substitution at codon 83 was identified in eight isolates. In addition to eighteen *qnrS1*-carrying isolates showing non-typical quinolone resistance, one carrying both the *qnrS1* gene and a *gyrA* mutation also showed a high level of resistance. Genotyping by multilocus variable number of tandem repeat analysis suggested a possible clonal expansion of NAL-resistant strains nationwide. Our data suggested that NAL-resistant isolates with single quinolone resistance determinant may potentially become fluoroquinolone resistant by acquiring secondary determinants.

Table 8 Primers used for polymerase chain reaction and their nucleotide positions and sizes of amplified products.

Primer	Sequence (5'---3')	Size (mer)	Product size (bp)
Sal-*gyr*A QRDR	Fw: GTTAGATGAGCGACCTTGCG	20	496
	Rv: GGAATTTTGGTCGGCATGAC	20	
Sal-*gyr*B QRDR	Fw: GCTGGAAAACCCATCTGACG	20	464
	Rv: CGATAGAAGAAGGTCAACAGC	21	
Sal-*par*C QRDR	Fw: CCCTGTTAATGAGCGATATG	20	519
	Rv: GCCGTTCAGCAGGATGTTCG	20	

QRDR, Quinolone resistance determining region

Table 9 Primer sequences for amplification of VNTR loci for MLVA typing

Locus	Primer sequences[a] (5'---3')	Repeat size (bp)	Reference
SE1-F	**NED-**AGACGTGGCAAGGAACAGTAG	7	(9)
SE1-R	**GTTTCTT**CCAGCCATCCATACCAAGAC		
SE2-F	**6-FAM-**CTTACGATTATACCTGGATTGTTGG	7	(9)
SE2-R	**GTTTCTT**GGACGGAGGCGATAG		
SE5-F	**VIC-**CGGGAAACCACCATCAC	6	(9)
SE5-R	**GTTTCTT**CAGGCCGAACAGCAGGAT		
SE7-F	CCGACCCAATAAGGAG	61	(15)
SE7-R	CTTACCGTTGGTAGTTTGTTA		
SE9-F	**PET-**CGTAGCCAATCAGATTCATCCCGCG	9	(9)
SE9-R	**GTTTCTT**TGAAACGGGGTGTGGCGCTG		

[a]Sequence of reverse primers (SE1, SE2, SE5, SE9) were modified by adding nucleotides at the 5' end for clear peaks by capillary electrophoresis.

Table 10 Distribution of minimum inhibitory concentrations and resistance determinants of *S.* Enteritidis in Thailand

Drugs	Resistance determinants		Total No.	Number of isolates according to MICs (mg/L)																			
	gyrA genotypes	*qnrS*		**0.032**	**0.047**	**0.064**	**0.094**	**0.125**	**0.19**	**0.25**	**0.38**	**0.5**	**0.75**	**1**	**1.5**	**2**	**3**	**4**	**8**	**16**	**24**	**32**	**64**	**≥256**
NAL	wild type (n=20)	+	18																	5	10	3		
		-	2																					2
	Mutants (n=138)																							
	Ser83Ile	-	8																					8
	Ser83Phe	-	2																					2
	Ser83Tyr	-	39																				1	38
		+	1																					1
	Asp87Asn	-	2																					2
	Asp87Gly	-	2																					2
	Asp87Tyr	-	83																			1		82
	Ser83Phe/Asp87Tyr	-	1																					1
NOR	wild type (n=20)	+	18											2	12	4								
		-	2										1	1										
	Mutants (n=138)																							
	Ser83Ile	-	8							1	1	1	1	4										
	Ser83Phe	-	2										1	1										
	Ser83Tyr	-	39							5		5	9	13	6	1								
		+	1														1							
	Asp87Asn	-	2											2										
	Asp87Gly	-	2							1		1												
	Asp87Tyr	-	83						1	2	1	28	34	11	2	3	1							
	Ser83Phe/Asp87Tyr	-	1																	1				
CIP	wild type (n=20)	+	18							1	16	1												
		-	2					1	1															
	Mutants-(n=138)																							
	Ser83Ile	-	8				2		1	2	3													
	Ser83Phe	-	2						1	1														
	Ser83Tyr	-	39		4	1	2	4	13	13	2													
		+	1													1								
	Asp87Asn	-	2					1	1															
	Asp87Gly	-	2	1			1																	
	Asp87Tyr	-	83	1	2	1	12	49	10	5		2		1										
	Ser83Phe/Asp87Tyr	-	1															1						

MICs, minimum inhibitory concentrations. CIP, ciprofloxacin; NAL, nalidixic acid; NOR,norfloxacin

Table 11 Characteristics of *S.* Enteritidis carrying *qnrS1*

ID	MICs (mg/L)			Resistance to other than quinolones	MLVA types	Source of isolate			*gyrA*
	NAL	CIP	NOR			Year	Specimen	Regions	
50	16	0.25	1	AMP/STR	5-5-11-7-3	2005	stool	BK	WT
92	16	0.38	1.5	AMP/STR	5-5-11-7-3	2005	blood	NE	WT
106	16	0.38	2.0	AMP/STR	5-5-11-7-3	2006	stool	N	WT
138	16	0.38	1.5	AMP/STR	5-5-9-7-3	2006	blood	C	WT
142	16	0.38	1.5	AMP/STR	5-5-11-7-3	2006	blood	NE	WT
77	24	0.38	1	AMC/AMP/STR	5-5-11-7-3	2005	blood	BK	WT
102	24	0.38	2	AMP/STR	5-5-11-7-3	2006	stool	N	WT
110	24	0.38	1.5	AMP	5-5-12-7-3	2006	stool	S	WT
114	24	0.38	1.5	AMP/STR	5-5-11-7-3	2006	stool	BK	WT
116	24	0.38	1.5	AMP/STR	5-5-11-7-3	2006	stool	BK	WT
123	24	0.38	1.5	AMP/STR	5-5-11-7-3	2006	stool	N	WT
128	24	0.38	1.5	AMC/TET/AMP/STR	5-5-11-7-3	2006	blood	NE	WT
156	24	0.38	1.5	AMP/STR	5-5-11-7-3	2007	stool	W	WT
160	24	0.38	1.5	AMP/STR	5-5-10-7-3	2007	stool	C	WT
177	24	0.38	1.5	AMP/STR	5-5-11-7-3	2007	blood	C	WT
117	32	0.38	2	AMP/STR	5-5-12-7-3	2006	stool	W	WT
131	32	0.38	1.5	AMP/STR	5-5-11-7-3	2006	blood	N	WT
134	32	0.5	2	AMP/TET	5-5-11-7-3	2006	blood	NE	WT
135	≥256	2	3	TET	5-5-10-7-3	2006	blood	S	Ser83Tyr

Abbreviations; AMC, amoxicillin+clavulanic acid; AMP, ampicillin; STR, streptomycin; TET, tetracycline. BK, Bangkok; C, Central provinces; E, Eastern provinces; N, Northern provinces; NE, Northeastern provinces; S, Southern provinces; W, Western provinces.

Table 12 Antimicrobial resistance patterns of *qnrS* positive *Salmonella* isolates and their transconjugants

	Strains	TSI	Indole	Zone size of disk diffusion (mm)		MICs (mg/L)	
				NAL	AMP	CIP	NOR
Exp-1	SE-50	K/A, H_2S^+	-	15	7	0.25	1.0
	E. coli Top 10	K/A, H_2S^-	+	30	20	<0.002	<0.016
	Conjugant	K/A, H_2S^-	+	24	<6	0.125	0.5
Exp-2	SE-110	K/A, H_2S^+	-	16	<6	0.38	1.5
	E. coli DH5α	K/A, H_2S^-	+	13	24	<0.016	0.094
	Conjugant	K/A, H_2S^-	+	<6	<6	2	6

Abbreviations; TSI, triple sugar iron agar slant; K/A, K over A, refers to an alkaline slant and acid butt.

Table 13 Amino acid substitutions in quinolone resistance determining region of *gyrA* and possession of *qnrS* among nalidixic acid resistant *S.* Enteritidis isolates in Thailand in 2004-2007

Amino acid substitution in *gyrA*	*qnrS1*	Total isolates (n=158)	Number of isolates				Number of isolates	
			2004 (n=36)	2005 (n=36)	2006 (n=43)	2007 (n=43)	Blood (n=81)	Stool (n=77)
Wild type	+	18		3	12	3	8	10
	-	2		1	1		2	
Ser83Ile	-	8		4	2	2	1	7
Ser83Phe	-	2		2			2	
Ser83Tyr	-	39	19	9	6	5	21	18
	+	1			1		1	
Asp87Asn	-	2	1			1	1	1
Asp87Gly	-	2	2				2	
Asp87Tyr	-	83	14	17	21	31	42	41
Ser83Phe + Asp87Tyr	-	1				1	1	

Table 14 Distribution of amino acid substitutions in *gyrA* and MLVA types of *Salmonella* Enteritidis isolates in Thailand

Amino acid substitutions in *gyrA*	5-5-11-7-3	5-5-9- 7-3	5-5-10-7-3	4-5-10-7-3	4-5-11-7-3	5-5-10-null-3	5-5-12-7-3	5-5-8-7-3	5-5-13-7-3	4-5-6-7-3	4-5-9-7-3	5-4-9-7-3	5-5-11-5-3	5-5-11-null-3	5-5-12-null-3
Wild type (n=20)	14	2	1				3								
Ser83Ile (n=8)						6								1	1
Ser83Phe (n=2)			2												
Ser83Tyr (n=40)	1	28	7					3				1			
Asp87Asn (n=2)			2												
Asp87Gly (n=2)				2											
Asp87Tyr (n=83)	67		3	4	2		1		3	1	1		1		
Ser83Phe + Asp87Tyr	1														
Total (n=158)	83	30	15	6	2	6	4	3	3	1	1	1	1	1	1

Table 15 Characteristics of *S.* Enteritidis with *gyrA* mutation at codon 83 (Ser83Ile)

ID	Source of isolate			MLVA type	Resistance profile*	MICs (mg/L)		
	Year	Specimen	Region			NAL	CIP	NOR
51	2005	Stool	BK	5-5-10-null-3	NAL/CIP/TET	≥ 256	0.25	0.75
54	2005	Stool	BK	5-5-10-null-3	NAL/CIP/TET	≥ 256	0.19	0.5
57	2005	Stool	NE	5-5-10-null-3	NAL/CIP/CTX	≥ 256	0.094	0.25
64	2005	Stool	S	5-5-10-null-3	NAL	≥ 256	0.094	0.38
100	2006	Stool	BK	5-5-11-null-3	NAL/TET	≥ 256	0.38	1
108	2006	Stool	BK	5-5-10-null-3	NAL/CIP/TET	≥ 256	0.25	1
163	2007	Stool	E	5-5-10-null-3	NAL/CIP/TET/SXT	≥ 256	0.38	1
181	2007	Blood	NE	5-5-12-null-3	NAL/CIP/TET	≥ 256	0.38	1

Abbreviations; BK, Bangkok; C, Central provinces; E, Eastern provinces; N, Northern provinces; NE, Northeastern provinces; S, Southern provinces; W, Western provinces; CIP, ciprofloxacin; CTX, cefotaxime; NAL, nalidixic acid; NOR, norfloxacin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline

*Analysed by disk diffusion method and interpreted according to CLSI, 2013

Table 16 Amino acid substitutions in quinolone resistance determining region (QRDR) of *gyr*A among *S*. Enteritidis isolates in different Thai regions

Region	Total mutation	Number of isolates with amino acid substitutions in QRDR of *gyrA*						
		Ser83Ile	Ser83Phe	Ser83Tyr	Asp87Asn	Asp87Gly	Asp87Tyr	Ser83Phe + Asp87Tyr
BK (n=54)	49	4	1	14	2	2	25	1
C (n=6)	2	-	-	-		-	2	-
E (n=8)	8	1	-	2	-	-	5	-
N (n=27)	23	-	-	9	-	-	14	-
NE (n=24)	20	2	1	7	-	-	10	-
S (n=19)	18	1	-	4	-	-	13	-
W (n=20)	18	-	-	4	-	-	14	-
Total (n=158)	138	8	2	40	2	2	83	1

Abbreviations; BK, Bangkok; C, Central provinces; E, Eastern provinces; N, Northern provinces; NE, Northeastern provinces; S, Southern provinces; W, Western provinces

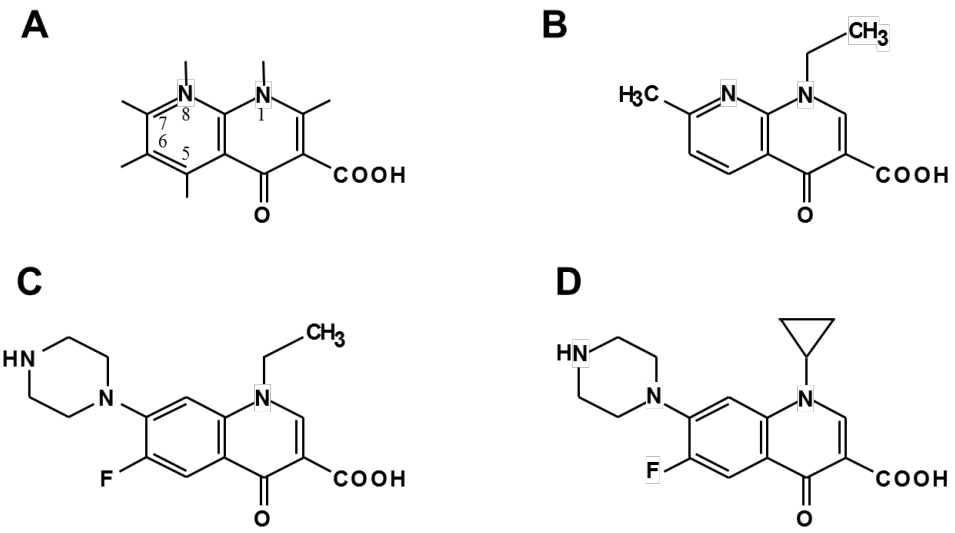

Fig. 7 Structure of quinolones A: Essential structure of quinolones, B: Nalidixic acid, C: Norfloxacin, D: Ciprofloxacin. Numbers in A denote positions susceptible to modification.

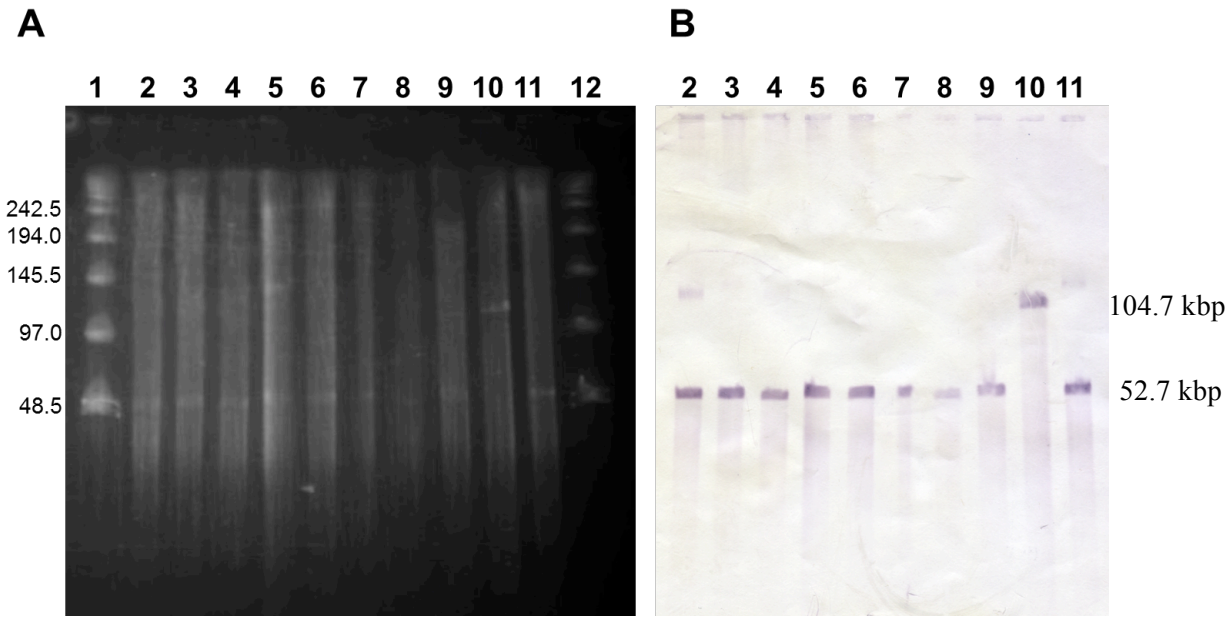

Fig. 8 Plasmid profiles of CIP resistant *E. coli* transformants, A: PFGE profiles of *S1*-nuclease digested total DNA, Lane 1 and 12, lambda ladder; Lane 2 - 11: ten selected CIP resistant *E. coli* transformants; B: Southern blot-hybridization of *S1*-nuclease digested DNA using a *qnrS1* specific probe

CONCLUSION

Salmonella Enteritidis (SE) is a predominant nontyphoidal *Salmonella* causing foodborne disease worldwide. Food animals as poultry and chicken eggs are regards as the main source of this pathogen. SE infections can cause self-limited gastroenteritis and also severe bloodstream infection that required antimicrobial treatment. In Thailand, SE is a major cause of invasive salmonellosis and significant increase of antimicrobial resistance rate was observed among SE isolated from chicken meat and clinical samples, since 1994. The genetic characteristics of SE strains distributed in Thailand are required to understand the epidemiological features and important for efficient monitoring and control measures.

This study revealed the genetic diversity and antimicrobial resistance determinants among clinical SE isolates from throughout Thailand. Total of 190 SE isolates collected from blood and stool samples, during 2004-2007. As SE was claimed to be highly clonal bacteria, multilocus variable number tandem repeat (MLVA) typing classified by five polymorphic loci of variable number tandem repeat (VNTR) has been used to discriminate the strains. To elucidate quinolone resistance determinants among SE isolates, point mutation in topoisomerase genes in the quinolone resistance-determining region (*gyrA* and *gyrB*) and plasmid mediated quinolone resistance (*qnr* gene) were analysed.

In chapter I, the retrospective study displayed the nationwide features of MLVA types, antimicrobial resistance and virulence determinants of clinical SE isolates in 2004 - 2007. Twenty MLVA types were identified in SE strains circulating in Thailand. Of these, three closely related MLVA types predominantly shared SE populations in Thailand. Over half of study isolates were resistant to nalidixic acid (83.2%), ciprofloxacin (51.1%) and amplicillin (50.5%). Multidrug resistance was observed in 25.5% of the isolates. The resistant isolates were spread throughout Thailand and no significant difference in resistance rate between blood and stool isolates. All SE isolates carried *Salmonella* difference fragment (*sdfI*), which has been proposed as specific DNA marker for SE identification. Moreover, SE strains were considered as invasive potentiality via harbouring the prophage-encoded virulence-associated genes (*sodC*1

and *sopE*) and virulence plasmid as *spvA*. The fact that a high incidence of strains resistant to certain antimicrobials correlated with dominant MLVA types may indicate the spread of these strains under selection pressures. Evidence showed that the usage of antimicrobials in human medicine and farming might act as selective pressures for SE that causes human infections.

In chapter II, the quinolone resistance determinants, including *gyrA* and *gyrB* point mutations and the presence of *qnr* genes were investigated among the quinolone resistance SE strains in Thailand. The quinolone resistance determinant was found to be mediated predominantly by *gyrA* mutations that caused amino acid substitutions Asp87Tyr and Ser83Tyr as well as a new amino acid substitution, Ser83Ile. A strong correlation between mutations and specific MLVA types suggested a possible clonal expansion nationwide in Thailand. In addition, the presence of a plasmid-mediated quinolone-resistant gene, *qnrS1*, raises concerns about a broad dissemination of resistant strains.

These findings raise concern about potential antimicrobial resistance of SE circulating in Thailand, particularly the becoming high resistance to current drug of choice against invasive salmonellosis. Based on our data, it is recommended to restrict the usage of quinolone for therapeutic and farming purposes in order to effectively control the emergence and spread of fluoroquinolone resistant SE. The molecular epidemiological data found in this study were baseline information for monitoring the SE strains in Thailand. Therefore, SE isolates collecting in more recent year are needed to be observed. Moreover, further investigation for the other antimicrobial resistance determinants has also been recommended.

ACKNOWLEDGEMENTS

The completion of my dissertation was attributed to guidance, encouragement and support of following persons. I would like to extend my thankful, here.

First at all, I would like to express my sincere gratitude to my Japanese advisor, Professor Yusuhiko Suzuki, for his excellence academic advice, extensive assistance and good flash inspiration throughout my research study and writing for publication. I would also like to extend my appreciation to my advisor in Thailand, Professor Orasa Suthienkul who always offers expertise and support during my study.

I would never have been able to finish my dissertation without the mentorship of Assistance Professor Chie Nakajima. I would like to gratefully thank for her invaluable guidance, kind assistance, and also warming friendship.

I would like to gratefully thank my committee members, Professor Hideaki Higashi and Associate Professor Norikazu Isoda, for their kindness and valuable suggestions.

I sincerely thank all staffs of Department of Microbiology, Faculty of Public Health, Mahidol University, especially Associate Professor Kanokrat Siripanichgon for their kind assistance and encouragement during my study.

I wish to sincerely thank Ms Yakari Fukushima, Ms. Haruka Suzuki for their help a lot in laboratory work. Special thank goes to Ms. Kanjana Changkaew, Ms. Siriporn Kongsoi and Ms. Ruchirada Changkwanyeun for their support in experiment and always being beside me in Japan. My appreciation goes to all staff members and students in the Hokkaido University Research Center for Zoonosis Control for their wonderful friendliness and sharing experience in research.

I am grateful to my parents and my family for their strong encouragement, forever love and understanding throughout my study.

Finally, I would like to express my deepest gratitude to JSPS RONPAKU Program for giving me the financial support and great opportunity as JSPS fellow. This work also was supported by the Ministry of Education, Culture, Sports, Science, and Technology, Japan (MEXT) and the Global Research Network on Infectious Diseases (J-GRID).

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