Title: Genetic Diversity and Antimicrobial Resistance Pattern of *Salmonella enterica* serovar Enteritidis Clinical Isolates in Thailand.

Running title: Antimicrobial Resistant *Salmonella* Enteritidis in Thailand

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Summary

Objective

To trace the history of antimicrobial resistance in *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis, SE) circulating in Thailand, we characterised clinical isolates obtained during 2004-2007.

Methods

Antimicrobial resistance profiles, multi-locus 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.

Results

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.

Conclusion

The usage of antimicrobials in human medicine or farm settings might act as selective pressures and cause the spread of resistant strains. Hence, a strict policy on antimicrobial usage needs to be implemented to achieve the control of resistant SE in Thailand.

Key words: *Salmonella* Enterilidis, MLVA typing, Antimicrobial resistance

1. Introduction

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 [1, 2]. Monitoring conducted

by the Foodborne Diseases Active Surveillance Network (FoodNet) in the United States found that since 2007, *Salmonella enterica* serovar Enteritidis (S. Enteritidis, SE) has displaced *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) as the first ranked gastroenteritis causative agent [3]. SE was reported as the predominant *Salmonella* serovar isolated from clinical samples and chicken meat [4] and also the major cause of invasive non-typhoidal salmonellosis defined as cases with positive blood salmonella culture [5, 6].

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 [5].

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 [8] and exported food products [9]. 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. Multi-locus 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 [10]. Subsequently, the method was optimised for *Salmonella enterica* serovar Typhi (*S.* Typhi) [11] and SE [12, 13] to simplify the interpretation of results, which achieved consistency and comparability across different laboratories [12, 14].

Recently, Hendriksen *et al* (2012) reported that clinical SE isolates collected in 2008 in

Thailand were highly resistant to AMP (60%), NAL (83%) and ciprofloxacin (CIP, 90%) [15]. In contrast, lower percentages of resistance to AMP (21%), NAL (55%) and CIP (0 %) were reported in the same country during 2001-2006 [5]. 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.

2. Materials and Methods

2.1. *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 [16] at the WHO National *Salmonella-Shigella* Center, belonging to the Ministry of Public Health of Thailand.

2.2. 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 [17]. *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.

2.3. DNA extraction

SE strains were cultured on Luria Bertani (LB) agar (Becton–Dickinson) at 37 °C for 18-24 h. A single colony of each strain was inoculated in 5 mL of LB broth and incubated with shaking at 37 °C for 4-6 h. 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.

2.4. Multiple-locus variable number tandem repeat analysis

Selected VNTR loci and primer sets for polymerase chain reaction (PCR) were synthesised in accordance with previous publications [12, 18, 19], 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 s, 50 °C for 20 s and 72 °C for 40 s, 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-Σ (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 [20]. 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 s, 50 °C for 20 s and 72 °C for 30 s 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).

2.5. Detection of SdfI and virulence-associated genes

All SE isolates were analysed for the presence of *Salmonella* Enteritidis specific fragment (Sdf I) 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 [21, 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 s, 55 °C for 10 s and 72 °C for 40 s and a final elongation at 72 °C for 5 min.

3. Results

3.1. 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 1). 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,

169 resistance to ≥ 3 antimicrobial categories [23], at least 25.5% of the resistant isolates were MDR
170 (Supplemental Table 1). However, no significant difference between blood and stool isolates was
171 observed for the overall resistance rate and frequency of multi-drug resistance (P = 0.09 and 0.5,
172 respectively). Drug resistant SE isolates were spread throughout Thailand with 58% in the central
173 region and 75-95% in other regions. The two most common resistance profiles, AMP/NAL and
174 AMP/CIP/NAL, were also found in all geographical regions with a wide range of prevalence (8-50%).
175 NAL, CIP/NAL, AMP/CIP/NAL/STR and CIP/ NAL/SXT/TET were prevalent profiles distributed in
176 all but one or two regions. MDR strains were found in all regions (Supplemental Table 2). The drug
177 susceptibility of two isolates from stool was not examined because of their extinction during storage.

178 **3.2. VNTR variations**

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

187 **3.3. Distribution of MLVA types**

188 The MLVA types of SE isolates were defined with five code numbers designated by the tandem
189 repeat number in loci of SE1, SE2, SE5, SE7 and SE9. For example, MLVA type 5-5-10-7-3 denotes
190 that a strain contained 5, 5, 10, 7 and 3 repeat units in those loci, respectively. Using these five
191 polymorphic VNTR loci, 20 MLVA types were identified in SE strains circulating in Thailand (Fig 2).
192 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 2).

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 3). The ratio of each MLVA type in Thailand during the study period is shown in Figure 4. 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.

3.4. Association of MLVA types and drug resistance

The association between drug resistance and MLVA types was analysed (Table 5). 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%).

3.5. 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.

4. 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. 1). AMP resistance rate in each year showed the surge reflected by the expansion of the 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 [5] and 2008 [15], 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 [5]. 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 by Hendriksen *et al* [15].

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 [24]. In Thailand, AMP is an accepted drug for systemic salmonellosis [25], and both enrofloxacin and amoxicillin are generally used in broiler farming [26]. In addition, SE was observed as the predominant serovar isolated in Thailand from 28% of retail chicken meat in 1997 [27] and 20% of frozen chicken during the period 1993-2002 [4]. 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 (Supplemental Table 1) and shown to be distributed in different regions (Supplemental Table 2). 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 [12, 18, 28] or European countries [13] possibly because of the high clonality of SE in Thailand (Fig. 3 and 4). Each VNTR locus showed different Nei's indices (Table 4). 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* [15]. Interestingly, the Nei's index of each polymorphic locus analysed in this study was lower than those in studies from other countries [12, 18, 28]. 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. 2) during the 4-year period (Fig. 4). 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 (Table 5). MLVA types 5-5-11-7-3 showed significant association with resistance to NAL ($P < 0.001$) and AMP ($P < 0.001$). The trend of AMP resistant SE during 2004-2007 (Fig. 1) was associated with that of MLVA type 5-5-11-7-3 (Fig. 4), 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 5). SE with this MLVA type showed a decreasing trend from 2004 to 2007 that related to SXT resistance (Fig. 4). The majority of MDR SE (88.0 %) 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 [22]. 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*.

In conclusion, this retrospective study displayed the nationwide features of MLVA types, antimicrobial resistance and virulence determinants of clinical SE isolates in 2004 - 2007. Three closely related MLVA types predominantly shared SE populations in Thailand. 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 cause human infections. Hence, a strict policy on antimicrobials usage should be implemented to achieve a strict control of resistant *Salmonella* in Thailand.

Conflict of Interest: There is no conflict of interest to be declared.

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Figure legends

Fig 1. 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 2. 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 3. 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 4. 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.

399 Table 1. Number of *Salmonella* Enteritidis isolates collected from different regions of Thailand during the period 2004-
400 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

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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	12
SE1-R	**GTTTCTT**CCAGCCATCCATACCAAGAC		2504728-47	
SE2-F[a]	**6-FAM**-CTTACGATTATACCTGGATTGTTGG	7	4617817-02	12
SE2-R	**GTTTCTT**GGACGGAGGCGATAG		4617615-30	
SE3-F[a]	CAACAAAACAACAGCAGCAT	12	2073240-59	12
SE3-R	GGGAAACGGTAATCAGAAAGT		2073547-27	
SE5-F[b]	**VIC**-CGGGAAACCACCATCAC	6	3073216-32	12
SE5-R	**GTTTCTT**CAGGCCGAACAGCAGGAT		3073427-10	
SE6-F[b]	CGGTGGCGGAGATTCTAATCA	33	3510975-95	18
SE6-R	ACGCCGTTGCTGAAGGTAAT		3511412-393	
SE7-F[a]	CCGACCCAATAAGGAG	61	2961466-79	19
SE7-R	CTTACCGTTGGTAGTTTGTTA		2961949-29	
SE8-F[c]	TTGCCGCATAGCAGCAGAAGT	87	2812703-23	12
SE8-R	GCCTGAACACGCTTTTTAATAGGCT		2813171-49	
SE9-F[c]	**PET**-CGTAGCCAATCAGATTCATCCCGCG	9	533460-36	12
SE9-R	**GTTTCTT**TGAAACGGGGTGTGGCGCTG		533132-53	
SE10-F[b]	GCTGAAGAAGCGGCAAAAC	45	774231-49	18
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.

414 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	21
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	22
Sdf-R	CGTTCTTCTGGTACTTACGATGAC		

415 * Originally designed primer based on *sop*E and *sod*CI sequences of *S.* Enteritidis strain P125109
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Table 4. 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*	0	0.51	0.41	0.54
SE5	0.65	0.68	0.73	0.64	0.77
SE6	0*	0	0.03	0	0.07
SE7	0.12	nd	0.50	0.48	0.60
SE8	0*	0	0.48	0.51	0.45
SE9	0.06	0.09	0.44	0.38	0.53
SE10	0*	nd	0.04	0	0.05

*n = 89

nd; not determined

a: Clinical strains isolated in 2008 (Ref.15)

b: Clinical strains isolated in various state in USA during the period 2000-2007 (Ref.28)

c: Clinical strains isolated in the period 1990-1999 (Ref.18)

d: Clinical strains isolated in the period 1998-2003 (Ref. 12)

427 Table 5. 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[η] (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

428 *Resistance to at least one antimicrobial

429 [η]Resistance to at least 3 classes of antimicrobials defined by Magiorakos AP et al.[23]

430 [#] Statistical significance ($P < 0.001$)

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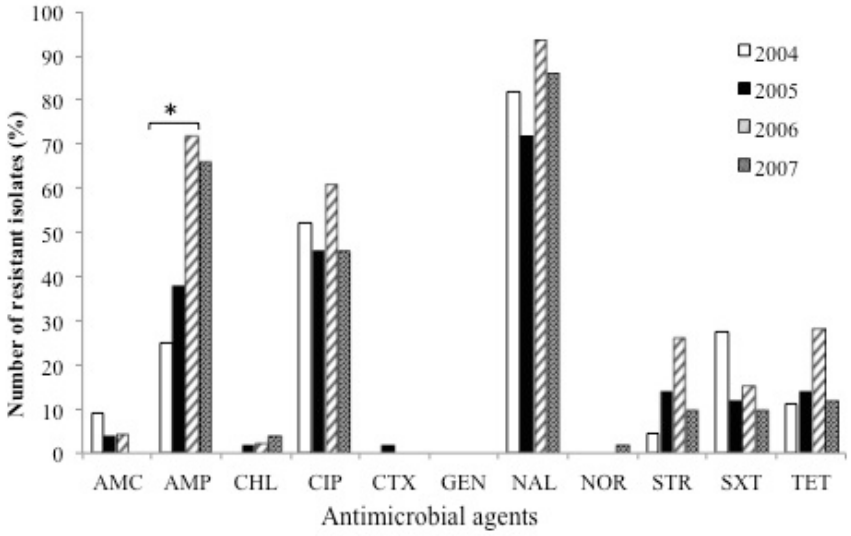
Utrarachkij et al. Fig. 1



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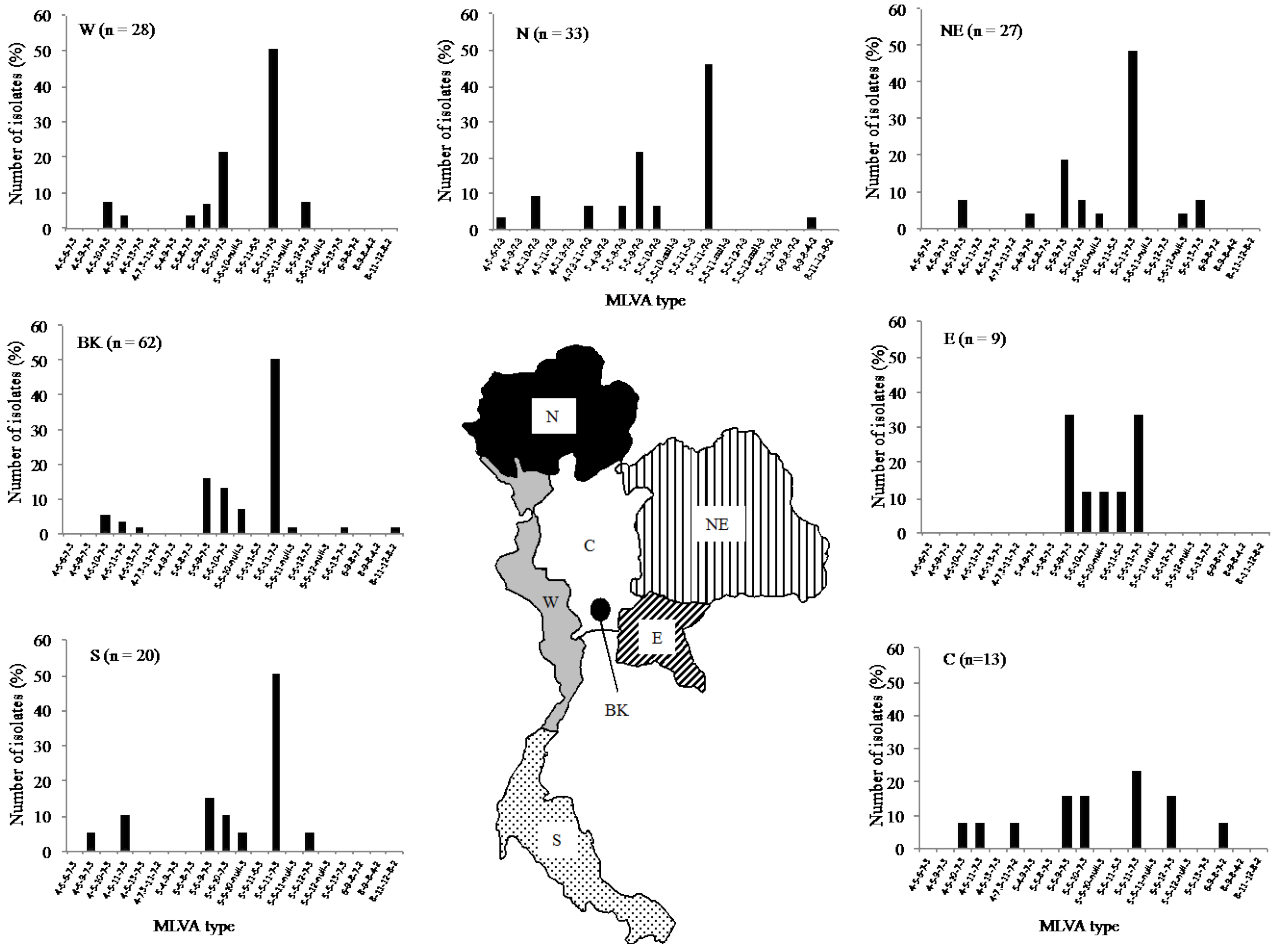
Utrarachkij et al. Fig. 2



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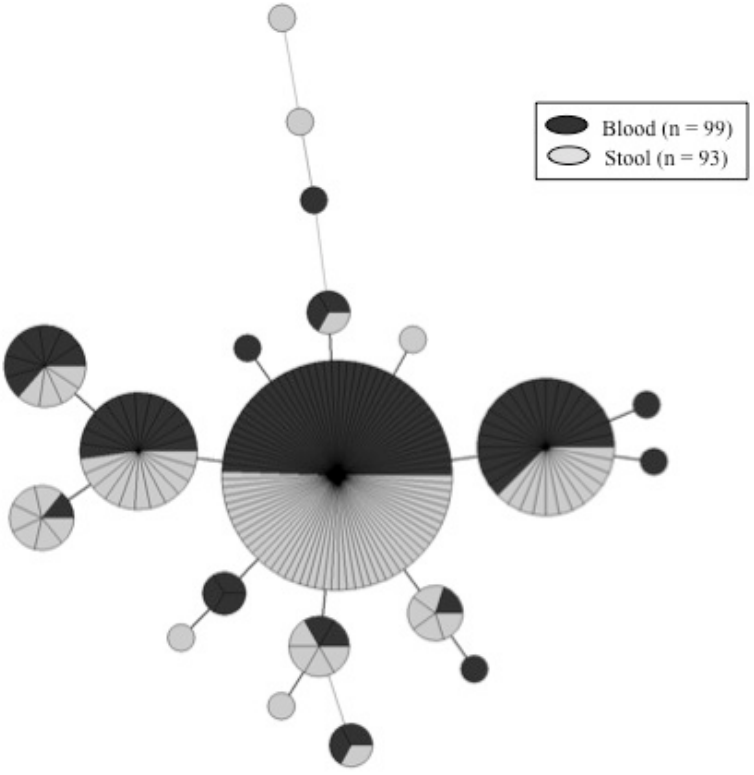
Utrarachkij et al. Fig. 3



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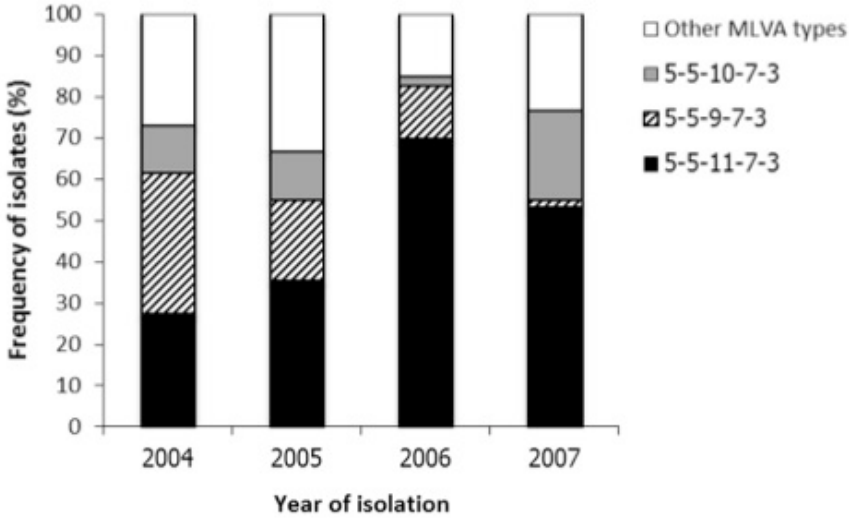
Utrarachkij et al. Fig. 4



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Supplemental Table 1. Frequency of antimicrobial resistance profiles in blood and stool *Salmonella* Enteritidis isolates in Thailand during 2004-2007.

Resistant profile*	Blood (N = 99)				Total blood N (%)	Stool (N = 91)				Total stool N (%)	Total N (%)
	2004	2005	2006	2007		2004	2005	2006	2007		
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.

Supplemental Table 2. 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.

**SE isolates were collected from patients in Bangkok and various provinces located in different Thai regions. Abbreviations of the regions are as follows: BK, Bangkok; C, Central; E, East; N, North; NE, North-East; S, South; W, West.