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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)

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Salmonella Enteritidis (SE) is the globally most common serovar causing human infection. In Thailand, SE was also the major cause of invasive non-typhoidal salmonellosis defined as cases with positive blood salmonella culture. Moreover, SE isolates from Thai patients and chicken meat exhibited significant increase in antimicrobial resistance since 1994 and multidrug resistance, including drug of choice as fluoroquinolone were observed in the past decade.

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 and exported food products. Therefore, the study on antimicrobial resistance profiling and genetic diversity of clinical SE isolates in Thailand can provide better epidemiological data for tracing of SE infection. This study aim to elucidate the situation of antimicrobial resistance determinants and genetic diversity among clinical SE isolates in throughout Thailand.

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. 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. These SE isolates were analysed for drug susceptibility profiling, virulence associated gene and multilocus variable number tandem repeat analysis (MLVA). Moreover, the quinolone resistance determinants, including point mutation in topoisomerase genes in the quinolone resistance-determining region (QRDR) and a plasmid mediated quinolone resistance (PMQR), *qnr* genes were investigated among quinolones resistant SE strains.

Chapter 1

Antimicrobial resistance profiles of SE in Thailand

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). The results revealed a high rate of resistance to NAL (83.2%), CIP (51.1 %) and AMP (50.5%) in clinical SE strains circulating in Thailand. 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.

Based on the definition of multidrug resistance (MDR), that is resistance to ≥ 3 antimicrobial categories, at least 25.5% of the resistant isolates were MDR. This study 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. This result may indicate that SE in Thailand has

survived under various antibiotic selective pressures that contribute to diverse antibiograms.

The presence of *sdfI* and virulence-associated genes

All SE isolates were analysed for the presence of *Salmonella* different fragment (*SdfI*) and virulence-associated genes *spvA*, *sodC1* and *sopE* by PCR method. All isolates carried *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 (*sodC1*, *sopE*) and plasmid virulence gene as *spvA*.

Distribution of MLVA types in SE isolates

Five polymorphic variable number tandem repeat (VNTR) loci including of four short repeat VNTR loci (SE1, SE2, SE5, SE9) and one long repeat locus (SE7) were selected for MLVA types of all SE isolates. The SE7 locus of isolates was amplified by enhanced PCR. The numbers of repeat units in SE7 locus was calculated from the estimated size of amplicons obtained by agarose gel electrophoresis. The short repeat loci were amplified using multiplex PCR, and the product size of each locus was simultaneously determined with multicolour capillary electrophoresis. 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.

With these five polymorphic VNTR loci, 20 MLVA types were identified in SE strains circulating in Thailand. Various MLVA types were identified in SE isolates from different regions. 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.

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. 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.

Association of MLVA types and drug resistance

Significant associations were observed between the MLVA types and the resistance to each antimicrobial. 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, an increasing trend in frequency of MLVA type 5-5-11-7-3 from 2004 to 2007, with a peak in 2006 was associated with that of AMP resistant SE. This result clearly demonstrated the clonal expansion of AMP resistant SE in Thailand.

Chapter 2

Distribution of minimum inhibitory concentrations (MICs) in quinolone resistant SE isolates

To determine the level of resistance in quinolone resistant SE isolates, MICs of NAL, NOR and CIP were determined by E-test according to the manufacturer's protocol. The distribution of MICs in 158 SE isolates with intermediate or fully resistance to NAL are analyzed. A high MIC of NAL (≥ 256 mg/L) was observed for 87.3% of the isolates, whilst the remaining 20 isolates presented MICs ranging 16-64 mg/L. Except for one, all isolates were susceptible to NOR. As for CIP, 145 isolates (91.8%) had decreased susceptibility (CIP^{DS}, $0.064 < \text{MIC} < 1$ mg/L), whilst only three isolates (1.9%) were resistant (CIP^R).

Topoisomerase gene mutations and presence of *qnr* genes in SE isolates

Point mutations in the QRDR of topoisomerase genes (*gyrA*, *gyrB*, *parC*) and the

presence of *qnr* genes (*qnrA*, *qnrB* and *qnrS*) in quinolone resistant SE isolates were analysed by PCR and sequencing. The results 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. Asp87Tyr was most frequently identified, followed by Ser83Tyr. Eight isolates had a new amino acid substitution at codon 83 (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. Transferability of the plasmid carrying *qnrS1* with the size of 104.7 kbp and 52.7 kbp was also confirmed by conjugation experiment and nuclease S1 analysis of CIP resistant transformants.

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 analysed. Single amino acid substitution in *gyrA* mutants associated with classical quinolone resistance (high-level resistance to NAL and decreased susceptibility to CIP). 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). The isolates harbouring *qnrS1* and wild-type *gyrA* exhibited non-classical quinolone resistance phenotype (susceptible or low level resistance to NAL with $\text{MIC} \leq 32$ mg/L and decreased susceptibility to CIP with $\text{MIC} \geq 0.125$ mg/L). 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). These evidences suggested that single mutation in *gyrA* or carriage of *qnr* alone does not confer a strong resistance to fluoroquinolones.

Relationship between quinolone resistance determinants and MLVA types

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. All eight isolates harbouring Ser83Ile were found to have similar MLVA types with no amplification of locus SE7. This strong correlation between mutations and specific MLVA types suggested a possible clonal expansion nationwide in Thailand.

Conclusion

This study displayed the features of genetic diversity and antimicrobial resistant determinants among clinical SE isolates in nationwide Thailand. SE isolates circulating throughout Thailand were highly clonal, of which three quarters of the isolates confined to closely MLVA types and almost all isolates possessed invasive potential. In context of antimicrobial resistance, endemic SE in Thailand showed high frequency of resistance to quinolone and ampicillin. Quinolone resistance of SE in Thailand was found to be mediated predominantly by *gyrA* mutations with the amino acid substitution of Asp87Tyr and Ser83Tyr as well as a new amino acid substitution, Ser83Ile. Plasmid mediated quinolone resistance as *qnrS1* was identified in 10% of studied isolates. Single codon *gyrA* mutants or carriage of *qnrS1* alone presented the resistance to NAL and/or decreased susceptibility to CIP, isolates carrying double codon mutation or both in *qnrS1* gene and a *gyrA* mutation exhibited a higher level of resistance. These suggested that NAL resistance strains with single resistance determinant may become fluoroquinolone resistance by acquiring secondary determinants. Significant associations between antimicrobial resistance determinants and specific MLVA types found in this study suggested the possible clonal expansion nationwide in Thailand. In addition, the presence of plasmid mediated quinolone resistant gene, *qnrS*, raises concerns about a broad dissemination of resistant strains. The alarming increase and potential of antimicrobial resistance in

nationwide clinical SE isolates point to the need for restrictive usage of antimicrobial drug in therapeutic and farming propose to control the emergence and spread of resistant SE.