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Title: Quinolone Resistance Determinants of Clinical *Salmonella* Enteritidis in Thailand

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Running title: Quinolone Resistant Salmonella Enteritidis in Thailand

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Abstract

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 multi-locus 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. Restricted therapeutic and farming usage of quinolones is strongly recommended to prevent the emergence of fluoroquinolone-resistant isolates.

Key words: *Salmonella* Enteritidis; quinolone resistance; gyrA; qnrS1; MLVA

Introduction

Nontyphoidal *Salmonella* is a global public health concern, particularly regarding antimicrobial resistance [19, 38]. 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 [5, 14, 32, 38]. NAL resistance can be the cause of reduced susceptibility to ciprofloxacin (CIP) [1, 32], 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* [16]. 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 [10] and geographical areas [28]. 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* [27, 35]. 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 [35]. At present, *qnr* gene variants, *qnrA, qnrB, qnrD*, *qnrS* and *oqxAB* have been reported in non-typhoidal *Salmonella* worldwide [6, (6), 17, 21, 22, 26, 33, (40)].

In Thailand, an increasing trend of quinolone resistance in *S.* Enteritidis was reported during 2001-2008 [15, 38]. 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 multi-locus 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 firstly categorised into susceptible, intermediately or fully resistant to NAL, CIP and NOR by the Clinical and Laboratory Standard Institute (CLSI) zone size criteria [7].

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) ≥ 32 mg/L, susceptible (S) ≤ 16 mg/L; NOR, R ≥ 16 mg/L, S ≤ 4 mg/L; and CIP, R ≥ 1 mg/L, S ≤ 0.06 mg/L, according to the CLSI guideline [7]. *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 1). 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: pre-denaturation at 96 °C for 1 min; 35 cycles of denaturation at 96 °C for 10 s; annealing at 52 °C for 10 s and extension at 72 °C for 30 s; 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 [35], the presence of these genes in isolates was examined by PCR using the primer sets designed by Robicsek *et al*. [34] 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 prior to PFGE as previously described[41]. 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 h in 0.5 x Tris–borate–EDTA buffer at 14 °C. Southern blot hybridizations were performed by standard methods[42]. In order 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.

Multiple loci variable number tandem repeat analysis

MLVA typing was conducted using previously designed primers for PCR amplification of five VNTR loci, SE1, SE2, SE5, SE7 and SE9 [4, 9], with some modifications (Table 2). 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/sulfamethoxazole (SXT), was determined by the disc diffusion method according to the CLSI guideline (15).

Results

Quinolone resistance phenotypes

The disc 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 3. According to the CLSI guidelines, 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 3).

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*, whilst 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 3). 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 (TCC) → isoleucine (ATC), 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 4). 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. 2). Transconjugation experiments using the representative strains, SE-50 and -110, demonstrated the increase of MICs. The MIC of CIP and NOR against recipient *E. coli* Top 10 were <0.002 and <0.016 mg/L, respectively, where as those against transconjugantes with SE-50 were 0.125 and 0.5 mg/L, respectively. Similar increase of MIC 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 6).

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 3. Among the 138 *gyrA* mutants, all but two isolates showed high-level resistance to NAL (MICs ≥ 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) [11]. 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 ($P = 0.003$), whilst 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 (Supplemental table 1). 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 (Supplemental table 2). Asp87Tyr was strongly associated with type 5-5-11-7-3, whilst 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 4). All eight isolates harbouring Ser83Ile were found to have similar MLVA types with no amplification of locus SE7 (Table 5). All the resistance determinant types and MLVA types were widely distributed in Thailand (Supplemental table 3).

Discussion

To elucidate the quinolone resistance acquisition mechanism, 190 *S*. Enteritidis clinical isolates investigated, and 156 were identified to be positive for quinolone resistance determinants. Amongst these, 138 isolates had mutations in *gyrA* and 19 isolates carried *qnrS1*. The MIC necessary to inhibit isolates by 50% (MIC_{50}) for NAL was ≥ 256 mg/L, whilst those of NOR and CIP were 0.75 and 0.125 mg/L, respectively (Table 3). These results confirmed the stronger activity of fluorinated quinolones as DNA gyrase inhibitors in GyrA mutant strains. Moreover, the higher potency shown by CIP, but not NOR, demonstrated the effectiveness of the cyclopropyl ring at R1, which is a structure solely found in CIP (Fig. 1). 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 [16, 25, 31]. 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 [18, 30].

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 [31], the United Kingdom [10], Norway [29] and Ireland [23]. Ser83Tyr was the second

predominant mutation (40/138) and was also observed in Norway [29]. In our study we found strong correlations between specific *gyrA* mutations and MLVA types (Supplemental table 2). 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 5). This is the second finding all over the world following that reported from Malaysia[39]. All isolates with this substitution presented high MICs of NAL (≥ 256 mg/L), NOR (0.25-1 mg/L) and CIP (0.094-0.38 mg/L) (Table 3). In addition, isolates with Ser83Ile possessed similar MLVA types, of which different alleles were observed only at a single locus in two isolates (Table 5). 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* [11]. 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 (T<u>T</u>C) substitution requires only a single base change. In contrast, a substitution to isoleucine (<u>AT</u>C) needs a secondary base substitution. The mutated codon of ATC causing Ser83Ile might be derived from a base substitution of serine (T<u>C</u>C) via phenylalanine (T<u>T</u>C) 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 [40]. Limited number of *S*. Enteritidis isolates from

neonatal calf diarrhea in Egypt carried *qnrS* [2]. In Asian countries, *qnrS* was identified in several clinical *Salmonella* serovers mainly from serovar other than Enteritidis as reported in Korea [20] and Taiwan [21]. The report from China also reported low percentage of *qnrS* in *S.* Enteritidis (0.8%, 1/126) [41]. In Thailand, *qnrS1* was previously detected in 16 *S.* Corvallis isolated from human and food animals [6] and recently observed in clinical *S.* Stanley and *S.* Anatum from poultry [37]. To our best knowledge, this is the first work reporting clinical *S.* Enteritidis carrying *qnrS1* in Thailand.

As *qnrS1* is a plasmid-mediated quinolone resistance 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. 2). The transferability of the plasmid carrying *qnrS1* was also confirmed by conjugation (Table 6). The AMP resistant phenotype of transconjugants (Table 6) supported the idea of the co-existence of *qnrS1* and the gene encoding β-lactamase, *bla*, in the same plasmid as has been reported in *Klebsiella pneumonia* ($bla_{\text{SHV-2}}$) [8] and *S. enterica* serovar Stanley ($bla_{\text{LAP-2}}$) [12]. 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} $\text{NAL}^{\text{S/LR}}$; Table 4), which is consistent with the observations reported by Gunell *et al* [13]. In contrast, other studies demonstrated that *qnrS* in *Salmonella* caused other resistance phenotypes, including susceptibility to both CIP and NAL (CIP^{S} NAL^{S}) [31] and decreased susceptibility to CIP and resistance to NAL (CIP^{DS} NAL^{R}) [20]. 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 [24] and the United Kingdom [17]. 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*. Indeed, fluoroquinolone resistance requires the presence of both *qnrS* and a single or double mutation in *gyrA*. 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 [37], an enzymatic inactivation encoded by *aac(6')Ib cr* and effux pump encoded by *qep* and *oqxAB* [27, 35] might be playing roles in developing the resistance to quinolones. Further investigation of other PMQR will be conducted by our future study.

In conclusion, the quinolone resistance of *S.* Enteritidis in Thailand 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. 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 *S.* Enteritidis.

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Figure captions

Figure 1. Structure of quinolones

A: Essential structure of quinolones, B: Nalidixic acid, C: Norfloxacine, D: Ciprofloxacine. Numbers in A denote positions susceptible to modification.

Figure 2. 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.