Original Article

Mutations in Topoisomerase Genes and Expression of AcrAB Multidrug Efflux System in Fluoroquinolone-Resistant *Salmonella enterica* from Pork and Patients

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Abstract

The aim of this study was to further examine the mechanisms of ciprofloxacin resistance in *Salmonella enterica* isolates from pork and patients. Twenty-four isolates including 18 ciprofloxacin- resistant isolates and six susceptible strains were used. Mutations in the quinolone resistance-determining regions (QRDRs) of *gyrA*, *gyrB*, *parC* and *parE* were examined. Expression of AcrAB-TolC efflux pump was quantitatively determined using qRT-PCR. While nucleotide modifications were found in *gyrA*, *gyrB* and *parC*, all mutations identified were novel. All 18 ciprofloxacin resistant isolates carried mutations in at least one target gene and all six isolates susceptible to ciprofloxacin did not carry mutations in their QRDRs. There were no associations between the number and type of mutations and ciprofloxacin resistance level among the ciprofloxacin *Salmonella* isolates. All the *Salmonella* isolates produced AcrB, ranging from 1 to 430 folds. A *Salmonella* strain susceptible to all antimicrobials tested overexpressed AcrB. The isolates with comparable AcrB demonstrated different susceptibility to ciprofloxacin. Therefore, the role of mutations in topoisomerase genes and the AcrAB-TolC efflux pump in decreased susceptibility to ciprofloxacin varied among the *S. enterica* clinical isolates.

Keywords: AcrAB-TolC, ciprofloxacin, fluoroquinolones, *Salmonella enterica*, topoisomerase genes

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บทคัดย่อ

การกลายพันธุ์ในยีน Topoisomerase และการแสดงออกของระบบมัลติดรักซ์ AcrAB ในเชื้อแซลโมเนลลา เอนเทอริกาดื้อยาฟลูออโรคิวโนโลนที่แยกได้จากเนื้อสุกรและผู้ป่วย

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การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษากลไกการดื้อยาซิโปรฟล็อกซาซินในเชื้อแซลโมเนลลา เอนเทอริกาที่แยกได้จากเนื้อสุกรและผู้ป่วย เชื้อจำนวน 24 isolates ประกอบด้วยเชื้อดื้อยาซิโปรฟล็อกซาซินจำนวน 18 isolates และเชื้อไวต่อยาจำนวน 6 isolates โดยตรวจหาการกลายพันธุ์ใน the quinolone resistance-determining regions (QRDRs) ของยีน *gyrA*, *gyrB*, *parC* และ *parE* และตรวจการแสดงออกของระบบ AcrAB-TolC ด้วย qRT-PCR พบการเปลี่ยนแปลงของนิวคลีโอไทด์ในยีน *gyrA*, *gyrB* และ *parC* โดยเป็นการกลายพันธุ์ที่ยังไม่เคยมีรายงานมาก่อน เชื้อดื้อยาซิโปรฟล็อกซาซินทั้ง 18 isolates มีการกลายพันธุ์ในยีนเป้าหมายอย่างน้อยหนึ่งยีนและเชื้อทั้ง 6 isolates ที่ไวต่อการดื้อยาซิโปรฟล็อกซาซินไม่มีการกลายพันธุ์ใดๆในส่วน QRDRs ไม่พบความสัมพันธ์ระหว่างจำนวนและชนิดของการกลายพันธุ์กับระดับการดื้อยาซิโปรฟล็อกซาซิน ของเชื้อแซลโมเนลลาที่ดื้อยาซิโปรฟล็อกซาซิน เชื้อทุกตัวมีการแสดงออกของระบบ AcrAB-TolC 1-430 เท่า เชื้อที่ไวต่อยาทุกชนิดที่ทดสอบจำนวน 1 isolate สามารถผลิต AcrB ได้ โดยเชื้อที่ผลิต AcrB ในระดับที่ใกล้เคียงกันมีความไวต่อซิโปรฟล็อกซาซินต่างกัน ดังนั้นบทบาทของยีน topoisomerase และระบบ AcrAB-TolC ต่อการดื้อยาซิโปรฟล็อกซาซินในเชื้อแซลโมเนลลามีความหลากหลาย

คำสำคัญ: ระบบ AcrABC-TolC ฟลูออโรคิวโนโลน *แซลโมเนลลา เอนเทอริกา* ยีน Topoisomerase

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Introduction

Up to date, *Salmonella enterica* remains one of the major causes of foodborne illness in humans worldwide (Padungtod et al., 2006). While Salmonella gastroenteritis is typically self-limited and antibiotics are not required in most cases, antibiotics are recommended for patients with increased risk of invasive salmonellosis. Initial antibiotic therapy of salmonellosis includes ampicillin, chloramphenicol and sulphamethoxazole-trimethroprim combination (Ling et al., 2003). However, failure of such conventional antibiotic therapy frequently occurs, particularly in children with severe invasive infections. Fluoroquinones (i.e. ciprofloxacin) have been the drug of choice for treating invasive salmonellosis after the failure of common antibiotic remedy. Unfortunately, fluoroquinolone-resistant *Salmonella* strains have rapidly developed and salmonellosis cases with fluoroquinolone treatment failures have been reported (Vasallo et al., 1998; Walker et al., 2000).

Food of animal origins, including pork, is an important protein source for humans in most parts of the worlds. In response to increasing demand for pork consumption, several antibiotics have been introduced to modern pig production farm. Fluoroquinolones is among antibiotics that are used for disease treatment and prevention in pigs. It has been shown that fluoroquinolone-resistant *Salmonella* is present in pigs and pork products (Padungtod et al., 2006) and, therefore, poses the risk of causing salmonellosis that does not responsed to the antibiotic treatment in humans.

Resistance to fluoroquinolones in *S. enterica* is mediated by several mechanisms, including mutations of topoisomerase genes, active efflux and topoisomerase protection by plasmid-encoded Qnr protein (Giraud et al., 2006). However, such resistance initially arises from mutation (s) in one or more topoisomerase genes (Piddock, 2002). While the mutations in the quinolone resistance-determining region (QRDR) of *gyrA* have mainly contributed to reduced susceptibility to fluoroquinolones in the clinical *Salmonella* human and animal isolates (Eaves et al., 2004), those in *gyrB*, *parC* and *parE* have rarely been reported. It was suggested that combination of mutations in the same or different topoisomerase gene resulted in increased fluoroquinolone-resistance level. For this instance, the *Salmonella* isolates with reduced susceptibility contained a single mutation in *gyrA* and the resistant isolates carried at least two mutations in *gyrA* and/or *gyrB* and/or *parC* and/or *parE* (Eaves et al., 2004).

In addition to topoisomerase mutations, overexpression of the AcrAB-TolC efflux pump has been shown to mediate reduced-susceptibility to fluoroquinolones in salmonellae (Chen et al., 2007). The efflux system comprises three main functional components (i.e. AcrA, a periplasmic membrane fusion protein; AcrB, an inner membrane transporters; and TolC, a universal outer membrane protein) as seen in most pumps in the Resistance-Nodulation-Cell-Division family. As the AcrAB-TolC efflux pump was originally found in *Escherichia coli*, there are considerable structural and functional similarities between the pump in *E. coli* and *Salmonella* (Pomposiello and Demple, 2000).

As seen in other antibiotics, fluoroquinolone usage differs greatly as to drug formulations, target animal species, label indications, and geographic regions. This could result in difference in type and expression of resistance mechanisms among clinical isolates of bacteria, including *Salmonella*. The *Salmonella* strains in the present study are partly described in our previous study, where the presence of mutations in the QRDR in *gyrA* and *parC* were examined in all ciprofloxacin-resistant isolates (MICs = 4-8 μg/ml) (Wannaprasat et al., 2011). In the present study, we aimed to further study the mechanisms mediated fluoroquinolone resistance, including mutations in other topoisomerase genes and expression of the AcrAB-TolC efflux system. The ciprofloxacin-susceptible isolates (MICs = 0.125 μg/ml) were additionally included for better comparison.

Materials and Methods

Bacterial isolates and antimicrobial susceptibility testing: Twenty-four *S. enterica* isolates previously isolated were included in this study (Wannaprasat et al., 2011). They were isolated from pork (n = 8) and humans (n = 16) in northern Thailand during 2005-2007. All of the pork isolates were originated from raw pork in retail markets, and all of the human strains were isolated from patients' stools at Suandok hospital of the Faculty of Medicine of Chiang Mai University. All the strains were isolated as described in ISO6579:2002 (E) (ISO, 2002) and determined for serovars. Only one colony of each serotype was collected for each positive sample.

All the *Salmonella* isolates were tested for their susceptibilities to antimicrobials by determining minimum inhibitory concentrations (MICs) using a two-fold agar dilution in the former study (Wannaprasat et al., 2011). Eighteen isolates were resistant to ciprofloxacin (MICs = 4-8 μg/ml) while six isolates were susceptible (MICs = 0.125μg/ml).

Determination of mutations in QRDRs of gyrA, gyrB, parC and parE genes: PCR-template DNA was prepared from all the *Salmonella* isolates (n = 24) using the whole cell boiled lysate protocol (Levesque et al., 1995). All the PCR amplifications were performed in Thermo-StartTM 2X Reddymix™ PCR MasterMix (Thermo Fisher Scientific, CA, USA). The mutation(s) in the QRDRs of *gyrA*, *gyrB*, *parC* and *parE* were investigated using PCR with specific primers as follows: *gyrA*, gyrAsalF(5′- GCTGAAGAGCTCCTA TCTGG-3′) and gryAsalR, (5′-GGTCGGCATGACG TCCGG-3′); *gyrB*, gyrBF (5′-GCGCGCTCGATTTAGC CG-3′) and gryBR, (5′-TGATAGCGCAGCTTGTCCG); *parC*, parCF (5′-GTACGTGATCATGGATCGTG-3′) and parCR (5′-TTCCTGCATGGTGCCGTCG-3′); and *parE*, parEF (5′-GCGATCGCGAATATCAGGCG-3′) and parER, (5′-CAGTTGTTCCAGTACGCCC-3′) (Chuanchuen and Padungtod, 2009).

All PCR amplicons were purified using Nucleospin Gel Extraction kit (Nucleospin®, Gutenburg, France) and submitted for nucleotide sequencing at Molecular Informatic Lab, NT, Hong Kong. Both strands of DNA sequence were compared with the published DNA sequence in GenBank (Genbank accession numbers AE008801, AE008878 and AE008846 for *gyrA*, *gyrB*, *parC* and *parE*, respectively) and analysed by Edit seq and Seqman (DNA-STAR) program.

Detection of acrB expression: All the *Salmonella* isolates (n = 24) were firstly screened for acrB transcription using reverse transcription-PCR (RT-PCR). Total RNA was extracted using QIAGEN RNeasy mini kit (Qiagen® Hilden, Germany) as described by the manufacturer and treated with DNaseI (Fermentas®, Mainz, Germany) according to the manufacturer's protocol. Transcription of acrB was determined by using conventional RT-PCR. Firstly, cDNA was synthesized from one μg of DNaseI treated RNA sample using ImProm-IITM Reverse Transcriptase (Promega, Madison, USA) as described by the manufacturer and PCR primers acrBF (5′-TGAAAAAAATGGACCCGTTCTTC-3′) acrBR primer (5′-CGAACGGCGTGGTGTCA -3′) (Nishino et al., 2006). The cDNA was stored at -20^{0}C and used as DNA template for PCR.

Relative quantitation of acrB transcripion level: All the *Salmonella* isolates expressing AcrB were tested for transcription level of *acrB* by quantitative real-time PCR (qRT-PCR) using Biotools QuantiMix EASY SYG Kit (Biotools B&M Labs S.A., Madrid, Spain) and primer pairs acrBF and acrBR. Firstly, cDNA was synthesized from one μg of total RNA as described above and was measured for its concentration. The cDNA was 1:100 diluted to yield the threshold cycle (Ct) within the limits of standard curve ($r^2 > 0.990$). The chromosomal DNA template from S. Typhimurium ATCC 13311 was used to make standard curve, plotting the average Ct values on the X axis and the log of given concentrations on the Y axis. The housekeeping gene, *rrs*, served as internal control and its transcription was determined using primers rrsF (5′-CCAGCAGCCGCGGTAAT-3′) and rrsR (5′-TTACGCCCAGTAATTCCGATT-3′). For each sample, the PCR amplifications were performed in triplicate in two separate experiments (n = 6, SD < 0.1). The Ct values were used for calculation of the average *acrB* cDNA copy number that was individually normalized using the average *rrs* copy number of the same sample. The *acrB* transcription level was expressed by comparison with that of *S. Typhimurium* ATCC 13311.

Results

Antimicrobial susceptibilities: All the *Salmonella* isolates exhibited resistance to at least one antibiotic. Most (n = 18) were resistant to ciprofloxacin (MICs = 4-8 µg/ml). Six isolates were susceptible to ciprofloxacin (MICs = 0.125µg/ml) and all other antibiotics tested. The resistance phenotype of all the isolates is shown in Table 2.

Mutations in the QRDRs of gyrA, gyrB, parC and parE: Three topoisomerase genes; i.e. *gyrA*, *gyrB*, and *parC*, were found to contain mutations. Of all twenty-four isolates, 18 ciprofloxacin-resistant isolates were found to contain mutations in at least one gene (Table 1). Two point mutations in *gyrA* (i.e. G-144-T and C-133-T leading to Met-48-Ile and Pro-45-Ser amino acid changes in GyrA, respectively) were observed. Only one point mutation in *gyrB* (i.e. T-188-C leading to Val-63-Ala in GyrB) was identified. All the resistant isolates carried *parC* mutations, of which up to 13 different nucleotide changes were found. None of the isolates carried mutations in *parE*. All six isolates susceptible to ciprofloxacin did not carry mutations in their QRDRs.

Eleven groups (Gr. I to Gr. XI) were defined based on type and number of genes carrying mutations (Table 2). None of the isolates carried mutations only in *gyrA*. Five isolates with a point mutation in *gyrA* additionally harbored mutations in gyrB and/or parC (Gr. I and Gr.VI to IX). Two isolates (one in Gr.1 and the others in Gr. VII) carried mutations in all three genes. Eight isolates (Gr. III, IV, VI and X) possessed mutations only in *parC*.

The *Salmonella* isolates with point mutations in all three genes (i.e. SA463 in Gr. I and SA614 in Gr. VIII, MIC = 8 µg/ml) were not more resistant to ciprofloxacin than those with mutations only in *gyrB* and *parC* (i.e.SA613 and SA622in Gr.II and SA603 in Gr. V, MIC = 8 µg/ml). Similarly, the isolates with *gyrA* and *parC* mutations (i.e. SA608 and SA609 in Gr. VI and SA615 in Gr.IX) exhibited the same ciprofloxacin resistance level (MIC = 4 µg/ml) with those carrying only *parC* mutations (Gr. III, IV, VI and X).

When considered mutations in *gyrB* and *parC*, the isolates with mutations in both genes (i.e. SA671 in Gr.II and SA603 in Gr. V) and those with *parC* mutation only (Gr. III, IV and VII) showed the same ciprofloxacin MIC (4 µg/ml).

Among those with *parC* mutation only, the ciprofloxacin MIC (4 µg/ml) of the *Salmonella* isolates with one mutation (i.e. SA578 and SA579 in Gr. III) was equivalent to that of the isolates with at least six point mutations (i.e. SA601 and SA602 in Gr. IV and SA610 and SA611 in Gr. VII). Likewise, SA624 in Gr. IV, SA612 in Gr. VII and SA616 in Gr. X showed the comparable ciprofloxacin MIC (8 µg/ml).

Expression of AcrAB-TolC efflux system: All the *Salmonella* isolates produced AcrB. The transcription level varied from 1 to 430 folds of that of serovar Typhimurium ATCC 13311 (Table 2). Among the ciprofloxacin resistant isolates, AcrB expression level was diverse, i.e. those with MIC of 8 µg/ml expressed 24-131 folds while those with MIC 4 µg/ml expressed 1-430 folds. Two isolates with the highest AcrB expression SA671 (i.e. 430 folds) and SA608 (i.e. 273 folds) demonstrated ciprofloxacin MIC of 4 µg/ml comparable to those with the lowest AcrB expression, e.g. SA601 (i.e. 2 folds) and SA602 (i.e. 3 folds). Likewise, four isolates producing AcrB equivalent to ATCC 13311 (i.e. SA609, SA610, SA611 and SA578) showed ciprofloxacin MIC of 4 µg/ml. Five ciprofloxacin susceptible strains (Gr. XI) overproduced AcrB (i.e. 6-221 folds). Among these isolates, SA736 was susceptible to all antimicrobials tested and produced AcrB up to 221 folds.

The isolates with comparable AcrB demonstrated different susceptibility to ciprofloxacin. SA666 and SA741, producing AcrB of 18 folds, were susceptible to ciprofloxacin while SA579 (19 folds) and SA612 (24 folds) were resistant to the antibiotic. When considered resistance to other antimicrobials, not all the AcrB-overexpressing strains were multidrug resistant. For example, SA721 was resistant to only tetracycline and SA736 was susceptible to all antimicrobials tested overproduced AcrB 18 and 221 folds, respectively.

Table 1 Mutations in *gyrA*, *gyrB* and *parC* genes in the ciprofloxacin-resistant *Salmonella* isolates (n = 18)

Gene	Mutation		
	Nucleotide change	Amino acid substitution	No.(%)
gyrA	G-144-T	Met-48-Ile	5 (27.8)
	C-133-T	Pro-45-Ser	1 (5.6)
gyrB	T-188-C	Val-63-Ala	6 (33.3)
parC	T-2-C	Met-1-Thr	6 (33.3)
	A-13-C	Ser-5-Arg	1 (5.6)
	G-31-C	Ala-11-Pro	12 (66.7)
	C-62-T	Ser-21-Leu	12 (66.7)
	T-86-C	Ile-29-Thr	12 (66.7)
	C-92-T	Thr-31-Met	7 (38.9)
	C-152-T	Thr-51-Ile	6 (33.3)
	G-173-A	Gly-58-Glu	1 (5.6)
	C-182-G	Ala-61-Gly	6 (33.3)
	A-212-G	Gln-71-Arg	12 (66.7)
	T-230-C	Leu-77-Pro	7 (38.9)
	T-230-G	Leu-77-Arg	4 (22.2)
	C-245-T	Thr-82-Met	14 (77.8)

Table 2 Antimicrobial resistance characteristics of the *Salmonella* isolates (n = 24)

Gr	Strain	Mutation			CIP MIC (µg/ml)	Resistance pattern[a]	Expression level of *acrB*
		gyrA	*gyrB*	*parC*			
I	SA463	Pro-45-Ser	Val-63-Ala	Ser-5- Arg, Ala-11-Pro, Ser-21-Leu, Ile-29-Thr, Thr-31-Met, Gln-71-Arg, Leu-77-Pro, Thr-82-Met	8	CHPC-CIP-SPC-SUL-TET-TRI	131
II	SA613	-	Val-63-Ala	Thr-82-Met	8	AMP-CHPC-CIP-STR-SPC-SUL-TET-TRI	49
	SA622	-	Val-63-Ala	Thr-82-Met	8	AMP-CHPC-CIP-STR-SPC-TET-TRI	63
	SA671	-	Val-63-Ala	Thr-82-Met	4	CIP-SPC-SUL-TET-TRI	430
III	SA578	-	-	Thr-82-Met	4	AMP-CHPC-CIP-SPC-TRI	1
	SA579	-	-	Thr-82-Met	4		19
IV	SA601	-	-	Ala-11-Pro, Ser-21-Leu, Ile-29-Thr, Gln-71-Arg, Leu-77-Pro, Thr-82-Met, Met-1-Thr, Thr-51-Ile, Ala-61-Gly	4	AMP-GEN-CHPC-CIP-STR-SPC-SUL-TET-TRI	2
	SA602	-	-	Ala-11-Pro, Ser-21-Leu, Ile-29-Thr, Gln-71-Arg, Leu-77-Pro, Thr-82-Met, Met-1-Thr, Thr-51-Ile, Ala-61-Gly	4	AMP-GEN-CHPC-CIP-STP-SPC-TET-TRI	3
	SA624	-	-	Ala-11-Pro, Ser-21-Leu, Ile-29-Thr, Gln-71-Arg, Leu-77-Pro, Thr-82-Met, Met-1-Thr, Thr-51-Ile, Ala-61-Gly	8	AMP-GEN-CHPC-CIP-STR-SPC-TET-TRI	26
V	SA603	-	Val-63-Ala	Thr-82-Met, Gly-58-Glu	4	AMP-CHPC-CIP-STR-SPC-TET-TRI	55
VI	SA608	Met-48-Ile	-	Ala-11-Pro, Ser-21-Leu, Ile-29-Thr, Thr-31-Met, Gln-71-Arg, Leu-77-Pro	4	AMP-GEN-CHPC-CIP-SPC-TET-TRI	273
	SA609	Met-48-Ile	-	Ala-11-Pro, Ser-21-Leu, Ile-29-Thr, Thr-31-Met, Gln-71-Arg, Leu-77-Pro	4	AMP-CHPC-CIP-SPC-SUL-TET-TRI	1
VII	SA610	-	-	Ala-11-Pro, Ser-21-Leu, Ile-29-Thr, Thr-31-Met, Gln-71-Arg, Leu-77-Pro	4	AMP- CHPC-CIP-SPC-STR-SUL-TET-TRI	1
	SA611	-	-	Ala-11-Pro, Ser-21-Leu, Ile-29-Thr, Thr-31-Met, Gln-71-Arg, Leu-77-Pro	4	AMP-CHPC-CIP-SPC-TET-TRI	1
	SA612	-	-	Ala-11-Pro, Ser-21-Leu, Ile-29-Thr, Thr-31-Met, Gln-71-Arg, Leu-77-Pro	8	AMP-CHPC-CIP-STR-SPC-SUL-TET-TRI	24
VIII	SA614	Met-48-Ile	Val-63-Ala	Ala-11-Pro, Ser-21-Leu, Ile-29-Thr, Thr-31-Met, Gln-71-Arg, Leu-77-Pro	8	AMP-GEN-CHPC-CIP-STR-SPC-SUL-TET-TRI	58
IX	SA615	Met-48-Ile	-	Ala-11-Pro, Ser-21-Leu, Ile-29-Thr, Thr-31-Met, Gln-71-Arg, Leu-77-Pro, Thr-31-Met, Gln-71-Arg, Leu-77-Pro, Thr-82-Met	8	AMP-CHPC-CIP-STR-SPC-TET-TRI	58
X	SA616	-	-	Ala-11-Pro, Ser-21-Leu, Ile-29-Thr	8	AMP-CHPC-CIP-STR-SPC-SUL-TET-TRI	106
XI	SA545	-	-	-	0.125	AMP-TET-SUL	101
	SA666	-	-	-	0.125	AMP-CHPC-SPC-SUL-TET-TRI	18
	SA717	-	-	-	0.125	AMP-SPC-SUL-TET-TRI	1
	SA721	-	-	-	0.125	TET	18
	SA734	-	-	-	0.125	AMP-GEN-CHPC-CIP-STR-SPC-TET	6
	SA736	-	-	-	0.125	Susceptible to all antimicrobial tested	221

[a] Amp: ampicillin; Chp: chloramphenicol; Gen: gentamycin; Spc: spectinomycin; Str: streptomycin; Sul: sulfamethoxazole; Tet: tetracycline; Tri: trimethoprim

Discussion

In comparison to other pathogenic Enterobacteriaceae, *Salmonella* infrequently exhibit high fluoroquinolone resistance level and it was suggested that such limitation was associated with a prohibitive fitness cost (Giraud et al., 2003; Giraud et al., 2006). This agreed with this study, where the ciprofloxacin-resistant isolates showed the MIC value of 4-8 µg/ml. Fluroquinolone resistance in *Salmonella* is commonly associated with a single point mutation between nucleotides 67 to 122 in the QRDR of *gyrA* (Giraud et al., 1999), leading to the more common GyrA amino acid changes at position Gly81, Ser83 or Asp87 (Marimon et al., 2004). In contrast, the strains in this study lacked these prominent mutations. However, our strains carried mutations in *gyrA*, *gyrB* and/or *parC*. Based on our knowledge, all the mutations identified are novel in ciprofloxacin-resistant *S. enterica*. Since these mutations have never been found, their actual contribution may not be stated. Further experiment such as site specific mutagenesis or study in a larger population may worth it.

In Gram-negative bacteria, a single *gyrA* mutation confers low ciprofloxacin resistance level and the additional mutations in *gyrA* or within the other topoisomerase genes results in the increased-resistance level (Liebana et al., 2002; Dimitrov et al., 2009). However, none of the isolates in the present study harbored only *gyrA* mutations. Therefore, it may not precisely assess the contribution of mutations in *gyrA* and other two genes.

No nucleotide changes in *parE* were identified, consistent with previous studies (Marimon et al., 2004). This is not beyond expectation and supports that *parE* mutation is much less common than mutations in the others. Mutations in *parC* are seldom seen in the ciprofloxacin-resistant *S. enterica* (Eaves et al., 2004) and role of these mutations is still not apparent. In contrast, *parC* mutations were identified in all the resistant strains in this study. While up to 13 different point mutations were observed in *parC*, some single strains simultaneously carried 9 *parC* mutations. Still, it cannot ascertain that all these mutations contributed to ciprofloxacin resistance of the bacterial hosts. However, the susceptible isolates did not carried the same mutations in any of the three topoisomerase genes, supporting that nucleotide changes observed were not

likely associated with strain variation and indicating the significance of mutations in topoisomerase genes in ciprofloxacin resistance. Taken together, there are no associations between the number and type of mutations and ciprofloxacin resistance level among the ciprofloxacin-resistant *Salmonella* isolates in this collection.

The isolates with the same ciprofloxacin MIC expressed AcrB at different level. Vice versa, the isolates with the same AcrB expression exhibited different ciprofloxacin MICs. These data indicated that there was no correlation between expression of the AcrAB-TolC efflux pump and ciprofloxacin resistance level and also the existence of other uncharacterized mechanisms. In this case, the presence of the plasmid-borne *qnr* genes was demonstrated but not examined in this study. Transcription of *acrB* was detected in all 24 *Salmonella* isolates, supporting the constitutive expression of the AcrAB-TolC efflux pump in *Salmonella*. The AcrAB-TolC pump was identified as a major mechanism in fluoroquinolone resistance in *E. coli* (Piddock et al., 2000). It was shown that inactivation of the pump resulted in loss of fluoroquinolone resistance in the strains with ***gyrA*** mutations (Oethinger et al., 2000). This may not always be the case since some ciprofloxacin resistant isolates in this study (i.e. SA578, SA609, SA610 and SA611) did not overproduce AcrB when compared to the reference strain. Concurrently, the ciprofloxacin susceptible isolates in Gr.5 (MIC = 0.125 μg/ml) produced AcrB from 6 to 221 folds. The contribution of the AcrAB-TolC pump in these strains should be minimal (if any) and may not be accurately determined due to high susceptibility to ciprofloxacin of the strains.

Based on the observations in this study, the role of AcrAB-TolC of the *Salmonella* isolates is greatly different from previous studies (Baucheron et al., 2002; Giraud et al., 2000). This discrepancy could be explained by differences in bacterial sources and settings of fluoroquinolone-resistance phenotype. In most previous studies, the contribution of the AcrAB-TolC pump was compared between the parent strains and their isogenic-resistant mutants originated from *in vitro* exposure to fluoroquinolones. In contrast, all the *Salmonella* strains in this collection were of clinical origins and their resistance phenotype was associated with several factors within the cells and in their environment. Therefore, AcrAB-TolC expression dynamics and its contribution to fluoroquinolone resistance level may somewhat vary.

In addition to fluoroquinolone resistance, the AcrAB-TolC efflux pump plays a major role in multiple antibiotic resistance in *Salmonella* (Baucheron et al., 2002; Guerra et al., 2003). Deletion of the *acrAB* operon resulted in increased susceptibility to multiple drugs, e.g. chloramphenicol, ceftiofur, tetracycline, cephalothin, trimethoprim-sulfamethoxazole (Giraud et al., 2000; Eaves et al., 2004). Despite these previous reports, the same way may not be true in our strains. The best evidences were overproduction of AcrB in SA736 that was susceptible to all antimicrobials (221 folds) and SA721 that was resistant to tetracycline only (18 folds).

Particular attention was paid to SA736 that was susceptible to ciprofloxacin and all other antimicrobials tested lacked mutation in topoisomerase genes and overproduced AcrB up to 221 folds. Its genetic and phenotypic property illustrated the inconsistent role of the AcrAB-TolC efflux pump, while substantiated the role of mutations in the target genes in fluoroquinolone resistance in the *Salmonella* clinical isolates. Therefore, further studies are warranted to elucidate the actual involvement of these mechanisms in the clinical isolates.

In conclusion, decreased susceptibility to ciprofloxacin in *S. enterica* clinical isolates seems to be associated with the accumulative effect of many resistance mechanisms and the role of the well-characterized mechanisms may not always be in harmony. As fluoroquinolones are one of the best choices for salmonellosis treatment, increasing resistance to this antibiotic class may devastate their future use in medical therapy. The latter could be true for not only *Salmonella* infection but also other pathogenic bacterial infections. Judicious use of antibiotics is mandatory and needs to be initiated with no delay.

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