Original Article

Mutations in QRDRs of DNA gyrase and topoisomerase IV genes in nalidixic acid and ciprofloxacin-resistant *Salmonella enterica* isolated from chicken meat, pork and humans

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Abstract

Twenty-eight nalidixic acid-resistant *Salmonella* isolates originated from chicken meat, pork and humans were examined for mutation in the quinolone resistance determining region (QRDR) of *gyrA*, *gyrB*, *parC* and *parE* genes. Four single-point mutations in *gyrA* (i.e. C248A, C248T, G259T and G259A) leading to amino acid substitutions Ser83Tyr, Ser83Phe, Asp87Tyr and Asp87Asn in GyrA, respectively, were identified in 11 quinolone-resistant strains. Amino acid change at position Ser83 was most frequently identified. Resistance to nalidixic acid was not always associated with resistance to ciprofloxacin. The presence of mutations in GyrA did not well correlate with ciprofloxacin resistance phenotype. No mutations were observed in *gyrB* and *parE*. A serovar Typhimurium resistant to nalidixic acid did not carry any mutations in QRDR of all genes tested. The results highlight the high frequency of mutation in *gyrA* in the quinolone-resistant isolates and the existence of alternative-quinolone resistance mechanisms.

Keywords: DNA gyrase, fluoroquinolone resistance, nalidixic acid resistance, *Salmonella enterica*, topoisomerase IV

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Introduction

Non typhoidal *Salmonella enterica infections* are a *major* threat to *global public health and* is commonly associated with consumption of contaminated food of animal origin (e.g. chicken meat, pork). Salmonellosis is usually self-limited and does not require antibiotic therapy. However, antibiotic treatment may be required for young children or the elderly. Fluoroquinolones have been recommended by WHO as empirical treatment for multidrug resistant enteric fever (WHO, 2007) and are often used to treat invasive *Salmonella* infection in humans (Cremet et al., 2011). Particular concern is that the increasing use of fluoroquinolones may lead to the emergence and spread of fluoroquinolone-resistant *Salmonella*. Recently, fluoroquinolone-resistant *Salmonella* has been increasingly reported (Lertworapreecha et al., 2013; Wang et al., 2015).

Quinolones are broad-spectrum antibiotics with four generations, of which nalidixic acid is the first-generation drug exhibiting a narrow-spectrum activity against Gram-negative bacteria. Ciprofloxacin is the second generation quinolone with a broad-spectrum activity against Gram-negative and Gram-positive bacteria (Fabrega et al., 2009). These antibiotics selectively inhibit topoisomerase II (DNA gyrase) and topoisomerase IV and prevent bacterial DNA replication during growth and reproduction. DNA gyrase has two A subunits and two B subunits that are encoded by *gyrA* and *gyrB* genes, respectively. Topoisomerase IV is composed of two C and two E subunits encoded by *parC* and *parE* genes, respectively. During DNA replication, DNA gyrase catalyses negative DNA supercoiling and topoisomerase IV is responsible for segregation daughter chromosome (Hopkins et al., 2005).

Three major mechanisms of quinolone resistance have been recognized including mutations in the quinolone resistance determining region (QRDR) of DNA gyrase and topoisomerase IV genes; reduced drug accumulation in bacterial cell by overexpression of efflux pump; impermeability of outer membrane and the presence of plasmid-mediated quinolone resistance genes (Jacoby, 2005). Among these, mutations in QRDR of DNA gyrase and topoisomerase IV genes are frequently present in quinolone-resistant bacteria (Wasyl et al., 2014; Garcia-Fernandez et al., 2015). QRDR is a small region located between amino acid 67-107 in GyrA and amino acid 426-447 in GyrB (Yoshida et al., 1991) and may be quinolone-binding site (Madurga et al., 2008). The amino acid substitutions in QRDR could result in reduced fluoroquinolone binding affinity, leading to decrease in fluoroquinolone susceptibility in bacteria (Vashist et al., 2009).

Quinolone resistant *Salmonella* has been previously reported in Thailand. However, most studies focused on the resistance phenotype (Minami et al., 2010; Sanpong et al., 2010; Lertworapreecha et al., 2013) and little published information on the quinolone resistance genotype is available. The present study provides an update on mutations in the QRDR of *gyrA*, *gyrB*, *parC* and *parE* genes in nalidixic acid and/or ciprofloxacin-resistant *Salmonella* from pork, chicken meat and humans in Thailand.

A total of 28 nalidixic-resistant *Salmonella* isolates that were originated from faecal samples of humans (n=7), pigs (n=16) and chicken (n=5) in 2010-2011 (Table1) were examined. The strains were isolated using ISO6579:2002 (E) (ISO, 2002) and serotyping at Center of Antimicrobial Resistance in Foodborne Pathogens (in cooperation with the World Health Organization), Faculty of Veterinary Science, Chulalongkorn University. These *Salmonella* isolates were previously tested against nalidixic acid (30 µg) using the CLSI guidelines for antimicrobial disc susceptibility testing (CLSI, 2008). All the isolates were stored at -80°C in 20% glycerol as our strain collection.

Materials and Methods

All the isolates were determined for minimum inhibitory concentration (MIC) of nalidixic acid and ciprofloxacin by two-fold agar dilution method as described by CLSI (CLSI, 2008). Clinical breakpoints for nalidixic acid and ciprofloxacin were ≥ 32 and ≥ 4 µg/ml, respectively. *Escherichia coli* ATCC® 25922, *Pseudomonas aeruginosa* ATCC®27853, and *Staphylococcus aureus* ATCC® 29213 were included as quality control strains.

The QRDRs of *gyrA*, *gyrB*, *parC and parE* genes were PCR amplified by using specific primer pairs as follows: *gyrA*, gyrA-F (5′-GCTGAAGAGCTCCTATCT GG-3′)/gyrA-R (5′-GGTCGGCATGACGTCCGG-3′); *gyrB*, gyrB-F (5′-GCGCGCTCGATTTAGCCG-3′)/gyrB-R (5′-TGATAGCGCAGCTTGTCCG-3′); *parC*, parC-F (5′- GTACGTGATCATGGATCGTG-3′)/parC-R (5′-TTCCTGCATGGTGCCGTCG-3′); and *parE*, parE-F (5′-GCCATCGCGAATATCAGGCG-3′)/parE-R (5′-CAGTTGTTCCAGTACGCCC 3′) (Chuanchuen and Padungtod, 2009). The PCR amplicons were gel purified using Nucleospin® Gel and PCR clean up (Düren, Germany) and submitted for DNA sequencing (First Base Laboratories, Selangor Darul Ehsan, Malaysia). All sequences obtained were compared with those in GenBank database using the Blast algorithm available at www.ncbi.nlm.nih.gov (Genbank accession numbers AE008801, AE008878 and AE008846 for *gyrA*, *gyrB*, *parC* and *parE*, respectively). Two *Salmonella* strains susceptible to nalidixic acid and ciprofloxacin were included as control strains.

Results

All the nalidixic acid-resistant *Salmonella* isolates had MIC value ranging from 32 to 256 µg/ml. Six isolates (4 human isolates and 2 chicken isolates) were additionally resistant to ciprofloxacin with MIC 4 µg/ml (Table 1).

Nucleotide sequencing analysis revealed that 11 isolates carried a single point mutation in *gyrA* including C248A, C248T, G259T and G259A, leading to amino acid substitutions Ser83Tyr, Ser83Phe, Asp87Tyr, Asp87Asn in GyrA, respectively (Table 1). Amino acid change at codon 83 (i.e. Ser83Phe and Ser83 Tyr) was most commonly identified (n=8). Four isolates resistant to both nalidixic acid and ciprofloxacin harbored Ser83Phe (i.e. A50, A53 and

E21) or Asp87Tyr (i.e. E21). Seven *Salmonella* isolates resistant to nalidixic acid but susceptible to ciprofloxacin carried amino acid change Ser83Tyr (i.e. A43, C64, C92 and D10), Ser83Phe (i.e. E37), Asp87Tyr (i.e. D63) or Asp87Asn (i.e. A7). Twenty-two isolates carried a single point mutation G283C in *parC*, leading to Val95Leu substitution in ParC, which was also found in the two susceptible-control strains. None of the isolates harbored mutations either in *gyrB* or *parE*.

Table 1 MIC values of nalidixic acid and ciprofloxacin in *Salmonella* isolates from humans, chicken meat and pork (n=28)

Source	Strain ID	Serotype	Sample type	Location	MIC (µg/ml)[a]		Mutation[b]
					Nalidixic acid	Ciprofloxacin	
Humans							
(n=7)	A43	Anatum	Rectal swab	Hospital	256	2	C248A(Ser83Tyr)
	A45	Weltevreden	Rectal swab	Hospital	256	0.125	-[c]
	A50	Enteritidis	Rectal swab	Hospital	256	4	C248T(Ser83Phe)
	A53	Enteritidis	Rectal swab	Hospital	256	4	C248T(Ser83Phe)
	A54	Corvallis	Rectal swab	Hospital	32	4	-
	A60	Rissen	Rectal swab	Hospital	256	4	C248T(Ser83Phe)
	C48	Typhimurium	Rectal swab	Hospital	128	1	-
Chicken							
(n=5)	B36	Anatum	Carcass swab	Slaughterhouse	32	4	-
	C64	Amsterdam	Carcass swab	Slaughterhouse	128	1	C248A(Ser83Tyr)
	D63	Virchow	Chicken meat	Market	128	0.5	G259T(Asp87Tyr)
	D70	Agona	Chicken meat	Market	128	0.125	-
	E21	Virchow	Chicken meat	Market	128	4	G259T(Asp87Tyr)
Pig							
(n=16)	A4	Anatum	Carcass swab	Slaughterhouse	32	0.125	-
	A5	Anatum	Carcass swab	Slaughterhouse	32	0.25	-
	A7	Albany	Carcass swab	Slaughterhouse	128	0.5	G259A(Asp87Asn)
	C77	Weltevreden	Carcass swab	Slaughterhouse	32	0.125	-
	C78	Stanley	Carcass swab	Slaughterhouse	128	0.125	-
	C90	Anatum	Retail pork	Market	32	0.125	-
	C91	Corvallis	Retail pork	Market	32	2	-
	C92	Anatum	Retail pork	Market	>128	1	C248A(Ser83Tyr)
	D1	Corvallis	Retail pork	Market	32	2	-
	D5	Rissen	Retail pork	Market	32	0.125	-
	D10	Anatum	Retail pork	Market	128	1	C248A(Ser83Tyr)
	D11	Rissen	Retail pork	Market	128	1	-
	D54	Stanley	Retail pork	Market	128	1	-
	E16	Anatum	Retail pork	Market	128	1	-
	E37	Anatum	Retail pork	Market	128	1	C248T(Ser83Phe)
	E82	Worthington	Retail pork	Market	32	1	-

[a] The clinical breakpoints for nalidixic acid and ciprofloxacin were ≥ 32 µg/ml and ≥ 4 µg/ml, respectively.
[b] nucleotide change in *gyrA* (amino acid substitution in GyrA)
[c] -, no mutation found

Discussion

Based on the CLSI ciprofloxacin breakpoint, only six nalidixic acid-resistant isolates in this collection were additionally resistant to ciprofloxacin. It indicates that resistance to nalidixic acid is not always associated with resistance to ciprofloxacin, consistent with previous studies (Hakanen et al., 1999; Kim et al., 2011). However, it has been suggested that CLSI interpretative criteria for ciprofloxacin (i.e. ≥ 4 µg/ml) should be reevaluated (Ryan et al., 2011). Several studies showed that the nontyphoidal and typhoidal *Salmonella* isolates had the ciprofloxacin MIC much lower than 4 mg/liter (Kim et al., 2011; Wasyl et al., 2014) and a breakpoint of ≥ 0.125 µg/ml was recommended (Aarestrup et al., 2003). Based on the nucleotide sequencing analysis, similar mutations in GyrA (e.g. Asp87Asn, Ser83Phe, Asp87Thr) were observed in the ciprofloxacin-susceptible and -resistant isolates. Seven isolates resistant to nalidixic acid but susceptible to ciprofloxacin carried amino acid mutation at positions Ser83 and Asp87 in GyrA. It is possible that mutations may reduce the ciprofloxacin susceptibility at certain extent but not enough to bring their MIC value above 4 µg/ml. These may be another support for the requirement of CLSI-ciprofloxacin breakpoint revision.

In addition, the isolates with high nalidixic acid MIC (128-256 µg/ml) exhibited varied ciprofloxacin MIC (0.125 to 4 µg/ml). Therefore, there was no correlation between nalidixic acid MIC and the raised ciprofloxacin MIC, in agreement with a previous study (Kim et al., 2011).

Mutations within the QRDR of *gyrA* are considered one of the main mechanisms of quinolone resistance. Amino acid substitutions at positions Ser83 and Asp87 in GyrA have been previously reported (Wasyl et al., 2014; Wang et al., 2015). The replacement of the hydrophilic hydroxyl group of serine by the hydrophobic residue of phenylalanine or tyrosine results in the loss of OH group of serine, leading to the loss of hydrogen-bonding interaction between amino acid residue and quinolone molecule and, eventually, reduced drug binding affinity with target site (Piddock, 2002). Similar phenomenon is applied for the amino acid change at Asp87. It was previously shown that a mutation at Ser83 conferred a higher quinolone resistance level, in comparison to that at position 87

(Ogbolu et al., 2012). However, it was not the case in this study. Further studies, e.g. site-directed mutagenesis, is required to better understand the contribution of mutations in these target genes.

All four *Salmonella* isolates resistant to both nalidixic acid and ciprofloxacin containing a single mutation at positions Ser 83and Asp87 had nalidixic acid MIC value of ≥ 128 µg/ml and ciprofloxacin MIC value of 4 µg/ml. This finding is consistent with a previous study showing that *S*. Typhi and *S*.Paratyphi A isolates carrying Ser83Phe had a high nalidixic acid resistance level (MIC > 256 µg/ml) with a ciprofloxacin MIC value of 4 µg/ml (Hirose et al., 2002).

Previous studies demonstrated that high resistance level to fluoroquinolones was associated with double mutations in *gyrA* at positions 83 and 87 coupled with *parC* mutation (Cui et al., 2008; Garcia-Fernandez et al., 2015). However, double mutations in *gyrA* gene were not observed in our study. Most quinolone-resistant strains in the present study (n=22) carried amino acid substitution Val95Leu in ParC, which was also found in the quinolone-susceptible strains. Therefore, this amino acid change may be a result of biological variation among the *Salmonella* strains.

The lack of mutation in *gyrB* and *parE* in this study supports that the quinolone-resistant strain of *Salmonella* with mutations in *gyrB* or *parE* is rare (Yang et al., 2012; Lertworapreecha et al., 2013). In fact, the contribution of amino acid change in ParE to quinolone resistance remains unclear. In addition, some quinolone-resistant *Salmonella* isolates (n=17) lacked QRDR mutation of the tested genes, suggesting the existence of alternative resistance mechanisms that were not characterized in this study, e.g. efflux pump systems and plasmid-mediated quinolone resistance. This warranted further studies to elucidate the quinolone resistance mechanisms in these *Salmonella* isolates. Furthermore, the findings of the present study support that it is important to keep monitoring quinolone resistance-associated DNA gyrase mutations, particularly in *gyrA*, in order to predict the emergence of new mutations in quinolone-resistant *Salmonella* strains.

In conclusion, the results of this study highlight the complex pictures of quinolone resistance mechanisms in *Salmonella* isolate. Continuous monitoring of fluoroquinolone usage and resistance in *Salmonella* and other bacteria is indispensable.

Acknowledgements

This work was financially supported by the Research Grant for Mid-Career University Faculty RSA 5680051 cofunded by Thailand Research Fund (TRF), the Faculty of Veterinary Science, and Chulalongkorn University. It was also partially supported by the 90th Anniversary of Chulalongkorn University fund. NS is a recipient of the Royal Golden Jubilee PhD program PHD/006/2553, cofunded by TRF and Chulalongkorn University.

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บทคัดย่อ

การศึกษาการกลายพันธุ์ที่ตำแหน่ง QRDRs ของยีน DNA gyrase และ topoisomerase IV ของเชื้อแซลโมเนลลา เอนเทอริกาที่ดื้อต่อยากลุ่มควิโนโลนที่แยกได้จากเนื้อไก่ เนื้อสุกร และคน

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เชื้อแซลโมเนลลา เอนเทอริกาที่ดื้อต่อยาในกลุ่มควิโนโลนจำนวน 28 isolates แยกมาจากเนื้อไก่ เนื้อสุกร และคน ตรวจหาการกลายพันธุ์ในตำแหน่ง quinolone resistant determining regions (QRDRs) ของยีน *gyrA*, *gyrB*, *parC* และ *parE* พบการเปลี่ยนแปลงของกรดอะมิโนแบบ single point mutation จำนวน 4 จุด (C248A, C248T, G259T และ G259A) ซึ่งทำให้เกิดการเปลี่ยนแปลงของกรดอะมิโนที่ตำแหน่ง Ser83Tyr, Ser83Phe, Asp87Tyr และ Asp87Asn บนโปรตีน GyrA ของเชื้อแซลโมเนลลาที่ดื้อต่อยากลุ่มควิโนโลนจำนวน 11 isolates โดยพบการเปลี่ยนแปลงของกรดอะมิโนที่ตำแหน่ง Ser 83 มากที่สุด นอกจากนี้ ไม่พบการกลายพันธุ์ในตำแหน่ง QRDRs ของยีน *gyrB* และ *parE* ทั้งนี้ ยังพบเชื้อแซลโมเนลลา ไทฟิมูเรียมจำนวน 1 isolate ที่ดื้อต่อยานาลิดิซิก แอซิด ไม่พบการเปลี่ยนแปลงของกรดอะมิโนบนทุกยีนเป้าหมาย ซึ่งอาจเกิดมาจากกลไกอื่น ๆ ที่มีผลต่อการดื้อต่อยาในกลุ่มนี้

คำสำคัญ: ยีน DNA gyrase การดื้อต่อยาฟลูโอโลควิโนโลน การดื้อต่อยานาลิดิซิก แอซิด แซลโมเนลลา เอนเทอริก ยีน topoisomerase IV

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