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Molecular Detection of Mutations within the Quinolone Resistance-Determining Regions in Non Typhoidal *Salmonella* Isolates from Malaysia

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Abstract

Introduction: The efficacy of chemotherapy in bacteraemia caused by non-typhoidal *Salmonella* (NTS) is compromised by antibiotic resistance. **Objective:** This study was undertaken to describe the mechanism of resistance among clinical NTS isolates. **Materials & Methodology:** Thirty of NTS were isolated from blood (n = 19), stool (n = 10) and bronchioalveolar lavage (BAL; n = 1) respectively. These isolates were tested for susceptibility testing by disc diffusion method against ampicillin, gentamicin, tetracycline, co-trimoxazole, nalidixic acid, ciprofloxacin and ceftriaxone. Epsilometer tests (E-test) for nalidixic acid and ciprofloxacin were performed for nalidixic acid resistant isolates by disc diffusion method. DNA sequencing was carried out on six of the nalidixic acid resistant *Salmonella Enteritidis* isolates to identify mutations within quinolones resistance determining regions (QRDR) of *gyrA*, *gyrB*, *parC* and *parE* genes. **Results:** Resistance rates of NTS isolates from blood, stool, and BAL were respectively 37%, 20% and 0% for ampicillin, 79%, 40% and 0% for tetracycline, 32%, 40% and 0% for co-trimoxazole, 37%, 10% and 100% for nalidixic acid. Eight isolates were resistant to nalidixic acid and had exhibited reduced susceptibility towards ciprofloxacin by E-test. Mutation within QRDR was detected in *gyrA* gene (n = 6; Asp 47 → His [3], Asp 51 → Asn [1], Asp 73 → Gly [1], and Gly 48 → Asp [1]) and double mutation was detected in *parE* gene (n = 3; Gly 48 → Asp [3], Glu 82 → Ser [3]). Out of six isolates, three isolates were found to have both *gyrA* and *parE* gene mutations. **Conclusions:** There was no mutation observed in *gyrB* and *parC* gene. Muta-

tion in *gyrA* gene was sufficient to induce decreased susceptibility to ciprofloxacin. Variation in amino acid sequences are novel, while detection of other gene mutation was uncommon.

Keywords

Non-Typhoidal *Salmonella*, Quinolones Resistance-Determining Regions, Ciprofloxacin

1. Introduction

Antibiotics are used for the therapy of invasive diseases or complicated bacteraemia caused by non-typhoidal Salmonella (NTS). The prevalence of multidrug resistant NTS in adult patients has gradually increased in the last decade, making the treatment of invasive salmonellosis a clinical dilemma [1] [2] [3] [4] [5]. Fluoroquinolones is a broad-spectrum antimicrobial agent that inhibits DNA gyrase in susceptible organisms and promotes breakage of double-stranded DNA. It is highly effective in urinary tract infections, gonorrhoea, invasive gastrointestinal infections, intra-abdominal infections and lower respiratory tract infections in adults [6]. It is also widely used in agriculture and veterinary medicine [2] [7] [8] [9].

In principle there are four types of mechanism of antimicrobial resistance including inhibition of cell wall synthesis, inhibition of protein synthesis, inhibition of nucleic acid synthesis, and alteration of cell membrane function by which bacteria evade the action of antimicrobial agents. They work independently or in combination ranging from reduced susceptibility, which is not always detected by current antimicrobial susceptibility tests, to clinically relevant resistance. Resistance to quinolones is mainly mediated by chromosomal mutations which results to the target alteration of DNA gyrase and topoisomerase IV.

In Gram negative bacteria, the GyrA subunit of DNA gyrase is the most frequent primary target while topoisomerase IV are secondary target for quinolones. However, the contrary is true for Gram-positive bacteria [10] [11]. Point mutations most often occur within the highly conserved domain of the N-terminus of the *gyrA* gene, also known as "quinolone resistance-determining region" (QRDR) [11] [12]. Most isolates resistant to nalidixic acid (NAL) have a single point mutation in the QRDR of *gyrA*, whilst resistance to ciprofloxacin is accomplished in isolates having double mutations in *gyrA* gene[12] [13].

Nalidixic aciddisc is used as "surrogate marker" to screen for reduced fluoroquinolone (FQ) susceptibility in *Salmonella*, as it has been shown that high resistance NAL is associated with reduced FQ susceptibility [4]. Nevertheless, the NAL screening may miss isolates that exhibit reduced FQ susceptibility by being susceptible or only displaying low-level resistance to nalidixic acid. Recent data have raised concern that NAL resistance may no longer be a reliable indicator for reduced ciprofloxacin (CIP) susceptibility as results of evolving Salmonella

FQ resistance mechanisms. There are reports of NAL-susceptible isolates with reduced FQ susceptibility [14] [15] [16] with the phenotype appears to be mediated by different resistance mechanisms outside the *gyrA* gene.

Mutations in *gyrB* results in CIP minimum inhibitory concentrations (MICs) of 0.125 - 0.5 μg/mL and NAL MICs of 2 - 16 μg/mL, both within the susceptible range [17]. Similarly, plasmid-mediated quinolone resistance (PMQR) determinants, *qnr* and *aac'-6-Ib-cr* genes [18], are associated with modest NAL MIC elevations (8 - 32 μg/mL) and increased resistance to CIP [19] [20]. These PMQR are commonly found in Europe and Asia [21] [22].

Systematic epidemiological studies are required to determine the effect of reduced fluoroquinolone susceptibility on human health and its implications for treatment options. *In vitro* resistance to nalidixic acid isolates has been associated with reduced efficacy of fluoroquinolones *in vivo* [23].

This study aims to identify the mechanism of chromosomal mutations involved in QRDR that leads to decreased CIP susceptibility among NTS isolates in Malaysia. Nevertheless, this information will serve as reference and surveillance tool for antibiotic resistance pattern that can be used at regional and international level. This could improve options for empirical antibiotic therapy and consequently, patient outcomes.

2. Materials and Methods

***Salmonella* strains:** A total number of 30 NTS isolates belongs to 28 patients were obtained from December 2011 to December 2012 from Hospital Sg. Buloh, Selangor, Malaysia for detailed investigation. The NTS isolates were collected and identified from blood, stool, and bronchioalveolar lavage (BAL) culture. The isolates were further serotyped based on Kauffmann-White scheme and confirmed to be NTS. Those that were resistant to nalidixic acid were species identified by polymerase chain reaction (PCR).

Antimicrobial susceptibility testing: Susceptibility testing of the NTS isolates was performed towards six antimicrobial agents including ampicillin, tetracycline, gentamicin, nalidixic acid, ciprofloxacin and ceftriaxone by disc diffusion (Beckton Dickinson, USA) method. The Epsilometer test (E-test) (Bio-Meriaux, France) for NAL and CIP were carried out for NAL resistant NTS isolate to determine MIC. The MIC determination was performed according to Clinical and Laboratory Standards Institute (CLSI), 2015 [24]. A NAL resistance is defined as a MIC of ≥ 32 μg/ml while reduced susceptibility to CIP is defined as a MIC of ≥ 0.125 μg/ml as previously described [13]. All NAL-resistant isolates determined by E-test were examined for the mutation by PCR and sequence analysis.

Detection of target gene mutations: Polymerase chain reaction was used to amplify and screen the QRDR of *gyrA*, *gyrB*, *parC* and *parE* genes as previously described [25] using established primers [26]. Bands of the correct size were excised and purified using Wizard SV Gel and PCR Clean-Up System kit (Promega, Madison, USA) and were sent to Institute of Medical Molecular Bio-

technology (IMMB), Faculty of Medicine, Universiti Teknologi MARA (Selangor, Malaysia) for sequencing. The sequences that were obtained were assembled using BioLign version 2.0.9 (http: //en.bio-soft.net/dna/BioLign.html) and the genes were compared to the respective genes in the Genbank using BLAST tool (http: //www.ncbi.nlm.nih.gov).

3. Results

The 30 NTS isolates were obtained from three types of specimen including blood (n = 19), stool (n = 10) and BAL (n = 1). Antimicrobial susceptibility testing by disc diffusion method revealed that NTS isolates from blood were resistant to ampicillin (36.8%), tetracycline (79.0%), co-trimoxazole (31.6%), and nalidixic acid (36.8%), while, 20.0% of NTS isolates from stool were resistant to ampicillin, 40.0% to tetracycline, 40.0% to co-trimoxazole and 10% to NAL. Non-typhoidal *Salmonella* isolate from bronchioalveolar lavage (BAL) was resistant to NAL and demonstrated susceptibility to other antibiotics. All NTS isolates from blood, stool, and BAL culture were susceptible to gentamicin, CIP, and ceftriaxone respectively.

The MIC of the eight NTS isolates by E-test revealed 75% (blood: 83.3% [5/6], BAL: 16.7% [1/6]) of NTS isolates were resistant to NAL, and 87.5% (n = 7/8) of isolates demonstrated reduced susceptibility to CIP. Of eight NTS isolates that were subjected to E-test, seven were serotyped as *Salmonella enterica* Serovar Enteritidis and remaining as *Salmonella enterica* Serovar Corvallis. All the *S.* Enteritidis were resistant to NAL and were isolated from the blood.

Sequence analysis revealed that six out of seven *S.* Enteritidis isolates had amino acid substitutions as a result of point mutations within QRDR of the *gyrA* gene. In three isolates, the aspartic acid at codon 47 was replaced with histidine (Asp 47 → His), while two other isolates had their aspartic acid at codon 51 and 73 substituted with asparagine (Asp 51 → Asn) and glycine (Asp 73 → Gly) residue respectively. In the remaining one isolate, glycine at codon 48 was resulted into aspartic acid substitution (Gly 48 → Asp).

In addition to it, amino acid substitutions were also observed due to a single base pair (T) insertion in *parE* gene from three isolates of *S.* Enteritidis. In all three isolates, glutamine at codon 81 was resulted into leucine substitution (Gln 81 → Leu) with glutamate at codon 82 was replaced by serine (Glu 82 → Ser). Hence, all the six isolates of *S.* Enteritidis had point mutations at *gyrA* gene, and three isolates had mutation in both *gyrA* and *parE* gene. However, none of the *S.* Enteritidis isolates had mutation detected at *gyrB* and *parC* gene. A NAL MIC elevations (16 μg/mL) with increased resistance to CIP (0.5 μg/mL; 0.75 μg/mL) were detected in the other remaining isolates of *S.* Enteritidis and *S.* Corvallis. The results are shown in Table 1.

4. Discussion

In recent years, the proportion of NTS strains with reduced FQ susceptibility has increased in many countries including the United States, United Kingdom and

Table 1. Determination of MIC and detection of target gene mutations.

	MIC (μg/ml)		QRDR mutation			
Isolates	**NAL**	**CIP**	***gyrA***	***gyrB***	***parC***	***parE***
3012005267	256	0.25	Gly 48 to Asp	-	-	
3012006354	256	0.125	Asp 47 to His	-	-	Gln 81 to Leu Glu 82 to Ser
3012037286	16	0.75	-	-	-	
3012037287	16	0.5	-	-	-	
3012038240	512	0.94	Asp 73 to Gly	-	-	Gln 81 to Leu Glu 82 to Ser
3012039138	512	0.125	Asp 47 to His	-	-	
3012034257	512	0.125	Asp 51 to Asn	-	-	
3012058507	512	0.125	Asp 47 to His	-	-	Gln 81 to Leu Glu 82 to Ser

NAL: nalidixic acid; CIP: ciprofloxacin. Asp: Aspartate, Asn: Asparagine, Gln: Glutamine, Glu: Glutamate, Gly: Glycine, His: Histidine, Leu: Leucine, Ser: Serine -: Mutation was not detected.

Southeast Asia [1] [2] [4] [27] [28]. This situation is worrisome, as FQ is the first line antibiotic to be administered in the treatment of invasive disease caused by NTS. Over the past 7 to 12 years, there has been a seven-fold significant increase in non-typhi *Salmonella enterica* isolates that are resistant to NAL, and 91% of these isolates showed decreased susceptibility to CIP [18]. Additionally, reports have documented poor FQ treatment outcomes for systemic infections caused by NAL-resistant and reduced CIP susceptibility isolates of NTS [24].

In our study, 75% of NTS isolates were resistant to NAL, and 87.5% of these isolates exhibited reduced susceptibility to CIP, with MIC CIP ranges from 0.125 to 0.94 μg/mL. These findings correspond with previous reports [1] [3] [4] [18] [19], and reveal the increasing resistance of NAL with decreased susceptibility to CIP amongst NTS isolates in Malaysia. The NTS isolates were resistant to common antimicrobials (ampicillin and trimethoprim-sulfamethoxazole) and this concurs with previous related reports that described high resistance rate of NTS isolates to ampicillin, chloramphenicol and trimetophrim-sulfamethoxazole [23], [29].

Point mutations were identified on the DNA-binding surface of the enzyme near the putative active site tyrosine 122 in N terminal region of the GyrA [12]. Previous studies reported that mutations in *gyrA* QRDR are most commonly found at codons Asp 87 and Ser 83 [25] [30] [31] [32], leading to amino acid substitution associated with the NAL resistance in *Salmonella* strains. However, our findings are unique as novel mutations were observed at codons Asp 47, Asp 51, Asp 73 and Gly 48.

Mutations within QRDR of *parC* and *parE* genes were rarely detected and reported among *Salmonella* isolates [25] [31] [32]. The finding of double mutations in *parE* gene in this study are remarkable, and may be associated with higher level of FQ resistance [30] [31]. In addition to it, *parE* gene mutations were identified in three *S.* Enteritidis isolates that were having *gyrA* gene muta-

tions too. The isolates used in this study were resistant to nalidixic acid but susceptible to ciprofloxacin. These findings are in parallel to previous reports which suggested that multiple mutations are required for higher level of FQ resistance, while single mutation is important for resistance to quinolones [23] [31] [32].

There is a possibility that an isolate of *S.* Enteritidis and *S.* Corvallis are harbouring the PMQR determinants, *qnr* and *aac-6'-Ib-cr* genes, as these isolates were susceptible to NAL with MIC: 16 μg/mL, and increased resistance to CIP (0.5 μg/mL; 0.75 μg/mL). However, another study needs to be carried out to identify and confirm the presence of PMQR determinant genes.

The data of this study impart the rate of nalidixic acid resistance and reduced ciprofloxacin susceptibility among non-typhoidal *Salmonella* clinical isolates in Malaysia. Novel amino acid exchanges found in this collection of isolates provides better understanding towards possible mechanisms that could be responsible for the nalidixic acid resistance and decreased ciprofloxacin susceptibility. Despite small number of *S.* Enteritidis isolates in this study, the results obtained would be an essential reference for epidemiological and public health purposes especially in Malaysia, as well as in Southeast Asia region. Indirectly, this information will be constructive in modifying and deescalating the initial antimicrobial treatment.

In conclusion, the rate of nalidixic acid resistance and reduced susceptibility to ciprofloxacin is considered high in Malaysia. The resistance of *S.* Enteritidis isolates were essentially caused by point mutations in *gyrA* and *parE* genes. Furthermore *S.* Enteritidis is the most frequently isolated serotype causing bacteraemia, as all resistant isolates were isolated from the blood culture. Therefore, regional surveillance of antimicrobial resistance among non-typhoidal *Salmonella* isolates collected from food, animals and humans need to be enhanced to improve the management of invasive bacteraemia, particularly caused by *S.* Enteritidis, and to mitigate the increasing resistance rate of flouroquinolones.

5. Ethics Approval

Ethics approval to conduct the study and review the data was obtained from Universiti Teknologi MARA (UiTM) Research Ethics Committee 600-RMI/ST/ DANA 5/3/Dst (343/2011).

6. Conflict of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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