Original Article

Antimicrobial resistance in non-typhi *Salmonella enterica* isolated from humans and poultry in Palestine

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Abstract

Introduction: The efficacy of chemotherapy can be compromised by drug resistance. This study was undertaken to describe the resistance profiles and fluoroquinolone resistance mechanism of non-typhoidal Salmonella (NTS) isolated from humans and poultry in West Bank, Palestine.

Methodology: One hundred and fifty-one isolates of NTS, obtained from humans (71) and poultry (80), collected between September 2005 and January 2007, were tested for susceptibility to ampicillin, gentamicin, tetracycline ceftriaxone, nalidixic acid and ciprofloxacin. Mutation patterns within gyrA were determined by direct sequencing or by digestion of PCR-amplified DNA fragments with the restriction enzyme HinfI.

Results: Resistance rates among human and poultry isolates were respectively 59% and 51% for ampicillin, 31% and 10% for gentamicin, 59% and 80% for tetracycline, 59% and 45% for nalidixic acid, and 30% and 15% for ciprofloxacin. All the isolates were susceptible to ceftriaxone. Mutations at positions 83 and/or 87 were detected in gyrA of isolates with resistance to nalidixic acid. Isolates which were resistant to nalidixic acid but susceptible to ciprofloxacin had a single gyr A gene mutation at point 87. This gene mutation was sufficient to induce a new phenotype (6 isolates) with decreased susceptibility to ciprofloxacin.

Conclusion: Mutations in gyrA at positions 83 or 87 were the most prevalent mutation pattern of fluoroquinolone resistant NTS isolates but other unknown mechanisms are also present. Continued surveillance of antimicrobial resistance among NTS isolates is needed to mitigate the increasing prevalence of quinolone resistance.

Key words: non-typhi Salmonella enteric; antibiotics; resistance; quinolones, gyrA

J Infect Dev Ctries 2012;6(2):132-136.

(Received 05 May 2010 – Accepted 31 May 2011)

Introduction

Infections with non-typhoidal *Salmonella* (NTS) are a significant cause of illness and death worldwide. About 1.4 million cases are observed in the United States annually, out of which 600 are fatal [1]. NTS are also among the most common causes of invasive bacterial childhood disease [2-3] for which antimicrobial chemotherapy can be lifesaving. Antimicrobial resistance to several classes of traditional first-line drugs has emerged in recent decades. Fluoroquinolones [4-5] are normally used to treat invasive gastrointestinal infections in adults. Unfortunately, NTS with reduced fluoroquinolone susceptibility (> 0.06mg/L) has increased during recent years in many countries [2,4-8].

Animals are the main reservoirs for NTS. The transmission of this microorganism occurs by the consumption of inadequately cooked or pasteurized foods of animal origin, including poultry, beef, fish, eggs, and dairy products [9]. The incidence of human salmonellosis varies with geographic, socioeconomic, and environmental factors [10]. The present study aimed to obtain a snapshot of NTS resistance in West Bank, Palestine. To the best of our knowledge this part of the world has not previously been surveyed for this type of resistance.

Methodology

Bacterial strains and study population

A total of 151 NTS isolates were obtained from humans (71) and poultry (80). The human isolates were collected from children attending outpatient clinics in Bethlehem and Al-Makassed Hospital in East Jerusalem between September 2006 and October 2007. The poultry isolates were provided by the Central Laboratory for Public Health at Ramallah, Palestine, collected between September 2005 and January 2007.

Table 1. Antibiotic resistance among nontyphoid *Salmonella spp.* isolated from clinical and food sources

	%Resistance							
Source	AMP	CHL	CIP	CRO	GEN	NAL	SXT	TE
Human Isolates (n = 71)	59.1	8.4	29.6	0	30.9	59.1	9.9	59.1
Poultry Isolates (n = 80)	51.3	5	15	0	10	45	5	80

Ampicillin (AMP), Tetracycline (TE), Ciprofloxacin (CIP), Nalidixic acid (NAL), Gentamicin (GEN), Chloramphenicol (CHL), Trimethpprimsulfa-methoxazole (SXT), Ceftriaxone (CRO).

Table 2. Patterns of susceptibility to quinolones among non-typhi *Salmonella enterica spp.* isolated from human and poultry

Source of Specimen	Pattern of Susceptibility to Quinolones		
	SS	RS	RR
Human Isolates (71)	27 (38%)	23 (32.4%)	21 (29.6%)
Poultry Isolates (80)	41 (51.3%)	24 (30%)	12 (15%)

SS: susceptible to nalidixic acid and ciprofloxacin, RS: resistant to nalidixic acid but susceptible to ciprofloxacin, RR: resistant to both nalidixic acid and ciprofloxacin.

Antimicrobial susceptibility testing

Antimicrobial susceptibility of NTS isolates was determined using the disc diffusion method according to Clinical Laboratory Standard Institute (CLSI) guidelines [11]. Tested antibiotics were ampicillin (10µg), tetracycline (30µg), ciprofloxacin (5µg), nalidixic acid (30µg), gentamicin (10µg), and ceftriaxone (30µg) (all from Oxoid, Bakingstoke, United Kingdom). According to CLSI guidelines, susceptibility profiles are S >19, I 14-18, R $\leq$ 13 for nalidixic acid and S > 21, 16-20, R $\leq$ 15 for ciprofloxacin. According to CLSI, fluoroquinolone-susceptible strains that test resistant to nalidixic acid may be associated with clinical failure or delayed response in fluoroquinolone treated patients [11].

gyrA *gene amplification, restriction and sequencing*

PCR and restriction of amplicons by *HinfI* enzyme was performed according to the procedure outlined by Kariuki [12]. Briefly, the template DNA was prepared from each strain by boiling a fresh colony in 200µl of sterile distilled water for 15 minutes at 95^{o}C, followed by centrifugation at 14,000 rpm for 2 minutes. PCR reaction conditions consisted of 50 ng of DNA and 100 nM of each primer, GyrA-f*;* ATGAGCGACCTTGCGAGAGAAATTACACCG and GyrA-r; TTCCATCAGCCCTTCAATGCTGATGTCTTC (Syntezza, Jerusalem, Israel) in a buffer composed of 10 mM Tris-HCL (pH 8.3), 50 mM KCL, 1.5 mM $MgCl_2$, 200 µM deoxynucleoide triphosphate mixture, and 1U of Taq polymerase (Promega, Madison, WI, USA) in a final volume of 25 µl. The amplification program was set in the Minicycler TM, (MJ Research, Waltham, USA) to run an initial denaturation of 4 minutes at 94^{o}C followed by 35 cycles, each at 94°C for 30 seconds, 55°C for 30 seconds, and 72^{o}C for 30 seconds, with a final extension step of 72°C for 10 minutes. Restriction was achieved by combining 2 µl *Hinf*I (5 U) (Gibco, New York, USA) with 10 µl PCR product in a total volume of 20 µl buffer and incubating for 1 to 2 hours at 37°C. After digestion, electrophoresis was carried out using 2% agarose at 80 volts for 10 minutes, then at 120 volts for 30 minutes against a 50 bp ladder, viewed on a UV viewer and photographed by a Polaroid camera.

The amplicons containing *gyrA* gene were purified using MinElute PCR purification kit (Qiagen, Hilden, Germany) and the inserts were sequenced by a dideoxy chain termination method on an ABI PRISM Model 301 Sequence Instrument, Foster City, CA, USA at Bethlehem University, Bethlehem, Palestine.

Results

Antimicrobial susceptibility

The results of the antimicrobial susceptibility testing among human and poultry NTS isolates were respectively 59% and 51% for ampicillin, 31% and 10% for gentamicin, 59% and 80% for tetracycline, 59% and 45% for nalidixic acid, and 30% and 15% for ciprofloxacin. All the isolates were susceptible to ceftriaxone. Of the 151 NTS isolates three main susceptibility patterns were identified for quinolones: 71 (47%) were sensitive to both nalidixic acid and ciprofloxacin (zone size $\geq$ 19 mm and $\geq$ 21 mm respectively), 47 (31%) were resistant to nalidixic acid (zone size $\leq$ 13 mm) but susceptible to ciprofloxacin (zone size $\geq$ 21 $\leq$ 13 mm and $\leq$ 15 mm) and 33

Figure 1. Scatter plot showing the relation between the zone diameters (mm) of ciprofloxacin and nalidixic acid for all isolates of non typhoid *salmonella spp*. The vertical and horizontal lines in the scatter plot indicate the recommended CLSI breakpoints for the two antibiotics. Interpretation of zone daiameters (CLSI, M100-S20): Nalidixic acid: S >19, I 14-18, R ≤ 13, Ciprofloxacin: S >21, 16-20, R ≤ 15

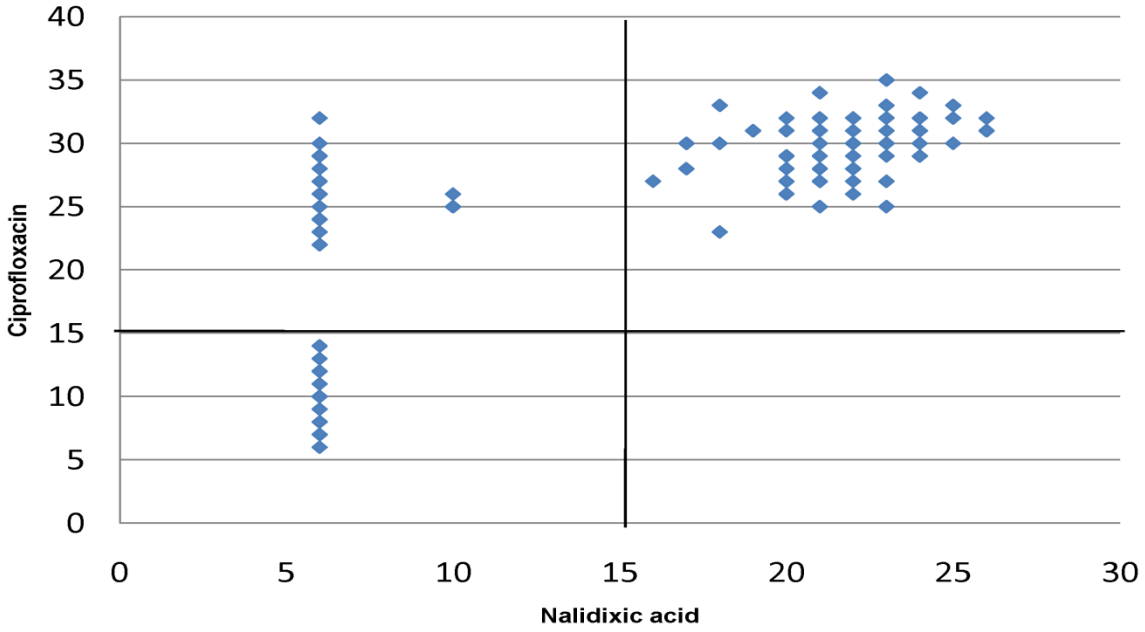
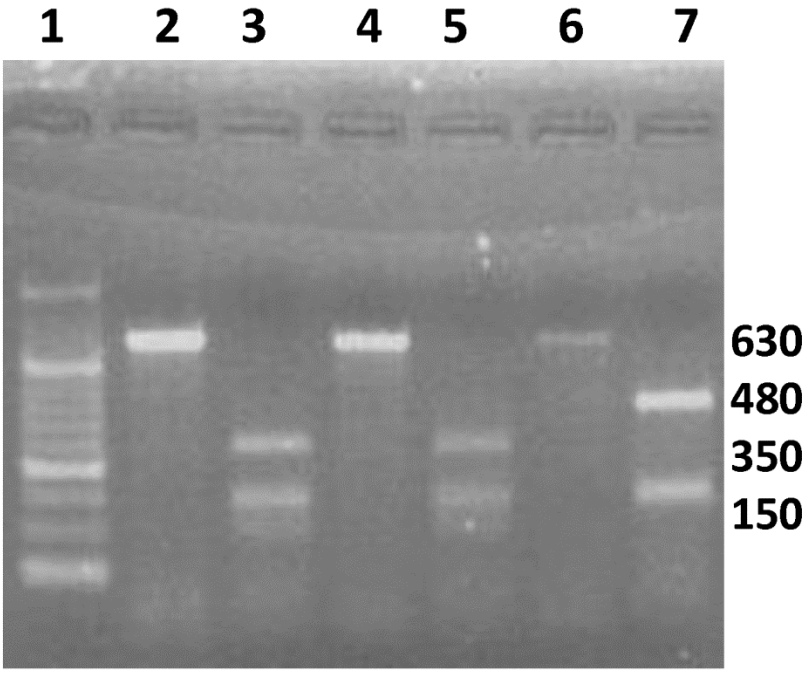


Figure 2. Agarose gel electrophoresis of HinfI restriction fragment length polymorphism: Lane 1, 50 bp ladder DNA; Lane 2, undigested 630-bp PCR product; Lane 3, digests from quinolone sensitive isolate; Lane 4, digests from quinolone resistant isolate



(21.8%) were resistant to both nalidixic acid and ciprofloxacin (zone size $\leq$ 13 mm and $\leq$ 15 mm respectively). Four isolates were intermediate for nalidixic acid (between 15 and 20mm zone size) but remained susceptible to ciprofloxacin. Since NTS is an invasive disease that is treated with fluoroquinolones, a scatterplot was constructed to show the relationship between the zone diameters of nalidixic acid and ciprofloxacin (Figure 1).

PCR products and point mutations in gyrA

The nucleotide sequences of the 630bp DNA fragment corresponding to the *gyrA* gene from nalidixic acid and ciprofloxacin- resistant isolates showed mutations in the codons corresponding to amino acids 83 (TCC to TTC) or 87 (GAC to TAC) or both in comparison with those of the quinolone-susceptible isolates, indicating a serine to phenylalanine substitution and tyrosine to aspartic acid substitution, respectively.

Hin*f1 restriction fragment length polymorphisms*

PCR products for all NTS isolates consistently had the mobility expected for a 630 bp DNA fragment (Figure 1, lanes 2). *Hin*fI digestion was predicted to yield three products of DNA fragments with sizes of 130, 150 and 350 bp. This was seen with DNA from isolates that are sensitive to quinolone (Figure 1, lane 32). However, mutation at the sequence corresponding to amino acid position 83, as found in isolates that are resistant to nalidixic acid and ciprofloxacin, removes one *Hin*fI site so that digestion generated only two fragments with sizes of 480 and 130 bp (Figure 2, lane 4).

Discussion

Antibiotic-resistant NTS, especially those with fluoroquinolone resistance, are increasingly isolated and are a serious problem in many areas. Many strains are multi-drug resistant: Finland [7], Mexico [13], Vietnam [14] and Israel [15] all have reported nalidixic acid resistance rates ranging from 20% to 54%, and even higher prevalence rates have been reported in the Belgium [2]. Data from the present study indicate an extremely high rate: 59.2% and 45% of nalidixic acid resistant NTS among human and poultry isolates respectively in Palestine. However, resistance to nalidixic acid may not predict resistance to fluoroquinolones in NTS, unlike the situation in *Salmonella* Typhi [16]. In this study high-level resistance of NTS to ciprofloxacin was shown in a lower percentage of the isolates: 29.6% and 15% in human and poultry isolates respectively. This rate of resistance to ciprofloxacin is probably, in part, a consequence of the administration of fluoroquinolones to food animals [8] and has major therapeutic implications, insofar as fluoroquinolone resistance is associated with multi-drug resistance [17-18]. Nearly half of the nalidixic acid-resistant isolates were also resistant to at least two or more of: ampicillin, tetracycline or gentamicin. Nevertheless, resistance to ceftriaxone was not related to resistance to other agents. Therefore, ceftriaxone may provide an alternative therapy for use in patient populations likely to be infected with multi-resistant NTS.

Studies on the quinolone resistance determining region revealed that mutation in *gyrA* led to resistance to fluoroquinolone [19]. To address this aspect, *gyrA* genes from quinolone isolates were amplified by PCR, and the sequence variation in the quinolone resistance determining region defined and compared to quinolone-sensitive isolates. Results showed that *gyrA* from the resistant isolates had mutations at codons for amino acids 83 or 87 or both. The first of these mutations led to replacement of serine-83 by phenylalanine, whereas the second mutation led to replacement of aspartic acid-87 by tyrosine. These results are comparable to the findings of other studies conducted in different countries [20-22]. In this study, the strains that were resistant to ciprofloxacin showed mutations at codons for amino acids 83 or 87 or both. Interestingly, a group of nalidixic acid-resistant isolates showed decreased susceptibility to ciprofloxacin (ciprofloxacin zone diameter < 25 mm) with a point mutation in the *gyrA* gene (Figure 1). Isolates resistant to nalidixic acid and ciprofloxacin had two mutations in the *gyrA* gene. Therefore, there must be an association between mutations in *gyrA* and low-level ciprofloxacin resistance. A limitation of the study is that only mutations in *gyrA* were looked at and it is possible, as in *S.* Typhi, that mutations in other topoisomerase genes could also be present [23].

In conclusion, the high frequency of fluoroquinolone resistance among NTS has clearly emerged as a serious problem in Palestine. There is considerable variation in the phenotype of fluoroquinolone resistance which may represent the influence of unknown resistance mechanisms. It is necessary to conduct continuous surveillance of this problem and link the minimum inhibitory concentration and molecular data to clinical outcome to generate accurate data and identify appropriate therapies for specific infections.

Acknowledgment

We are grateful to Dr. Kamel Adwan (Department of Biology and Biotechnology, An-Najah N. University, Nablus, Palestine) for his efforts in reviewing the manuscript and making necessary corrections.

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Conflict of interests: No conflict of interests is declared.