*Brief Original Article*

# Increased quinolone resistance among typhoid *Salmonella* isolated from Egyptian patients

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## Abstract

Introduction: Typhoid fever is endemic in Egypt; and quinolones are the empirical treatment of choice. There are very limited data reporting quinolone resistance among Egyptian typhoidal *Salmonella* isolates. We previously reported that all typhoidal *Salmonella* were sensitive to quinolones. This study aimed to isolate and identify typhoidal *Salmonella* from cases suffering from enteric fever at Minia Governorate, Egypt, determine their quinolone resistance patterns, compare them to those reported 20 years ago, and test *gyrA* mutation as a possible mechanism for quinolone resistance.

Methodology: Stool samples from Widal-positive subjects were screened by culture on suitable media and were identified biochemically. The identified isolates were tested for resistance against three representatives of the first three quinolone generations, namely nalidixic acid (NAL), levofloxacin (LEV), and norfloxacin (NOR). The *gyrA* gene was amplified and sequenced to detect point mutation(s) conferring quinolone resistance.

Results: Out of 230 stool samples (from patients with Widal anti-O titers of ≥ 1/160), 40 isolates were *S. enterica* serovar Typhi (97.5%) and Paratyphi A (2.5%). Six (15%) isolates were resistant to at least one of the quinolones, compared to 0% in 1993. In this regard, 15%, 7.5%, and 2.5% of the isolates were resistant to NAL, both NAL and LEV, and all three quinolones tested, respectively. Sequencing of the *gyrA* gene revealed point mutations at position 83 and/or 87 of the *gyrA* gene only among the resistant isolates.

Conclusion: There has been an increase in quinolone-resistant typhoidal *Salmonella* in Egypt over time.

**Key words:** *gyrA* gene; mutation; PCR; *Salmonella* Typhi; typhoid fever; *Salmonella* Paratyphi; quinolone resistance

*J Infect Dev Ctries* 2014; 8(5):661-665. doi:10.3855/jidc.4111

(Received 09 August 2013 – Accepted 22 November 2013)

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## Introduction

Enteric fever is caused predominantly by *Salmonella enterica* subspecies *enterica* serovar Typhi and Paratyphi A. Typhoid, caused by *S.* Typhi, is considered a major worldwide health problem; there have been 21.6 to 26.9 million cases with at least 250,000 deaths annually [1,2,3]. Typhoid fever is endemic in Egypt. At the time of publication, fluoroquinolones are the antibiotics of choice for treatment [4].

The primary target of fluoroquinolones in *Salmonella* is DNA gyrase, which consists of two subunits, A and B, which are encoded by *gyrA* and *gyrB* [5]. Resistance to fluoroquinolones has emerged in the region and represents a significant threat to typhoid fever treatment [6]. A single point mutation in the quinolone resistance determining region (QRDR) of *gyr*A can mediate the non-fluorinated quinolone (NAL) resistance and reduce susceptibility to fluoroquinolones (*e.g.*, ciprofloxacin [CIP]) [7,8]. Some of the more common point mutations found to be associated with resistance to quinolones in *S.* Typhi occur in the *gyrA* gene at amino acid position 83 and/or 87 [9,10].

The spread of multidrug resistance in countries of high endemicity such as Egypt is very serious [11,12,13,14]. There are limited data, if any, reporting quinolone resistance among Egyptian isolates of typhoidal *Salmonella* [15]. In this regard, we previously reported that all typhoidal *Salmonella* isolated from patients at Minia Governorate were sensitive to quinolones [16]. Here, we isolated and identified typhoidal *Salmonella* from patients suffering from typhoid fever at Minia Governorate, determined their quinolone resistance, compared it to that reported 20 years ago [16], and tested *gyrA* mutation as a possible mechanism for quinolone resistance.

## Methodology

*Isolation and identification of typhoidal Salmonella*

Stool samples were collected from 230 inpatients suffering from fever and attending Minia Fever Hospital between August 2011 and June 2012 (127 females [55.2%]; 103 males [44.8%]). Only 80 (34.7%) of the patients were rural residents, while 150 (65.3%) were urban residents. All patients had positive Widal tests (Biomed, Hannover, Germany) with anti-O titers of ≥ 1/160, and had a clinical picture suggestive of acute typhoid fever. The study protocol was approved by the dean of the Faculty of Medicine and the director of the Fever Hospital. Each subject consented prior to participation. The stool samples were enriched in selenite F broth and subcultured on MacConkey's agar and/or *Salmonella-Shigella* agar (all from Oxoid, Basingstoke, UK) to isolate lactose non-fermenting colonies. The *Salmonella* isolates were identified by Gram stain and biochemical tests (action on triple sugar iron agar, motility, urease production, oxidase, indole, methyl red, Voges Proskauer, citrate utilization, and gelatin liquefaction tests) as previously described [17].

*Antimicrobial susceptibility testing*

The antibiotic sensitivity test to representative members of the first three generations of quinolones was performed using the Kirby-Bauer disk diffusion method [18]. The following antimicrobials were tested: nalidixic acid (NAL, 30 μg), norfloxacin (NOR, 10 μg), and levofloxacin (LEV, 5 μg (Bioanalyse, Ankara, Turkey), and data were interpreted according to CLSI guidelines [19].

*DNA extraction, amplification, and sequencing of gyrA gene*

DNA was extracted from the study samples using a DNA extraction kit according to the manufacturer's instructions (Intron Biotechnology, Gyeonggi-do, South Korea). DNA was used immediately or stored at –20°C. QRDR of *gyrA* gene was amplified by PCR using the following primer set [20]: forward 5' CGGTACACCGTCGCGTACTT 3' and reverse GCCTTTAGGCAGACCGCTTT (Eurofins, Germany). Sequencing of the DNA amplicons was carried out using an automated DNA sequencer (ABI 3100 Genetic Analyzer, Darmstadt, Germany). DNA sequences of amplified regions were analyzed using commercial software (Chromas software, BioEdit, version 7.05). Sequences were compared using basic local alignment search tool (BLAST) analysis with nucleotide sequence database of *gyrA* under accession number AB071870 (*S. enterica* serovar Typhi *gyrA*).

## Results

*Prevalence of S. enterica serovar Typhi and Paratyphi*

Out of 230 subjects with anti-O Widal titers of ≥ 160, 40 (17.4%) isolates were identified as typhoidal *Salmonella* by the biochemical identification of stool isolates. *S.* Typhi was predominant among these isolates (97.5%), followed by *S.* Paratyphi A (2.5%). The prevalence of typhoidal *Salmonella* was 9% and 24% among males and females, respectively, while it was 13% and 26% among urban and rural residents, respectively. The mean age of all the study subjects was 30.7±10.9 years, while the mean age of those having positive *Salmonella* stool cultures was 28.2±13.7 years. The highest isolation rate was in the 11-20 year age group (35%), followed by the 21-30 (20%), 31-40 (15%), and 41-60 (10%) age groups, respectively, suggesting that the prevalence of typhoidal *Salmonella* infection decreases with increasing age.

*Quinolone resistance of typhoidal Salmonellae*

The susceptibility of the 40 typhoidal *Salmonella* isolates to a range of quinolones and fluorquinolones was examined by disk diffusion method. A total of 34 isolates (85%) were sensitive to all tested quinolones; only six isolates (15%) were resistant to NAL, three isolates (7.5%) were resistant to both NAL and LEV, and one isolate (2.5%) was resistant to all three quinolones tested.

*Molecular detection of quinolone resistance mechanism using gyrA sequencing*

Mutations in the *gyrA* gene that confer resistance to quinolones were determined by PCR and nucleotide sequencing of *gyrA*. This revealed mutations at position 83 and/or 87 of the *gyrA* gene (which confers resistance) among the quinolone-resistant isolates, but not among the sensitive isolates (Figure 1). Two examples of resistant isolates (70 and 106) are shown. Isolate 70, which was resistant to all three quinolones, had mutations at positions 83, 87, and 110, while isolate 106, which was resistant to NAL only, contained mutations at positions 83, 95, 110, and 119. Sensitive isolates (190 and 120) did not show mutations at the reported sites that confer resistance. As reported previously [10], a mutation occurring at position 83 of the DNA gyrase changed Ser to Tyr, Ala, or Phe, and a mutation at position 87 changed Asp to Asn or Gly (Table 1) in the resistant strains.

Importantly, both sensitive and resistant isolates had other mutations (at positions 95, 110, and 119) that did not change the amino acids of the protein (Table 1) and did not confer resistance to quinolones as reported [10].

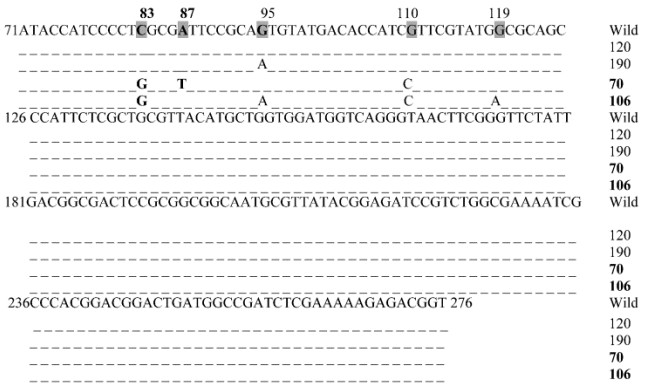
## Discussion

In this study, 40 typhoidal *Salmonella* isolates were collected from the stools of 230 Widal-positive patients suffering from acute typhoid fever, of which 15% were resistant to nalidixic acid compared to 0% in our 1993 study [16]; 7.5% were resistant to both NAL and LEV, and 2.5% were resistant to all three quinolones (NAL, NOR, and LEV). DNA sequencing revealed point mutations at position 83 and/or 87 of the *gyrA* gene only among the resistant isolates; these mutations are known to confer quinolone resistance.

The objective of this study was to test a sufficient number of typhoidal *Salmonella* to examine their resistance to quinolones and to monitor changes over time. The main methods of diagnosing acute typhoid fever in Egypt are leucopenia with relative lymphocytosis [21] and a positive Widal test with a titer > 1/80 [22]. We cultured the stools of 230 patients who had acute symptoms and Widal titers suggestive of acute typhoid fever. Blood culture may have been a more productive sample, but many of our patients refused to donate another blood sample after Widal testing and so, for ethical reasons, we obtained only stool samples for the isolation of typhoidal *Salmonella*.

We found a higher frequency of isolation from females (24%) than from males (9%), which may be attributed to the higher frequency of women than men handling and preparing food in our community, which may make females more likely to get infected than males. These data are in agreement with a previous report from Egypt [4]. However, our data disagree with another report from Egypt [14], where 62% of the cases were males. Another report from the United Kingdom [23] was, also, contradictory to our findings; 55% of adult typhoid cases were males.

**Figure 1.** Nucleotide and amino-acid sequencing of *gyrA* gene revealed mutations at position 83 and/or 87 of the *gyrA* gene among the quinolone-resistant isolates but not the sensitive ones




At the time of publication, fluoroquinolones are the antibiotic of choice for treatment of typhoid fever in Egypt. Resistant typhoid strains, however, have been recently reported [9,24]. There are limited data, if any, reporting quinolone resistance among Egyptian typhoidal *Salmonella* isolates [15]. We showed that NA resistance increased from 0% in 1993 [16] to 15% in this study, which was similar to what was found (15%) in a report from Vietnam [25] and another (16%) from Japan [26]. However, our data were contradictory to the data reported from Nepal (76% resistance) [27], India (51% resistance) [28], the United States (39% resistance) [29], and South Africa (0.05% resistance) [30]. LEV resistance in our study (7.5%) was lower than that reported in Nepal (73%) [31]. Together, our data show there has been an increase in resistance to the fluoroquinolones used to treat typhoid fever. These data suggest that quinolone-resistant typhoidal *Salmonella* increases over time in Egypt. Similar observations have been reported elsewhere [32,33].

Since fluoroquinolones are the antibiotics of choice for treatment of typhoid fever in Egypt; an alternative treatment for patients who had a resistance to quinolones is necessary. Culture and susceptibility

**Table 1.** Amino acid sequence of *gyrA* gene (at the sites conferring resistance) among the quinolone-sensitive and -resistant typhoid *Salmonella* from Minia, Egypt

| Position in *gyrA* gene | 83 | 87 | 95 | 110 | 119 |
|---|---|---|---|---|---|
| Wild type strain AA | Ser | Asp | Pro | Cys | Ala |
| Sample 120 | - - - | - - - | - - - | - - - | - - - |
| Sample 190 | - - - | - - - | - - - | - - - | - - - |
| Sample 70 | **Tyr** | **Asn** | - - - | - - - | - - - |
| Sample 106 | **Tyr** | - - - | - - - | - - - | - - - |

test-guided therapy would be ideal for these patients. Alternative empirical treatments could include azithromycin and newer generations of cephalosporins (*e.g*., cefotriaxone or cefotamine) [12]. In this regard, until recently, there was no resistance among Egyptian isolates of *Salmonella* to cefotriaxone [4]. Also, there are some data from Egypt that show reemergence of chloramephenicol- and ampicillin-sensitive typhoid isolates [34], suggesting that these cheap antimicrobials could be reintegrated as a treatment option for typhoid patients. The concept of recycling old antibiotics, to which the isolates may regain sensitivity, has been recently introduced into clinical practice [35].

This study investigated the association of quinolone resistance with point mutations in the *gyrA* gene of typhoid isolates from Minia Governorate. The sequencing of the PCR-amplified QRDR of *gyrA* of the resistant strains revealed mutations at position 83 and/or 87 of the gene only among resistant isolates, and this was reflected on the protein level. Similar data have been previously reported [36].

In conclusion, there has been an increase in quinolone-resistant typhoidal *Salmonella* in Egypt over the last 20 years. Continuous surveillance for antimicrobial resistance should be continued to provide suitable treatment guidelines for Egyptian patients with typhoid fever. Importantly, improved sanitation and vaccine development, rather than new antibiotics, is a long-term solution to this disease.

## Acknowledgements

We would like to thank the staff members of Minia fever hospital for helping with the collection of samples. Also, we would like to thank Prof. M.A. Elrehany (Biochemistry department, Minia Faculty of Medicine) for his help with the PCR testing.

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**Conflict of interests:** No conflict of interests is declared.