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ORIGINAL ARTICLE

Characterization of Fluoroquinolones-resistant *Salmonella enterica* serovar Typhi in Egypt

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ABSTRACT

Background: *Typhoid fever is endemic in developing countries including Egypt, producing major public health problems with high mortality and morbidity. The emergence of resistant serovar Typhi (S. typhi) to commonly recommended antimicrobials is alarming in developing countries. Fluoroquinolones have been the empirical drug of choice for multidrug-resistant MDR typhoid. However, there have been several alarming reports of fluoroquinolones therapeutic failure in typhoid patients. Resistance of S. typhi to fluoroquinolones commonly results from target site mutation.* ***Objectives:*** *Determination of antimicrobial resistance pattern of S. typhi isolated from Egyptian patients with typhoid fever admitted to or attended Mansoura University Hospitals (MUHs) and Mansoura Fever Hospital, detection of quinolones resistant strains and using PCR-RFLP and sequencing techniques for testing mutation at QRDR of gyrA gene in the isolated strains.* ***Methodology:*** *Blood and Stool samples from clinically suspected typhoid patients were screened by culture on suitable media and were identified biochemically. The identified S. typhi isolates were tested for susceptibility to antimicrobials using the Kirby Bauer disk diffusion method. Minimum inhibitory concentrations of ciprofloxacin were determined by E test. Interpretation of all results was done according to the CLSI guidelines 2015. Mutations in gyrA gene were detected by PCR-RFLP and sequencing methods.* ***Results:*** *Out of 500 blood and stool samples, 57 isolates were S. typhi (96.6%) and only two were S. paratyphi A (3.4%). Of the 57 S. typhi, 80.7% were resistant to nalidixic acid, 50.9% had ciprofloxacin MIC 0.125-0.5 µg/ml and 19.3% had ciprofloxacin MIC >1 µg/ml. Ser 83 mutation in gyrA was detected in 63.1% of the isolates.* ***Conclusion:*** *Increased emergence of fluoroquinolones -resistant typhoidal Salmonella in Egypt which is caused mainly by point mutation at codon 83 (Ser83-Phe substitution TCC→TTC) in QRDR of gyrA gene.*

INTRODUCTION

Typhoid fever caused by Salmonella enterica serovar Typhi *(S. typhi)*, remains as a global and endemic public health problem, especially in developing countries due to lack of personal hygiene and sanitation and limited access to safe water[1]. World Health Organisation (WHO) has estimated 21 million new cases of typhoid occur each year, resulting in approximately 216,000 deaths[2].

In developing countries, its annual incidence ranges from 12 to 622/100,000 persons[3]. Egypt remains a country with intermediate incidence of 13 to 59 per 100,000 cases of enteric fever, below nations such as India and Indonesia which claim more than100 cases per 100,000 persons[4].

Typhoid fever commonly spreads by ingestion of contaminated water or food. The basis of this disease treatment is an antimicrobial therapy[5]. For several decades after its introduction in 1948, Chloramphenicol was the drug of choice but has been set away in many countries because of the emergence of plasmid-mediated resistance and the rare but fatal side effect of bone marrow aplasia [6].

Trimethoprim–sulphamethoxazole and ampicillin were used to cover chloramphenicol resistance in the 1970s, only to be discarded because of the emergence of plasmid-mediated multidrug resistance that covered all three drugs (WHO termed as multidrug resistant [MDR] *S. typhi*) [1,7].

In the 1990s, Ciprofloxacin was used as the drug of choice in treating multidrug resistant strains of *S. typhi*[8]. The fluoroquinolones, including ciprofloxacin, were preferred to third-generation cephalosporins because they were available for oral use, less expensive, low rate of post treatment carriage and rapid clearance of fever and symptoms[9,10].

However, in the past decade, strains of *S.typhi* that show decreased ciprofloxacin susceptibility have emerged due to the improper use of antibiotics in

treating the infectious diseases, and patients infected with them have not responded to fluoroquinolone therapy as previously or have failed to clear organisms in stool cultures [11]. In this situation, third generation cephalosporin group of antibiotic (ceftriaxone) is recommended for the treatment of typhoid [12].

The targets of quinolones and fluoroquinolone action are DNA gyrase and topoisomerase IV. Both are tetramers composed of two pairs of identical subunits, GyrA and GyrB encoded by *gyrA* and *gyrB* in DNA gyrase and ParC and ParE encoded by *parC* and *parE* in topoisomerase IV[13]. Mutations in quinolone resistance-determining region (QRDR) of *gyrA* gene, which is located in the gene region between amino acids 67 and 122, have been responsible for resistance to quinolones[14].

Quinolones resistance has been associated with DNA gyrase and topoisomerase IV alterations. In Salmonella, the most common point mutation found to be associated with resistance to quinolones occur in the QRDR of the *gyrA* gene resulting in substitution at theSer-83 position, often to Tyr or Phe [15,16].

Other mechanisms involved in resistance to these antimicrobials include altered expression of porins or lipopolysaccharide leading to decreased penetration of fluoroquinolones within bacteria, expression of efflux pumps for eliminating the compound from the cell, and presence of the plasmid-mediated Qnr protein, which protects topoisomerase from the action of quinolones[17,18].

PCR-restriction fragment length polymorphism (PCR-RFLP) is one of the molecular methods used for the detection of point mutations within QRDR region of *gyrA of S. typhi*. Following PCR, the amplified DNA is digested with restriction enzyme *Hinf*I, which detects the C-to-T or C-to-A nucleotide change that give rises to the serine-to-phenylalanine or the serine-to- tyrosine substitutions at position 83 respectively [19].

METHODOLOGY

Study design:

The study is a cross-sectional descriptive study that was held on patients clinically suspected as having typhoid fever in different departments of Mansoura University Hospitals (MUHs) and Mansoura Fever Hospital. It was conducted over a period of eighteen months from March 2015 till October 2016.

Study population: This study was carried out on 250 febrile patients admitted to or attended MUHs and Mansoura Fever Hospital wards and outpatient clinics presented with suspected typhoid fever.

Patient's inclusion criteria:

- Patients were selected on the basis of high index of clinical suspicion like continuous high grade fever long-lasting 3 or more days, toxic appearance, abdominal discomfort, relative bradycardia, splenomegaly and hepatomegaly.

Patient's exclusion criteria:

- Febrile patients who were diagnosed for other known febrile illness (such as abdominal abscess, malaria, amebic hepatic abscesses, appendicitis, brucellosis and tuberculosis).
- Also patients who had received antibiotic treatment for their symptoms within two weeks before coming to the hospital were not included in this study.

Clinical samples:

A total of 500 clinical samples were collected including 250 blood and 250 stool samples.

Microbiologic studies:

Samples were processed in Microbiology Diagnostics and Infection Control Unit (MDICU) in Medical Microbiology and Immunology department, Faculty of Medicine, Mansoura University. The collected blood and stool specimens were cultivated on blood agar, MacConkey's agar media, xylose lysine deoxycholate (XLD) agar, salmonella-shigella (SS) agar and eosin methylene blue (EMB) agar plates, after being processed. All agar plates were incubated aerobically at 37°C for 24 hours. Non-lactose fermenting colonies of *S. typhi* were identified by Gram stain, colony morphology. Biochemical reactions as kligler iron agar (KIA), lysine iron agar (LIA), citrate utilization test, urease test and motility-indole-ornithine (MIO) agar were carried out and confirmed by API 20E (Bio-merieux SA, France) [20].

Antibiotic Susceptibility Testing:

Antimicrobial susceptibility of the identified *S. typhi* isolates was performed using disk diffusion method on Mueller–Hinton agar to determine its sensitivity to different antibiotics as recommended by the Clinical Laboratory Standards Institute's (CLSI) guidelines**.** Also the MIC of all the isolates to ciprofloxacin was determined using E test strips (Oxoid, UK), and interpreted according to the CLSI guidelines [21].

Amplification of *gyrA* gene by PCR:

PCR was carried out for all *S. typhi* isolates using primers specific for QRDR of *gyrA* gene.

- ***DNA extraction:*** Genomic DNA extraction was carried out for all isolated *S. typhi* using Thermo Scientific, Gene JET Genomic DNA Purification Kit.
- ***Nucleic acid amplification by PCR:*** *gyrA* gene specific primers were used *(LGC, Biosearch technologies) gyrA* F: 5`- **ATG AGC GAC CTT GCG AGA GAA ATT ACA CCG** -3`/ *gyrA* R: 5`- **TTC CAT CAG CCC TTC AAT GCT GAT GAT GTC TTC** [22]**.** The cycling parameters were as follows: hot start denaturation at 95 °C for 2 min, followed by 35 cycles consisting of template denaturation at 94°C for 45 seconds, primer annealing at 60 °C for 45 seconds, extension

reaction at 72 °C for 40 seconds and final extension at 72 °C for 7 min [16].

- ***Band detection:*** It was done on agrose gel 1.5% on comparison to DNA standard marker: 100 bp DNA Ladder #SMO373 (Thermo Scientific Inc.): expected band size was 630 bp for *gyrA* gene.

Digestion of the PCR amplicons by restriction endonuclease enzyme (*HinfI*): The PCR products of *S. typhi gyrA* isolates were digested with restriction enzyme *HinfI* (Biolab New England):

***Procedure*:**

Four µl of 10X buffer Tango was added to 36 µl of sterile, deionized water to give a final concentration of 1X. One µl (10 units) of the restriction enzyme was added and mixed well. Ten µl of the PCR product was added to the diluted restriction enzyme. The mixture was then incubated at 37°C for 1 hour. For visualization of digestion products, agarose gel electrophoresis was carried out to check for RFLP.

- ***DNA sequencing for QRDR of gyrA gene*** (gifted by Colors Research-Labs, Cairo, Egypt)

To confirm the mutations that were established by RFLP, the QRDR region of *gyrA* gene of certain strains were sequenced using the forwared primer, purified PCR products as the template and the the BigDye Terminator Sequencing Kit *(Applied Biosystems, USA).* DNA Sequence analysis was performed on ABI PRISM® 310 Genetic Analyzer.

Statistical analysis:

Data were collected and statistically analyzed using SPSS (Statistical package for social science) 21. Chi square test was used to compare between groups. P< 0.05 was considered to be statically significant.

RESULTS

Out of 250 the blood samples cultured, *Salmonella spp.* were isolated from 46 blood samples corresponding to an isolation rate of 18.4%. Also only 20 (8%) out of 250 stool samples showed positive culture results for *Salmonella* isolates.

S. typhi was the most frequently isolated serotype (57 strains corresponding to 96.6%) followed by *S. paratyphi* A (2 strains corresponding to 3.4%) as determined by API 20E.

There was a higher frequency of isolation of *Salmonella* from males (78%) than from females (22%) which is statistically highly significant (*P*<0.0005).

Salmonella infections were more prevalent in patients with the age group 11-20 years (50.8%) compared to the other age groups which is statistically highly significant (*P*<0.0005) as shown in table (1).

Table 1: Age distribution among patients with *Salmonella* infections.

Age	Salmonella isolates		*χ2*	*P** Value*
	No= 59	**%**		
≤ 10 years (n= 42)	9	15.3	38.203	<0.0005
11-20 years (n= 72)	30	50.8		
21-30 years (n= 53)	11	18.7		
31-40 years (n= 50)	6	10.1		
> 40 years (n= 33)	3	5.1		

*P<0.0005** Monte Carlo test*

Antimicrobial susceptibility testing of *S. typhi* isolates revealed that the most effective tested antibiotics were ceftriaxone (100%), followed by azithromycin (92.9%), while the least effective antibiotics were tetracycline (3.5%) and ampicillin (7%). Determination of MIC of ciprofloxacin by the E-test revealed that 11 *S. typhi* isolates (19.3%) were resistant to ciprofloxacin, 29 isolates (50.9%) were intermediately resistant and 17 isolates (29.8%) were sensitive to ciprofloxacin.

Amplification of QRDR of *gyrA* gene for all *S. typhi* isolates by PCR produced a 630bp product. In isolates sensitive to quinolone, *HinfI* enzyme cuts the 630bp of *gyrA* gene product at two sites (one of which lies at Ser83) so they showed a *HinfI* restriction pattern I consisting of three fragments of sizes 130, 150 and 350 bp.

However, mutation at the sequence coding for amino acid Ser83, as found in isolates that are resistant to nalidixic acid and ciprofloxacin, disturbs one *HinfI* restriction site, so that digestion generated only two fragments with sizes of 480 and 150 bp (pattern II) as shown in table (2).

Table 2: RFLP characterization of all *S. typhi* isolates in relation to ciprofloxacin MIC.

S. typhi					
ciprofloxacin MIC	**Sensitive (n=17)**	**Resistant (n=40)**	***Total* (n=57)**	***χ2***	***P** Value***
Restriction Pattern I	17	4	21 (36.9%)	41.529	< 0.0005
Restriction Pattern II	0	36	36 (63.1%)		

*P<0.0005** Chi-square test*

This table shows that all ciprofloxacin sensitive *S. typhi* (17) isolates showed restriction pattern I due to the absence of mutation at Ser83 however among the 40 isolated ciprofloxacin resistant *S. typhi*, 36 (26 intermediate resistant and 10 resistant) isolates showed restriction pattern II due to presence of mutation at Ser83. The other 4 ciprofloxacin resistant (3 intermediate resistant and 1 resistant) isolates exhibited restriction pattern I which is attributed to any other mechanism rather than Ser83 mutation.

Looking at RFLP patterns, there was a statistically highly significant difference between ciprofloxacin sensitive and ciprofloxacin resistant *S. typhi* isolates ($P < 0.0005$).

Sequencing results confirmed that ciprofloxacin sensitive *S. typhi* isolates showing restriction pattern I have no mutation at Ser83 (TCC) and ciprofloxacin resistant *S. typhi* strains showing restriction pattern II possess a mutation at codon 83 leading to replacement of serine-83 by phenylalanine (TCC→TTC).

DISCUSSION

Typhoid fever continues to be a serious public health problem, especially in the developing countries of the tropics, due to a combination of factors including poor sanitation, improper sewage disposal and lack of clean water system which cause the transmission of *S. typhi* [23].

The chromosomal-mediated drug resistance phenomenon against fluoroquinolones in *S. typhi* has been reported recently and attributed mainly to a single point mutation in the QRDR of the topoisomerase gene *gyrA*, which encodes DNA gyrase [10].

In our study *Salmonella spp.* were isolated from 46 blood samples corresponding to an isolation rate of 18.4%. Higher blood isolation rate was reported by [24] in India, they found that blood culture was positive in 66% of cases. Higher isolation of *Salmonella* from blood samples was also documented by [20] in Bangladesh, where the blood isolation rate represented 23.3%. This may be attributed to that the endemicity of enteric fever is higher in these regions.

Lower blood isolation rate for Salmonella was detected[25], who found that blood cultures of eleven patients (4.1%) were positive for salmonella species. Also in a study of [26], Out of 1,202 blood samples cultured, only 86 (7.2%) showed positive culture results for *Salmonella* isolates.

Our study also revealed that Salmonella were isolated from 20 stool samples corresponding to an isolation rate of 8%. Higher rates of stool isolation were documented by[27] in Egypt, where 17.3% of stool samples showed positive culture results for *Salmonella* isolates. Higher rates were also described by [28] in Kuwait, they found that the percentage of *Salmonella* isolation accounted 15.5% from human patients' stools.

In contrast, a study of [29] recorded only a rate (3.9%) of Salmonella positive stool cultures but in diarrheal human. This variation among different studies might be due to difference in geographical location, nature of patient population, endemicity of the etiological agents, and seasonal variation.

The low yield of cultures in this study could have been due to the dependence on a single blood and stool cultures results as opposed to multiple cultures that are known to increase culture yields [30]. It was not possible to perform multiple cultures due to follow up of the patients was difficult since most of the patients recruited in the study were outpatients.

Clear in this study that *S. typhi* was the most frequently isolated serotype (57 strains) representing 96.6% followed by *S. paratyphi A* (2 strains) representing 3.4% of all Salmonella isolates.

These results are so close to the results in Egypt, which reported that 97.5% of their Salmonella isolates were *S. typhi*, while 2.5% were *S. paratyphi A*.[27] Also, in Islamabad the cultures positive for *S. typhi* (78.7%) were more common than those positive for *S. paratyphi A* (21.3%) and in these studies *S. paratyphi B* was not isolated[31].

Contrary to our finding, a higher isolation of *Paratyphi A* was reported in India *Paratyphi A* was isolated 1.5 times more than *S. typhi*.[32] Also of the total Salmonella isolates, 64.13% were *S. paratyphi A*, and 35.87% were *S. typhi*. These Variations between studies could be resultant from geographic differences in serotype distribution[33].

In our study, a higher frequency of isolation of Salmonella from males (78%) than from females (22%) was reported, which may be attributed to the more outdoor exposure of males in our community, which may make males more likely to get infected than females.

These data are in agreement with the results in Egypt[34] they rerported a higher rate of Salmonella infection among males (62%) than females (38%). Also, this finding was consistent with the results in Nepal[35], who have also shown higher prevalence of Salmonella infection among males than females accounting for 68%.

However, our data disagree with the report in Bangladesh[36], who mentioned that females (54.25%) were more susceptible to Salmonella infection than males (45.73%).

In the current study, the highest Salmonella isolation rate was in the 11-20 year age group (50.8%), followed by the 21-30 (18.7%), 3-10 (15.3%), and 31-44.5 (15.3%) age groups, respectively, suggesting that enterica highest attack rates occur in school going children and young adults as they may not be having enough caution for their foods and drinking water due to their busier schedule than that of other age groups. This

was also as stated by[37], who isolate Salmonella from the age group (11-20 y) by a percentage of 20%, followed by the 21-30 y age group by a percentage of 16%.

Regarding resistance to ciprofloxacin, 70.2% ciprofloxacin-resistant *S. typhi* isolates was detected by MIC testing in our study, which could possibly explained due to the widespread use of fluoroquinolones in past decades. Such data are going with the results of [38], who found that 70.5% of their isolated *S. typhi* and all (100%) their Salmonella Paratyphi A isolates were resistant to fluoroquinolones.

Our results are also supported by [23,39] but with higher frequencies of ciprofloxacin resistance accounting for 80.4% and 81% respectively.

Conversely, higher rates of ciprofloxacin sensitivity (90% and 78%) were reported[40-41].

Results of this study also displayed 100% sensitivity for ceftriaxone when tested against our isolates. This finding was in agreement with many studies in Egypt which reported that none of their *S. typhi* isolates was resistant to ceftriaxone [34,42]. This means that ceftriaxone remains a viable parenteral choice for treatment of typhoid in Egypt.

Also in another study ceftriaxone was effective for treatment of 52 patients with acute typhoid significantly associated with a shorter time of defervescence[43].

Similarly, in a study to determine the proportion of *S. typhi* isolates with reduced susceptibility to ciprofloxacin from six countries in the Middle East and Central Asia (Egypt, Uzbekistan, Pakistan, Qatar, Jordan and Iraq), nearly all 654 *S. typhi* isolates collected between 2002 and 2007 from Egypt were susceptible to ceftriaxone (99.7%), except for two from Alexandria, which showed intermediate resistance [44].

Regarding PCR-RFLP: Pattern I corresponded to *S. typhi* strains having no mutation at ser83 (wild type) which was observed for all the quinolone sensitive (17) isolates. This is in harmony with many studies that confirmed the absence of mutation at *gyrA* QRDR in quinolone susceptible strains [45-47].

On the other hand Pattern II in our study represents *S. typhi* strains with *gyrA* Ser83 mutation (mutant type) which have been observed in 63.1% (36/57) of all the isolates and in 78.3% (36/46) of the Nalidixic acid resistant clinical isolates of *S. typhi.*

This finding is in line with that previously reported that documented 84% of mutation in the Nalidixic acid resistant *S. typhi* isolates but 92.1% of the isolates had *gyrA* Ser83 mutation[49].

Also analysis of isolates for *gyrA* gene mutations by PCR –RFLP using *HinfI* restriction enzyme for digestion of *gyrA* PCR Product reported that 94% (125/133) of the clinical isolates having mutation at Ser83 with a high level of resistance to nalidixic acid.

In order to confirm mutations associated with resistance to quinolone, sequencing of the QRDR of the *gyrA* gene of specific strains was performed with an automated DNA sequencer using the dideoxynucleotide chain termination method. In this study, the amino acid substitution at codon 83 led to replacement of serine-83 by phenylalanine (TCC→TTC), which was also recorded by [51].

However another substitution at codon 83 led to replacement of serine-83 by tyrosine (TCC→ TAC) was detected in Egypt[27], their tested *gyrA* mutation as a potential mechanism for quinolone resistance in *S. typhi* isolated from stool of Egyptian typhoid patients. Similarly, Ser83-Tyr substitution was also noticed in other studies. [52, 53]

Four isolates were resistant to ciprofloxacin but with no detected mutation at ser83 in QRDR of the *gyrA* gene which proposes other probable resistance mechanisms such as other mutations in topoisomerase genes (*gyrB, parC* and *parE*) or over-expression or decreased permeability of the efflux pumps.

CONCLUSION

In conclusion, these data clearly show increased resistance to quinolones among typhoidal *Salmonella* in Egypt which makes the concept of using fluoroquinolones as first-line drugs for empirical therapy of enteric fever questionable. PCR-RFLP using *HinfI* digestion, provides beneficial and simple method to identify *gyrA* mutation in Salmonella strains that causes very high-level resistance to nalidixic acid and decreased susceptibility to fluoroquinolones leading to ciprofloxacin therapy failure.

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