Research Article

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Journal of Infectious Diseases and Treatment
ISSN 2472-1093

2017
Vol. 3 No. 1: 2

DOI: 10.21767/2472-1093.100029

Changing Trends in Antibiogram and Molecular Analysis of Quinolone Resistant *Salmonella typhi* Isolates in Pakistan

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Citation: Saleem MZ, Arshad A, Qayyum M, et al. Changing Trends in Antibiogram and Molecular Analysis of Quinolone Resistant *Salmonella typhi* Isolates in Pakistan. J Infec Dis Treat. 2017, 3:1.

Abstract

Multidrug resistance is the ongoing burning issue in *Salmonella typhi* around the whole globe. This study was conducted to investigate changing trends in antibiotics resistance and mechanism of resistance against Nalidixic acid among multidrug resistant *Salmonella typhi* strains isolated from Islamabad Capital Territory, Pakistan.

Methodology: Prior to blood sampling, demographic data of patients was recorded. A total of 103 clinical isolates from blood of typhoid patients were identified using microbiological techniques such as colony morphology, Gram's staining and confirmed using standard biochemical techniques. For molecular confirmation, hyper variable region VI of flagellin *fliC* gene was targeted using PCR. Antibiogram of the isolates was tested by Kirby Bauer disc diffusion method using fifteen regularly used antibiotics. Efficacy of antibiotics was evaluated in respect to various seasons, age groups and gender of patients. Relevant genes *gyrA* and *gyrB* were targeted and quinolone resistance determining region (QRDR) of these genes was sequenced to analyze mutations.

Results: Antibiogram study demonstrated that 90.3% isolates were multidrug resistant. 75.7% isolates were sensitive to cefipime while 80.58% were resistant to nalidixic acid. 66.02% isolates were found to be ciprofloxacin resistant exposing reduced susceptibility of *salmonella typhi* to fluoroquinolones. Molecular studies revealed a single point mutation with substitution of serine-83 by phenyl-alanine. This single point mutation seems to be responsible for resistance against nalidixic acid in *S. typhi*.

Conclusion: The incidence of typhoid is high in Islamabad with significant resistance of *S. typhi* isolates against currently administrated antibiotics due to the presence of one point mutation. Therefore, it must be mandatory for the health care professionals to test for the antibiogram before prescribing appropriate antibiotics.

Keywords: Antibiogram; *Salmonella typhi*; Resistance, Susceptible; Antibiotics

Received: February 13, 2017; **Accepted:** February 25, 2017; **Published:** March 09, 2017

Background

Typhoid fever is one of life threatening illnesses. *Salmonella enterica* serovar Typhi commonly known as *S. typhi* is the causative agent of sporadic outbreak of typhoid fever. When left untreated, it results in serious infection among children and adults triggering bacteremia and inflammatory obliteration of organs mainly intestine [1].

Typhoid is most prevalent disease with more than 100 cases per 100,000 persons per year in South central Asia and Southeast Asia [2]. *Salmonella* is responsible for 3 million worldwide deaths, 16 million annual typhoid cases and 1.3 billion gastroenteritis cases [3]. Asian regions are at the hit list of *Salmonella* with almost 80% deaths and cases while rest in Africa and Latin America. The proportion, epidemics and fatality of typhoid fever is larger in

different parts of Southeast Asia, including Pakistan, India, China, Bangladesh, Indonesia, Malaysia, Vietnam and Tajikistan [4,5].

In Pakistan, typhoid fever is 4th major killer disease. In the area of higher transmission and inappropriate treatment, fatality rate of typhoid patients touches 30% instead of 5% common mortality. By using suitable drugs, this exaggeration can be reduced to less than 0.5% [6] but inappropriate use of the antibiotics have led to the advent of multidrug resistant *S. typhi* (MDRST) throughout Pakistan. Eventually, it appears as a leading cause of long-lasting stay in hospitals, additional cost of fitness care and some severe difficulties triggering morbidity and mortality [7,8]. In some cases, these drug-resistant organisms spread up to risky level due to cross infections of hospitalized patients [9].

Mechanism developed antibiotics resistance is due to genetic diversity and adaptability of *S. typhi.* This adaptation is caused by either plasmid acquisition or chromosomal mutations [10,11]. In MDRST, plasmid mediated resistance is more common [12]. These plasmids belong to incompatibility complex group *Inc*HI. These are responsible for resistant gene transfer in bacteria and these are originated from Southeast Asia [13,14].

Due to these MDRST, fluoroquinolone such as ciprofloxacin became first line of drug for action [15-17]. In acute infections, these drugs are positively used to shorten duration of symptoms and fecal elimination of salmonellae. Over the years, resistance has been increased even to quinolones in different parts of the world particularly in Asia [18]. Nalidixic acid (NA) is a quinolone used as a sample for *in vitro* screening tests. Unfortunately, NA resistant strains are displaying reduced susceptibility to ciprofloxacin across the globe [19]. In this case, azithromycin is set as an alternative drug for treatment of typhoid fever but now it has lost its tribute due to resistant strains onsets [20].

Therefore, it is important to study the resistance pattern of *S. typhi* isolates from the patients. At the same time, a lot of effort is needed to find genetic variations causing resistance. Keeping in view the above stated reasons, the basic aim of this research work was to find resistance patterns along with their genetic reasoning in order to lay out future recommendations for proper use of suitable antibiotics.

Materials and Methods

Sample collection

Blood samples were collected from suspected typhoid patients in different diagnostic centers and hospitals. All samples were collected using standard sterile measures. Prior to the blood sampling, age, gender and different clinical/laboratory examination findings were recorded.

Isolation of colonies

Two ml of blood was inoculated into a bottle containing 16.0 ml trypticase soy broth (TSB) and kept at 37˚C for 72 hours. One ml of TSB (BD211768) from primary cultivated sample was poured on both blood and MacConkey agar (Oxoid, USA) plates. The plates were kept at 37°C for 24 hours. The plates which indicated growth were separated. Colonies were taken from these plates and re-cultivated by primary, secondary and tertiary streaking on fresh plates of Mac Conkey agar. Fresh streaked plates were kept overnight at 37˚C.

Microbiological and biochemical identification

All isolates were identified by standard microbiological procedures including colony morphology, Gram staining and were verified by various biochemical reactions specifically triple sugar iron tests. For further confirmation of *S. typhi*, API 20E strips were used.

Molecular identification of isolates

For molecular identification, DNA was extracted by standard Phenol-chloroform method. The reliability of extracted DNA was mediated by agarose gel electrophoresis in which 1% agarose gel was used. Eagle Eye UV trans-illuminator was used to photograph bands showing purity of DNA samples. Furthermore, these samples were analyzed for quality using spectrophotometer. Hyper variable region VI of flagellin *fliC* gene was targeted using two sets of primers **(Figure 1a)**. These primers were ST 1 (5'-ACTGCTAAAACCACTACT-3') and ST2 (5'-TGGAGACTTCGGTCGCGTAG-3') as reported by Song et al. [21] and Frankel [22].

PCR reagents comprised template DNA (5 ng/µL), dNTPs (0.7 nmol/µL), PCR buffer without $MgCl_2$ (10X), $MgCl_2$ (25 mM), forward primer (25 pmol/µl), reverse primer (25 pmol/µl), Taq Polymerase (5 U/µL) and Tris buffer of pH: 8.3 (10 mM). 50 µl volume was attained by adding distilled water to reagents. PCR conditions were used in way of initial denaturing temperature of 94°C for 2 minutes followed by 30 cycles of denaturing temperature of 94°C, annealing temperature of 50°C and extension temperature of 72°C for a minute each and a final extension of 72°C for 5 minutes.

Sensitivity testing

For sensitivity testing, Kirby-Bauer disc diffusion method [23] was performed according to the guidelines of Clinical and Laboratory Standards Institute, formerly National Committee for Clinical Laboratory Standards (NCCLS) [24,25]. In this method, antibiotics ability was tested to inhibit growth of bacteria by forming a zone of inhibition around the disk. Penicillin, carbapenum, cephalosporin, aminoglycosides, macrolides/lincosamides, quinolones, fluoroquinolones and some other miscellaneous groups of antibiotics were applied to petri plates of *S. typhi*. The illustrative drugs among above mentioned antibiotics group were ampicillin (10 µg), imepenum/meropenum (10 µg), cefixime (5 µg), ceftrixone (30 µg), ceftazidime (30 µg), cefipime (30 µg), gentamicin (10 µg), erythromycin (15 µg), azithromycin (15 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), ofloxacin (5 µg), co-trimaxazole (1.25/23.75 µg) and chloramphenicol (30 µg). Isolates were considered as multidrug resistant when resistant to more than two classes of antibiotics at least.

Genes for antimicrobial resistance

The strains found resistant to ciprofloxacin and nalidixic acid were selected for molecular characterization. A large number of genes are reported previously for phenotypic resistance but in this study, *gyrA* and *gyrB* were selected. Primers used for targeting

gyrA **(Figure 1b)** gene were StgyrA-1 (TGTCCGAGATGGCCTGAAGC) and StgyrA-2 (TACCGTCATASGTTATCCACG) as mentioned by Griggs et al. [26]. In the same way, primers for *gyrB* **(Figure 1c)** were StgyrB-1(CAAACTGGCGGACTGTCAGG) and StgyrB-2 (TTCCGGCATCTGACGATAGA) as mentioned by Ling et al. [27].

These genes were amplified by using PCR. 50 µl of reaction mixture contained similar reagents as mentioned for *fliC* gene. PCR conditions were followed in way of initial denaturing temperature of 92°C for 2 minute followed by 30 cycles of denaturing temperature of 92°C and annealing temperature of 62°C for a minute each while extension temperature of 74°C for 2 minutes. Final extension temperature was of 74°C for a minute.

Obtained amplified products were sequenced from Macrogen, Korea. The nucleotide sequence for these genes was blast at NCBI. Proteomic server was used for comparison of nucleotide sequences with reference *S. typhi* str. CT 18.

Statistical analysis

Statistical package for social science (SPSS version 16) was used for statistical analysis in which analysis of variance with interaction was applied to distinguish the impact of antibiotics groups and their representative drugs. Meanwhile, resistance patterns of isolates in terms of different environmental and demographic variables like season, age and gender were also tested.

Results

Epidemiology

In this study, suspected typhoid patients were categorized in different age groups from ≤ 2 years up to 80 as shown in **Table 1.** These were divided into infants, children, sub-adults/adults and post adults. These patients were synchronized for research purpose on the basis of investigated persistence of symptoms.

Isolation strength

Among 103 isolates, 55 were obtained from male patients while remaining 48 were female patients. In this study, 59.2% patients belonged to urban area while remaining 40.8% were rural which revealed typhoid fever to be more common in urban areas of Rawalpindi and Islamabad Capital Territory **(Table 1).**

Drug sensitivity

Six time periods were framed to regulate the consequences of disc diffusion method, performed, throughout the year. Each time constraint comprised a phase of two months. These time frames were designed on the base of seasonal variations of study area to reveal seasonal outbreaks of *S. typhi* strains. These seasons included summer, monsoon, autumn, winter and spring.

After applying all selected antibiotics, it was found that cefipime, a fifth generation cephalosporin, was most effective and sensitive drug to 87 (84.47 %) isolates. 78 (75.73%) isolates were found sensitive to ceftriaxone which had nearly similar outcome of 77 (74.76%) to cefixime. As far as resistance is concerned, 90.3% isolates were confirmed to be MDRST. 83 (80.58%) isolates were resistant to nalidixic acid while 68 (66.02%) out of 103 were found to be resistant to ciprofloxacin and imepenum/meropenum. Nalidixic acid and ciprofloxacin were not suitable drugs because of highest resistance percentage. Cephalosporins were confirmed to be suitable drugs as shown in **Figure 2.**

Statistical analysis for drug sensitivity

By applying statistics, it was proved that resistance to different antibiotics groups was independent of seasons. There was a significant difference ($P \leq 0.05$) between antibiotics group's resistance and seasons **(Figure 3).** A high non-significant difference ($P=0.302$ or $P \geq 0.05$) was professed between antibiotics group's resistance and age groups **(Figure 4).** In the same way, high non-significant difference ($P=0.976$ or $P \geq 0.05$) was also confirmed between antibiotics group's resistance and gender **(Figure 4).**

LSD was applied to weigh peculiar effectiveness and multiple comparisons of drugs with specificity to impact of season and age categories. Cephalosporin was unique group with a high significant difference ($P=0.000$) from other applied groups. Fluoroquinolones were adjac

ent in their actions to penicillin, carbapenum, macrolides and miscellaneous antibiotics ($P \geq 0.05$) while diverse from cephalosporin and aminoglycosides ($P \leq 0.05$). Cefipime, among cephalosporin, was effective drug having significant difference ($P=0.000$) in action from other applied drugs except third generation cephalosporins like cefixime, ceftriaxone and ceftazidime used in Kirby-Bauer disk diffusion method. Monsoon and autumn were the seasons of typhoid outbreak. Results for summer, winter and spring season were similar while monsoon and fall were different from other seasons of the study area in respect to antibiograms of *S. typhi* **(Figure 3).** Excessive antibiotics resistance with high significant difference ($P = 0.000$) was found in patients having age between 10-20 years **(Figure 5).**

Genes for drug resistance

Isolates resistant to nalidixic acid were selected for their molecular analysis to find genetic mechanism of their resistant behavior. The reason behind selection of isolates was their highest resistance to nalidixic acid in the study region. For analysis of quinolone resistance, *gyrA* and *gyrB* was checked and then amplified by using regular PCR. Amplification of *gyrA* was indicated by single product of 347 bps. On the other hand, *gyrB* was amplified showing a single product of 345 bps.

Analysis of sequencing

In this study, a common single point mutation was detected among all selected isolates which was substituting Serine-83 (TCC) with Phenyl-alanine (TTC). This single point mutation is associated with resistance against nalidixic acid in *S. typhi* [28].

Discussion

Typhoid fever remains a major communal health problem in most developing countries such as Pakistan. It is due to factors blend including deprived cleanness and health care set-up. Antimicrobial resistance is a major public health problem generated in *Salmonella typhi*. Some of the reasons of antibiotic resistance include improper use of antibiotics as well as poor access to the doctors. These reasons ultimately boost the intolerable selling practice of antibiotics [29].

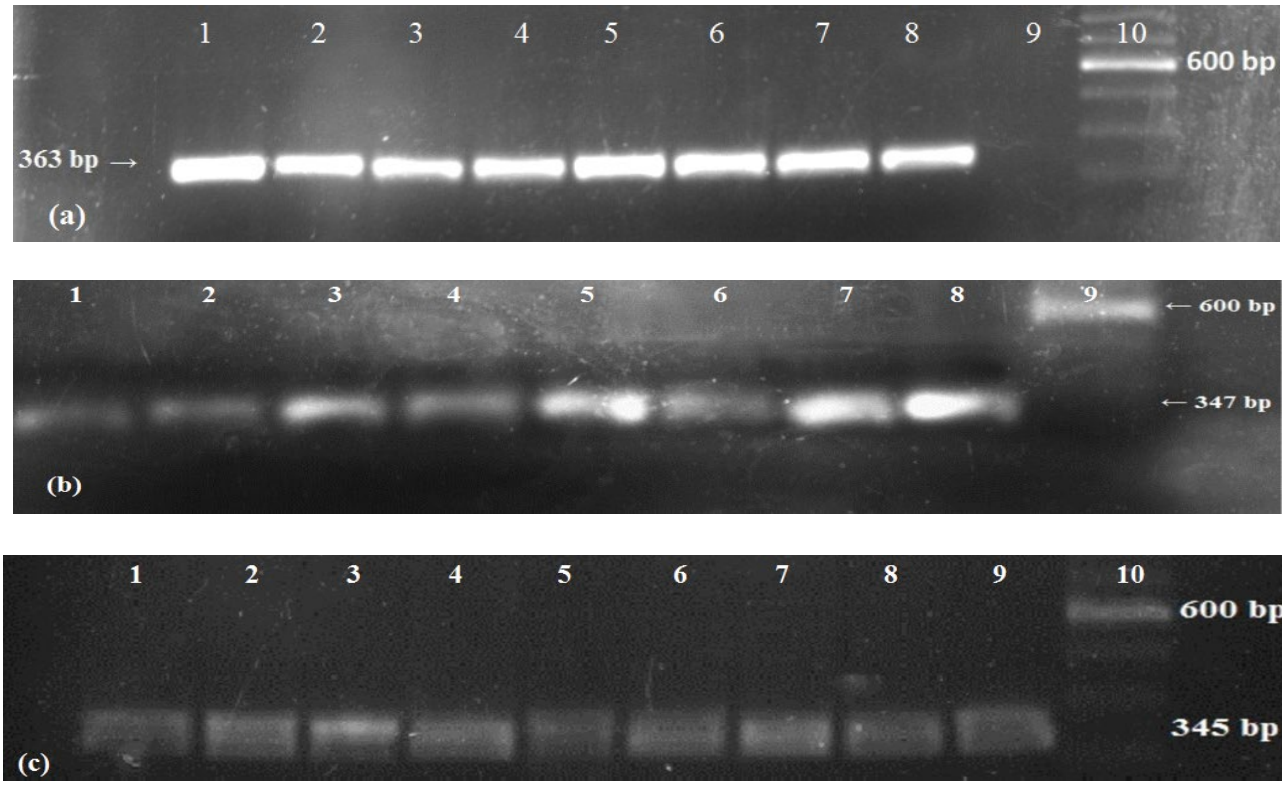

Figure 1 **1a *fliC* gene.** Figure shows molecular identification of *S. typhi*. Lane 1-8: Amplicons of *fliC* gene. Lane 10: Gene ruler (Invitrogen). **1b) *gyrA* gene.** PCR results for *gyrA* gene. Lane 1-8: Amplicons of *gyrA* gene. Lane 9: Gene ruler (Invitrogen). **1c) *gyrB* gene.** PCR results for *gyrB* gene. Lane 1-9: Amplicons of *gyrB* gene. Lane 10: Gene ruler (Invitrogen).

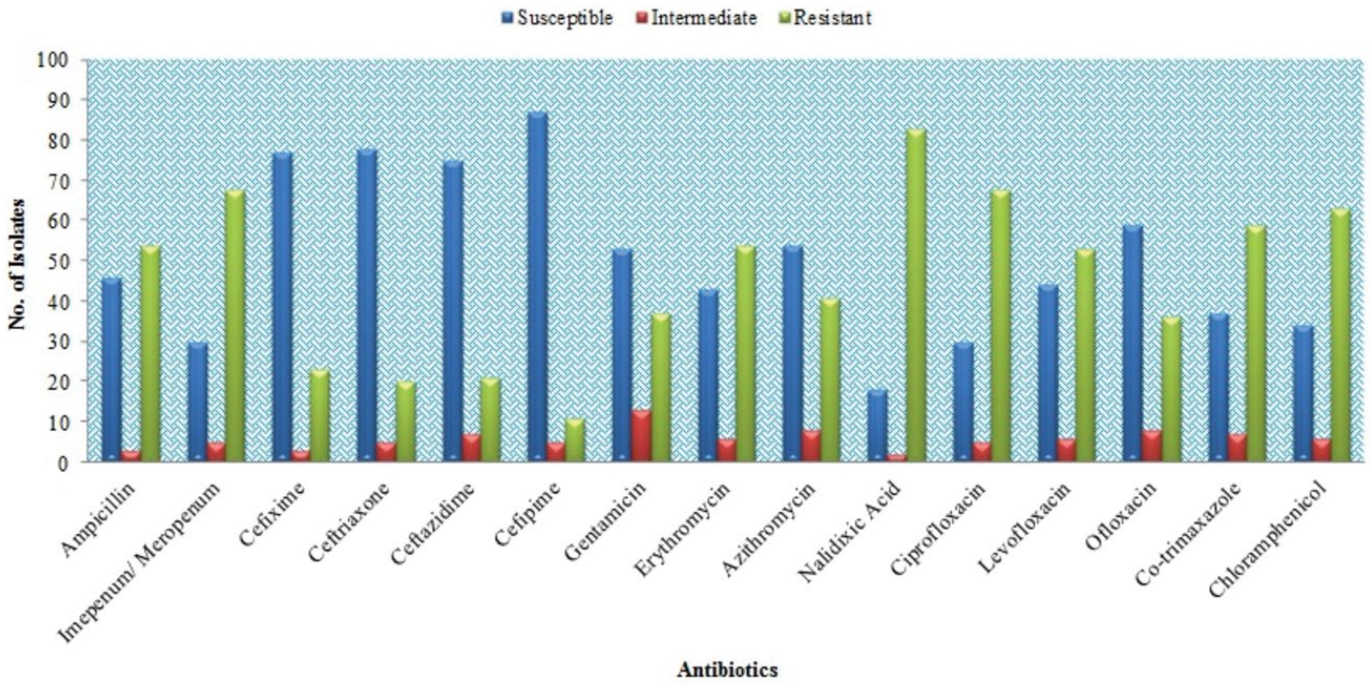

Figure 2 Antibiogram studies of *S. typhi* throughout the year from May, 2014 to April, 2015.

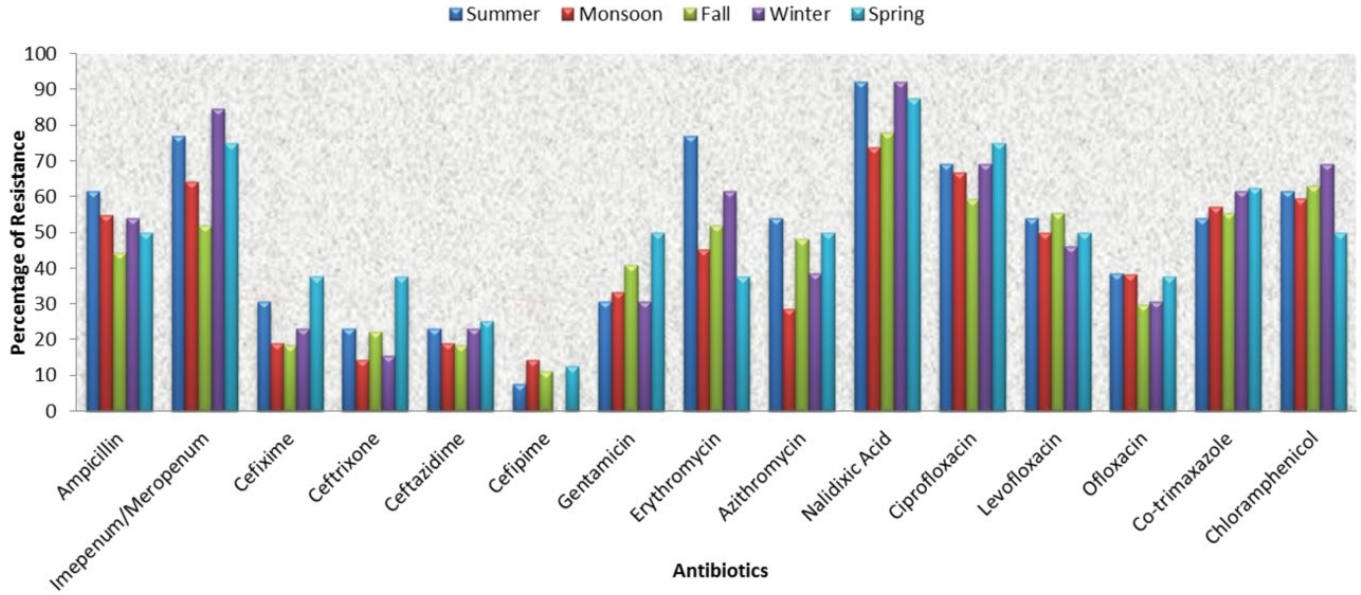

Figure 3 Antibiotics resistant isolates relative to seasons ($P \leq 0.05$).

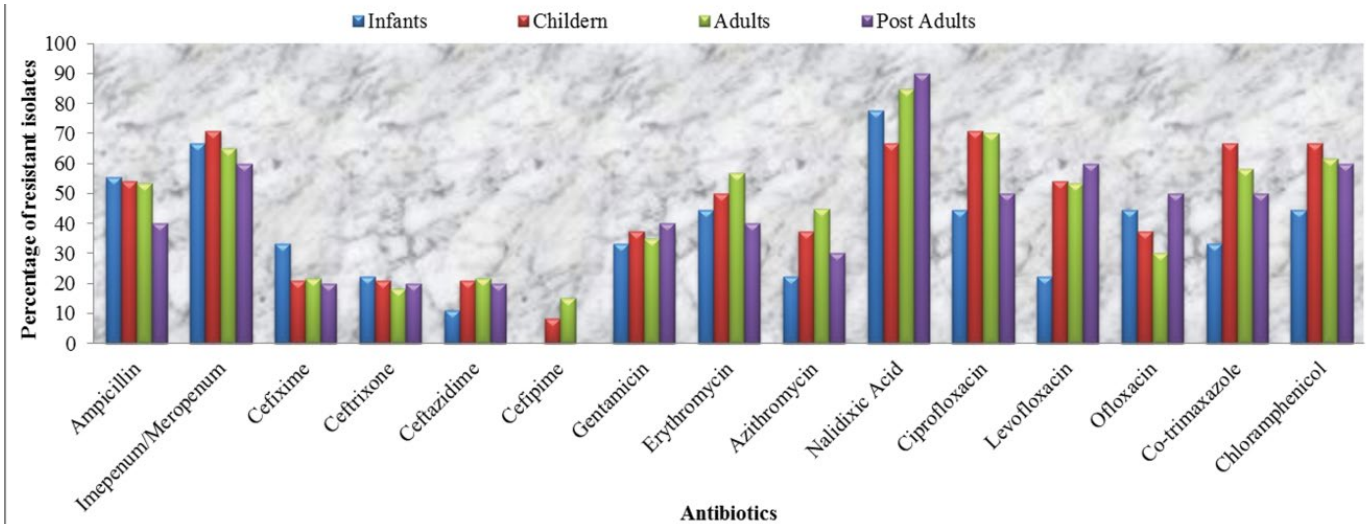

Figure 4 Antibiotics resistant isolates relative to different age groups of patients ($P=0.302$ or $P \geq 0.05$).

There is high burden of antibiotics resistance in Asia. In Bangladesh, resistance to ciprofloxacin and ofloxacin was only 8% in 2000 [12] but 71% resistance patterns were observed in 2005. Resistance to fluoroquinolones reached to 90% in 2009 [30]. In 2014, it was found that nalidixic acid is most inapt antibiotics followed by cefixime, levofloxacin and ciprofloxacin in Bengladesh. Antibiogram study at Gulbarga University, India revealed complete sensitivity of all isolates to imipenum. Highest resistance was against nalidixic acid, ampicillin and chloramphenicol i.e., 31.57%, 29.47% and 28.42%, respectively. 10% isolates were multidrug resistant [2]. In Nepal, 26.4% isolates were confirmed to be MDR in a study from year 2000 to 2005. High resistance of 77% was found against nalidixic acid. 30% isolates were resistant to ampicillin while resistance to chloramphenicol and co-trimaxazole was 27%. No isolate was resistant to ciprofloxacin during the five year study [31]. In a study conducted in China in 2006-2007, a high frequency of antibiotics resistance was recorded against different antibiotics. Trimethoprim resistance was among 96% isolates, similarly, resistance to sulfamethoxazole (95%), ampicillin (95%), gentamicin (95%), streptomycin (91%), ciprofloxacin (96%), nalidixic acid (95%) and chloramphenicol (84 %) was recorded [18].

In 2011, a study was conducted in NIBGE, Pakistan to check antibiograms of *S. typhi*. 30% and 10% isolates were resistant to nalidixic acid and ciprofloxacin respectively. Meanwhile, resistance to cefixime and ceftriaxone were observed in 13.3% and 3.3% isolates respectively [32]. Previously, several dramatic

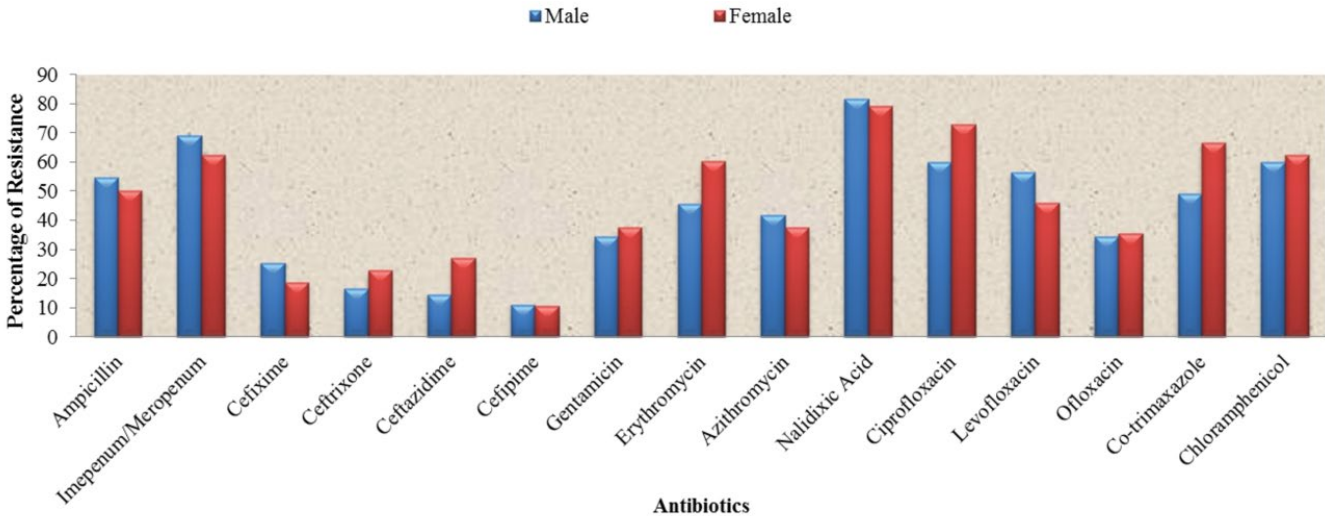

Figure 5 Antibiotics resistant isolates relative to gender of patients (*P=0.976).*

Table 1 Gender, age, locality and socio-economic status of patients.

Age	Isolates		Gender		No. of Patients		Socio-economic Status		
(Year)	No.	%	Male	Female	Rural	Urban	Lower	Middle	Upper
Infants									
≤2	9	8.74	5	4	2	7	1	8	-
Children									
02-Oct	24	23.3	14	10	14	10	10	12	2
Sub adults & adults									
Oct-20	21	20.39	15	6	13	8	12	8	1
20-30	10	9.7	7	3	4	6	3	6	1
30-40	14	13.6	3	11	2	12	4	6	4
40-50	15	14.56	6	9	5	10	4	9	2
Post adults									
50-60	6	5.83	2	4	2	4	2	3	1
60-70	3	2.91	3	-	-	3	-	3	-
70-80	1	0.97	-	1	-	1	-	1	-
Total	**103**	**100**	**55**	**48**	**42**	**61**	**36**	**56**	**11**

changing trends in antibiotics resistance are discussed for typhoid salmonellae in Rawalpindi/ Islamabad. From year 1996 to 2000, no isolates were found to be resistant to fluoroquinolones like ciprofloxacin in a study conducted in Armed Forces Institute of Pathology, Rawalpindi but in session 2001-2003, some of the ciprofloxacin resistant isolates were attained from native population [33].

This study was conducted to evaluate quantitative relation of antimicrobials resistance and their use as well as to assess fluctuating tendencies of antibiotics resistance in *S. typhi.* Among 15 different antibiotics applied, quinolone/fluoroquinolones i.e., nalidixic acid and ciprofloxacin and cephalosporin like cefixime, ceftrixone, ceftazidime and peculiarly cefipime were monitored adequately due to their current alarming status. Other conventional antimicrobials comprised ampicillin, imepenum/ meropenum, gentamicin, erythromycin, azithromycin, levofloxacin, ofloxacin, co-trimaxazole and chloramphenicol.

At culmination of this comprehensive study, nalidixic acid, ciprofloxacin, imepenum/ meropenum, chloramphenicol, co-trimaxazole, ampicillin, erythromycin and levofloxacin were drugs against which more than half of isolates were resistant. Nalidixic acid and ciprofloxacin were observant drugs with highest resistance percentage of 80.58% and 66.02% respectively. An introduced fifth generation cephalosporin, cefipime, was found reliable drug against 87 *S. typhi* isolates. Other third generation cephalosporins were also dependable drugs with less number of resistant isolates. Ceftrixone, cefixime and ceftazidime were suitable drugs for 78, 77 and 75 isolates, respectively. Among 103 samples, 93 were confirmed to be MDR exhibiting 90.2% MDRST isolates. These outcomes have exposed astonishing intensification in MDRST as well as fluoroquinolone resistant *S. typhi* isolates for last ten years in the Islamabad capital territory.

Point mutations in quinolone resistance determining regions (QRDR) of target genes i.e., *gyrA* and *gyrB* is responsible factor

for resistance in quinolone and reduced susceptibility to fluoroquinolones [34]. In *gyrA* gene, the most common reported point mutations are substitutions at Ser-83 position to Ala, Tyr or Phe and substitutions at Asp-87 to Asn, Tyr or Gly [35].

In present study, the basic cause for nalidixic acid resistance was confirmed to be associated with single point mutation with amino acid alteration from Ser83- position to Phe, a similar mutation found at NIBGE, in year 2011. Likewise, it was concluded that MIC of ciprofloxacin needs to be improved.

Conclusions

On the basis of current study, it is concluded that cefipime and third generation cephalosporin like ceftriaxone are suitable drugs to be prescribed instead of fluoroquinolones in Islamabad, the capital territory of Pakistan. It is understandable that the occurrence of infection is apparently high. Blood isolates of *S. typhi* were more resistant to most of the commonly prescribed antibiotics like ciprofloxacin. Therefore it must be compulsory upon the committed medical doctors, clinical microbiologists and public health officials to familiarize themselves with the information of local antimicrobial resistance patterns of *S. typhi*. It would help for directing experimental and pathogen- specific therapy. The proper health education like antibiotic sensitivity testing as well as the accurate remedy and dosage of antibiotics will help to restrict the expansion of antibiotics resistance by *Salmonella*.

Acknowledgements

Authors would like to thank Higher Education Commission, Pakistan for research support. We also thank Islamabad Diagnostic Center and Kulsoom International Hospital, Islamabad for providing support to make this

research effort possible. Meanwhile, authors pay gratitude to NIBGE, Faisalabad for providing their technical support.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

For this type of study formal consent is not required. This article does not contain any studies with human participants or animals performed by any of the authors.

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