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Original Article

Emergence of Plasmid-mediated quinolone-resistant SalmonellaTyphi clinical isolates from Punjab, Pakistan

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ABSTRACT

The present study evaluated the prevalence of fluoroquinolone resistance and the underlying mechanisms among Salmonella Typhi clinical strains from Punjab, Pakistan. A total of 174 Salmonella Typhi strains were isolated from the blood culture samples. The strains identification was done by using the API 20E system and VITEK®, while serovar validation was done by agglutination assays using antisera. Molecular characterization was done by PCR using the primers targeting the fliC-d gene of Salmonellaenterica serovar Typhi. Antimicrobial susceptibility testing was performed by disc diffusion procedure and the minimum inhibitory concentration using the broth microdilution technique. Moreover,plasmid-mediated quinolone resistance genes were amplified through a polymerase chain reaction. Alarming rate of ciprofloxacin resistance (90.8%) were observed with high MICs ranging from <0.06 µg/mL to > 2.0 µg/mL.Overall, the qnrS gene was detected among every ciprofloxacin-resistant isolate, of which maximum frequency of qnrS genes was detected among isolates showing MICs <1.0 µg/mL, while 14 qnrSgene-positive isolates were showing very high MICs values 2.0 µg/mL.Moreover, the ciprofloxacin-resistantisolates, as well as the ten isolates showing intermediate resistance (MIC; 0.5 µg/mL), were negative for qnrA and qnrB genes. This study highlights that the quinolone resistance among S. Typhiis at a critical level that necessitates the need for alternative therapeutic measures and the development of new antibiotics.

INTRODUCTION

Typhoid fever is considered the fourth commonest cause of death inPakistan and remained one of the major bacterial causes of febrile sickness in low and middle-income republics, South Central and Southeast Asia.Currently, it is a significant public health threat in Pakistan and continues to be prevalent around the globe[1].Over the past 30 years,the evolution of antibiotic resistance has thrived against the most frequently used regimens for treating typhoid fever[2].Unfortunately, the increased prevalence of multidrug-resistant (MDR) isolates is limiting the therapeutic value of mostantibiotic groups. Consequently, the choice of antimicrobial therapy is becoming difficult for typhoid treatment due to the endemicity of these superbugs and the resistance to the preferredantimicrobials such asampicillin andchloramphenicol.The increasing rate of resistance to these first-line antimicrobials led to the use of fluoroquinolones in clinical settings[3]. Subsequently, these MDR SalmonellaTyphi strains have become

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increasingly resistant to fluoroquinolones as well and treatment failures following fluoroquinolones therapy have been reported around the globe[4]. The mechanism behind drug resistance is the genetic diversity as well as the adaptability of S. Typhi strains which is either due to the presence of plasmid-associatedmutations in the chromosomal region[5]. It is quite imperative to note that the isolates having the MICs for ciprofloxacin as low as 0.125 µg/mLarelinked with a considerable number of treatment failures[6]. Distinct resistance mechanisms have been defined for the members of the Enterobacteriaceae family.The mutations observed in the quinolone resistance-determiningregions(QRDRs) were believed to be the mechanisms of resistance[7].While plasmid-mediated mechanism, qnrA was initially identified during the late 1990s and later on many other related mechanisms have been exposed comprisingdiverseqnr variants, aac(6=)-Ib-cr, qepA, and oqxAB. These mechanisms of resistance classicallyreducedthe susceptibility of isolates to the ciprofloxacin (MIC range; 0.125 to 1.0 µg/mL) andas well as nalidixic acid (MIC range; 8 to 32 g/ml) [8,9]. Therefore, it is extremely important to evaluatethe antimicrobial-resistant paradigm of S.Typhi, to layout future treatment recommendations for the appropriate use of effective drugs. The present study was planned to assessthe efficacy of fluoroquinolone against SalmonellaTyphi isolates. In this study, ciprofloxacin susceptibilitywas evaluated. Moreover, we also determined the MIC of the indigenous S. Typhi isolates.Moreover, we have tried to delineate the mechanisms underlying fluoroquinolone resistance among the S. Typhi isolates with acquired resistance mechanisms, including PMQRs.

METHODS

Salmonella Typhi Isolates: During the period January-December 2018, a total of 174*Salmonella enterica*(serovar Typhi) strains were isolated from the blood culture samples obtained from the patients suffering from typhoid fever from tertiary care hospital at Lahore (Punjab), Pakistan.The strains identification was done by API20E system (Biomérieux, France) and VITEK® according to manufacturer instruction, while serovar validation was done by agglutination assays using antisera (Denka Seiken Co Ltd, Japan).

Molecular characterization: The FavorPrep™ Genomic DNA Extraction Kit (Favorgen Biotech Corporation) was used for bacterial DNAextraction, the eluted DNA was kept at –20 °C until further testing. For molecular identification, PCR techniquewasused as described previously[10].The amplicons were run on a 1.2% agarose gel having the DNA binding dye i.e.,ethidium bromide and visualized under a gel documentation system.

Antimicrobial Susceptibility Testing: Kirby Baur's disc diffusion procedure was used for antimicrobial susceptibility profiling as per CLSI 2018 guidelines.The ciprofloxacin, levofloxacin and ofloxacin discs (Oxoid, UK) were tested using Mueller-Hinton agar. *Escherichia coli*(ATCC 25922) was used as reference strains for quality control.

Minimum Inhibitory Concentration (MIC): The MICs testing was done by using the broth microdilution technique forciprofloxacin using Mueller-Hinton broth in a microtitration plate. The ciprofloxacin (0.016 to 16 g/ml) was used andthe results were interpreted as per CLSI guidelines. *Escherichia coli* ATCC 25922 were used as reference strains for quality control.

PCR amplification of PMQR: The PMQR genes (*qnr*A, *qnr*B, *qnr*S) were amplified by using the alreadyestablished protocol as described previously[11].

RESULTS

In the present study, typhoid suspects were categorized in different age groups (Figure1), gender-wise male and female were 83(47.7%), 91(52.5%) respectively. Department wise maximum typhoid cases were noticed in OPD 49 (85.2%) followed by peads emergency 44 (25.2%), peads 39 (22.4%), ICU 35 (20.1%), postoperative 5 (2.8%) and emergency 3(1.7%). Alarmingly very high ciprofloxacin resistance was observed against SalmonellaTyphi isolates, 158(90.8%) isolates were resistant to ciprofloxacin, with MICs ranging from <0.06 µg/mL to >2.0 µg/mL; only 10(5.7%) isolates were intermediately susceptible with aMIC of 0.5 µg/mL and only six (3.4%) isolates were sensitive (Table:1). About 75 (43.1%) isolates were ESBL positive. Overall, the qnrS gene was detected among every ciprofloxacin-resistantisolate, of which maximum frequency of qnrS genes was detected among isolates showing MICs <1.0 µg/mL, while 14 qnrSgene-positive isolates were showing very high MICs values 2.0 µg/mL.Moreover,the ciprofloxacin-resistantisolates, as well as the ten isolates showing intermediate resistance (MIC; 0.5 µg/mL), were negative for qnrA and qnrB genes (Table:2).

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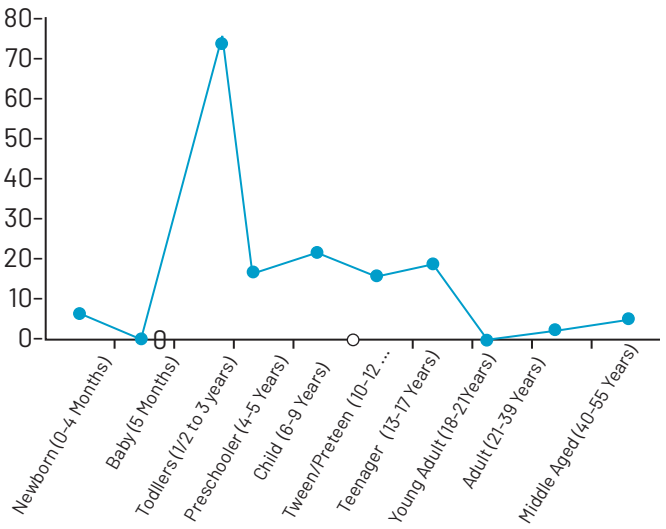

Figure:1 Age group-wise frequency distribution of typhoid cases n=174

	MIC 50 (µg/mL)	MIC 90 (µg/mL)	No. of isolates from which the MIC (µg/mL) were										
			≤ 0.06	0.125	0.25	0.5	1	2	4	16	32	64	≥ 128
MIC of Ciprofloxacin	1	2	6	0	0	10	130	28	0	0	0	0	0

Sensitive(6), Intermediate(10)Resistant(158)

Table 1: The MIC distribution of ciprofloxacin for the 174Salmonella Typhiisolates

Ciprofloxacin Susceptibility	MIC (µg/mL)	Isolates		PMQR resistance determinants		
		Number	%	*qnrA*	*qnrB*	*qnrS*
Sensitive	**≤ 0.06**	6	3.4%	-	-	-
Intermediate	0.125	0	0%	-	-	-
	0.25	0	0%	-	-	-
	0.5	10	5.7%	-	-	-
Resistant	1	130	74.7%	-	-	130
	2	28	16.1%	-	-	28

Table 2: Comparison of MICs of ciprofloxacin with the presence of PMQR genes among Salmonella Typhiisolates

DISCUSSION

The Salmonella enterica serovar Typhi (S.Typhi) is accountable for 3 million deaths and around 16 million typhoid cases/year and 1.3 billion gastroenteritis cases. Unfortunately, 80% of S.Typhi associated morbidity and mortalities occur among the Asian populations,although the burden of disease is increasing across sub-Saharan Africa . The proportion, epidemicsand fatality of drug-resistant strains are increasing in India, Bangladesh and Pakistan. Within Pakistan, in low resource areas fatality rate of typhoid cases reached up to 30% as substitute <5 common mortality. This increased rate of infections can be reduced with good hygienic habits, proper sanitation systems, appropriate and timely treatment of infected patients . Subsequently, inappropriate drug prescribing behaviors, poor hygienic attitudes, lack of good sanitation systems have yielded the emergence of drug-resistant strains into the two largest provinces of Pakistan (Sindh and Punjab). In the past, Salmonellosis was being treated with Ampicillin, chloramphenicol and co-trimoxazole, but currently, fluoroquinolones are widely used.Despite, considerable progress has been in preventing drug resistance, multidrug-resistant(MDR) isolates have emerged, some of which have spread globally.During the period of 1970-1980s, the emergence of MDR SalmonellaTyphi strains started and as a consequence, the WHO in 2003encouraged the clinical use of fluoroquinolones as a most clinically dependable antibiotic for treating MDR typhoid cases. The clinicians were particularly reliant on the fluoroquinolones, however, the extensive fluoroquinolone practice has been associated with the rise of isolates with raised MICs as predicted. Consequently, over the years emergence of fluoroquinolone-resistant S.Typhi strains is being observed across the globe, particularly in Asian countries . The outbreak of fluoroquinolone resistance SalmonellaTyphi isolates has been observed in current and last year and the number is increasing gradually. In this study, alarmingly very high (90.8%) ciprofloxacin resistance was observed against Salmonella Typhi isolates, with MICs ranging from <0.06 µg/mL to > 2.0 µg/mL.Among PMQR genes, it is astonishing that every fluoroquinolone-resistant isolate was qnrS gene-positive, of which maximum frequency of qnrS genes was detected among isolates showing MICs 1.0 µg/mL, while 28 (16.1%)qnrSgene-positive isolates were showing very high MICs values 2.0 µg/mL.Moreover, every ciprofloxacin susceptible isolates were negative for qnrA and qnrB genes. The clinical impact of PMQR is not clear yet, however, as indicated above, isolates showing ciprofloxacin MICs as low as 0.125 g/ml have been frequently associated with treatment failures. Moreover, among patients having Salmonella Typhi infection with the isolates with reduced susceptibility to ciprofloxacin (MICs, 0.125 to 1 g/ml) is reported to increase the duration of the symptoms including fever and is commonly associated with a higher chance of treatment failures as compared to the patients infected with ciprofloxacin susceptible strains (MICs, 0.125 g/ml).Moreover, it was observed that the PMQR mechanisms are likely to provide an advantageous background for the mutational events in the chromosome particularly in the QRDR regions which ultimately lead to high-level resistance

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to the fluoroquinolones . It is therefore recommended that the MIC should be performed routinely to figure out the isolates with decreased susceptibility to fluoroquinolones. After 2012, the breakpoints for disc diffusion and MIC for ciprofloxacin, ofloxacin and levofloxacin were revised although before that were common for all the members of Enterobacteriaceae .The CLSI guidelines have lowered the breakpoint to define resistance against ciprofloxacin for the extraintestinal Salmonella from 4 µg/mL to 1.0 µg/mL. Moreover, the isolates having MIC ranging between 0.125 to 0.5 µg/mL were classified as intermediate. This further stresses the need to do more research on the increased dosage and duration of fluoroquinolones treatment for the management of Salmonellainfections.

CONCLUSSION

This study indicates the emergence of plasmid-mediated resistance against ciprofloxacin among the S. Typhi isolates.The findings suggest that alternative options shall be considered for typhoid treatment.

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