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First Detection of Extensively Drug-Resistant *Salmonella* Typhi Isolates Harboring *VIM* and *GES* Genes for Carbapenem Resistance from Faisalabad, Pakistan

Quratul Ain,[1] Mahnoor Tahir,[1] Amna Sadaqat,[2] Ambreen Ayub,[1] Asad Bashir Awan,[1] Muhammad Wajid,[1] Aamir Ali,[1] Mazhar Iqbal,[1] Abdul Haque,[3] and Yasra Sarwar[1]

Rapid emergence of resistance in *Salmonella enterica* serovar Typhi (*Salmonella* Typhi) against most of the available therapeutic options for typhoid has rendered its treatment more difficult. This study sought to determine the current scenario of antimicrobial resistance in local isolates of Faisalabad following several treatment failure reports. Out of 300 clinical specimens collected in 2018, 45 isolates were identified as *Salmonella* Typhi. To assess changes, we compared their antibiogram profile with 31 *Salmonella* Typhi strains isolated in 2005. The isolates collected during 2018 showed a significant rise in antimicrobial drug resistance as compared with isolates revived from the cultures of 2005, including 15 multidrug-resistant (MDR), 20 extensively drug-resistant, and 14 pan drug-resistant isolates compared with only 8 MDRs from 2005. Notably, in 2018 isolates, resistance to azithromycin was seen in 75% of the isolates. Extended-spectrum beta-lactamase production was detected in 47% of *Salmonella* Typhi isolates and 18% isolates showed resistance against carbapenems. The sequences of two carbapenemase genes, VIM and GES, found in *Salmonella* Typhi were submitted in NCBI. The carbapenem resistance is rare in Enterobacteriaceae and probably first time reported in *Salmonella* Typhi. H58 haplotype was identified in the 2018 *Salmonella* Typhi isolates and PCR–restriction fragment length polymorphism method identified 16.7% of H58 strains that belonged to lineage I, 19.4% of H58 strains that belonged to lineage II, and the remaining 63.9% that belonged to the node. The regional difference in the antimicrobial resistance trend needs effective epidemiological studies.

Keywords: multidrug resistance, extensive drug resistance, pan drug resistance, efflux pump, H58 subtyping

Introduction

Typhoid is a potentially fatal systemic illness caused by *Salmonella enterica* serovar Typhi (*Salmonella* Typhi). Approximately, 21 million cases are reported each year in low- and middle-income countries. In Pakistan, its incidence is increasing drastically, with emergence of drug resistance making the situation difficult to control.[1] The endemicity in the developing countries may be credited to different causes, including poor cleanliness and unwise utilization of antibiotics and inadequate sustainability of vaccines.[2]

Salmonella Typhi started to show resistance to all first-line drugs in 1974 in Vietnam,[3] and subsequently in different parts of the world.[4] Multiple drug resistance in *Salmonella* Typhi has been recorded in Pakistan since 1988, which has gradually attained significantly high proportions.[5] The antimicrobial resistance has spread because of the clonal dispersal of individual multidrug-resistant (MDR) *Salmonella* Typhi isolates or from the exchange of the plasmid to different *Salmonella* Typhi isolates.[6] The exposure of bacteria to antibiotics may result in the development of resistance during the course of treatment.[7]

Fluoroquinolones, particularly ciprofloxacin, have been extremely effective in fighting typhoid for over a decade, however tragically, decreased susceptibility and resistance in clinical isolates have developed now.[8] Decreased

[1]National Institute for Biotechnology and Genetic Engineering College, Pakistan Institute of Engineering and Applied Sciences (NIBGE-C, PIEAS), Faisalabad, Pakistan.
[2]Pediatric Medicine Unit, Allied Hospital, Faisalabad, Pakistan.
[3]Akhuwat Faisalabad Institute for Research in Science and Technology, Faisalabad, Pakistan.

susceptibility to the fluoroquinolones is linked typically to the point mutations in *gyrA* and *gyrB* encoding DNA gyrase, and with a lesser frequency in *parC* and *parE* encoding DNA topoisomerase IV.[9,10]

After the increase in the ciprofloxacin resistance in *Salmonella* Typhi, cephalosporins, particularly ceftriaxone and cefixime, have been widely utilized for the treatment of typhoid fever.[11] In spite of the fact that resistance to the third-generation cephalosporins in nontyphoidal *Salmonellae* had been accounted since 1989, the resistance in *Salmonella* Typhi is still on the rise. The main instances of decreased susceptibility or ceftriaxone resistance were reported in Sindh, Pakistan in 2018.[12]

For the clinical treatment of infection caused by Enterobacteriaceae, particularly by the MDR isolates producing extended-spectrum beta-lactamases (ESBLs), carbapenems such as imipenem, meropenem, and ertapenem, are the best antimicrobial agents.[13] But in recent years, incidence of carbapenem-resistant Enterobacteriaceae (CRE) is rising as the use of carbapenems has become common in clinical practice.[14] Since most of the carbapenem-resistant bacteria generally resist numerous commonly used antimicrobial agents, they have progressed toward becoming pan-resistant isolates, thereby representing an incredible danger to the life of patients.[15] Consequently, it is of paramount importance to understand the mechanism of drug resistance development in carbapenem-resistant Enterobacteriaceae isolates and to adequately control the subsequent nosocomial disease.

In underdeveloped countries, genome sequencing and single nucleotide polymorphism (SNP) typing are labor-intensive and expensive methods for regular use in molecular biology laboratories. That is why, we used inexpensive PCR–restriction fragment length polymorphism (PCR-RFLP) procedure to identify H58 *Salmonella* Typhi isolates and subtyped them based on the occurrence of particular deletions and SNPs between lineages I and II.[16]

Antimicrobial resistance emerges usually due to the presence or mutations of specific genes. The most commonly reported genes related to resistance have been accounted for each group of antibiotics.[17,18] The current study focused on exploring molecular mechanisms of increasing resistance against various generations of quinolones/fluoroquinolones and cephalosporins, whereas production of ESBLs and resistance to carbapenems, azithromycin, and aztreonam were also investigated. We have checked the current status of antimicrobial resistance, with nature/type and rate of resistance in local isolates from Faisalabad District (a population of >10 million) by comparing with 13-year-old culture stocks. We have also used a PCR-RFLP approach to identify and subtype H58 *Salmonella* Typhi. This study revealed a significant paradigm shift in nature and extent of antimicrobial resistance. The generated data may contribute in future planning to effectively manage the life-threatening *Salmonella* Typhi infections.

Methodology

Sample collection

Three hundred blood samples of suspected typhoid patients from local hospitals of Faisalabad District were collected in sterile culture vials. These samples were collected during 2018 starting from March until October. A questionnaire cum proforma approved by the Institutional Ethics Committee was used for recording the history of patients along with their consent. The inclusion criteria for the selection of patients were fever for 3–20 days and showed any three or four of the following symptoms: enlarged spleen, headache, rose spots, malaise, abdominal discomfort, constipation and if untreated, followed by diarrhea, fatigue, delirium, and agitation. Patients belonged to both sexes and all ages. The blood samples were cultured in tryptic soy broth (TSB) medium (Oxoid, United Kingdom); 2 mL of blood soon after collection was added in 16 mL of TSB (1:8) without any anticoagulant and incubated at 37°C for 48–72 hours. A loopful culture from this mixture was streaked on MacConkey's agar (Oxoid) and *Salmonella Shigella* agar (Oxoid). Based on colony morphology, the bacterial isolates from the agar plates were subjected to biochemical identification.

Thirty-one clinical isolates of *Salmonella* Typhi collected during 2005–2008 were revived from the glycerol stock cultures already available in National Biological Resource Center, the culture pool of National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad. These bacterial isolates were preserved in 30% glycerol and were kept at −20°C for further use.

Biochemical identification and molecular confirmation of Salmonella *Typhi isolates*

Freshly isolated cultures were subjected to biochemical testing by inoculating triple sugar iron agar (Oxoid) slants using stab and streak method. The agar slants were incubated at 37°C for 24 hours and the results were interpreted according to manufacturer's guidelines. These isolates, and the archived *Salmonella* Typhi isolates, were revived from stocks, and harvested for DNA extraction by phenol:chloroform method.[19]

The quality and quantity of DNA was analyzed by agarose gel electrophoresis and Nanodrop. These isolates were confirmed by regular and nested PCR targeting *fliC-d* gene, using the PCR conditions as previously reported.[20]

Antimicrobial sensitivity testing

Antimicrobial susceptibility testing was done by Kirby-Bauer disc diffusion method and interpreted according to the Clinical Laboratory Standard Institute (CLSI) guidelines.[21] Briefly, the 6-hour bacterial growth having optical density equal to ~0.5 McFarland standard was spread on Muller–Hinton agar (Oxoid) plates and the antimicrobial discs were aseptically placed. The plates were incubated at 37°C for 18–24 hours and the zones of bacterial growth inhibition were interpreted according to CLSI guidelines.

The antimicrobials were selected on the basis of clinical use and structural similarities. Among the 15 drugs tested, there were 6 cephalosporins (cefoperazone, ceftriaxone, cefixime, cefepime, ceftazidime, and cefotaxime), 4 fluoroquinolones (nalidixic acid, ciprofloxacin, pefloxacin, and levofloxacin), representing first, second, and third generations, three carbapenems (imipenem, meropenem, and ertapenem), one monobactam (aztreonam), and one macrolide (azithromycin).

Determination of minimum inhibitory concentration using E *test*

Minimum inhibitory concentration (MIC) is the lowest concentration of an antibacterial agent required to completely inhibit the visible growth of bacteria. It is determined by using different antimicrobial concentrations either by serial dilution method or *E*-test, which utilizes strips impregnated with a predefined concentration of antibiotic. The *Salmonella* Typhi isolates, which exhibited an intermediate resistance behavior against clinically important antimicrobials, ceftriaxone, cefotaxime, ciprofloxacin, and levofloxacin, were tested for MIC of these drugs. The MIC was determined by using *E*-test (Oxoid). Briefly, a strip having a gradient of antimicrobial concentration is applied on Muller–Hinton agar plate having bacterial inoculation. After overnight incubation, the results were interpreted according to the reference of CLSI.[21]

Screening for ESBL phenotypes

Phenotypic identification of ESBL-producing *Salmonella* Typhi isolates was performed according to CLSI guidelines for combination disc method.[22] Three combinations of antimicrobials were used, ceftazidime alone and with clavulanic acid; cefpodoxime alone and with clavulanic acid; and cefotaxime alone and with clavulanic acid. An isolate showing enhanced zone of ≥5 mm with antimicrobial combined with clavulanic acid as compared with a zone of antimicrobial alone was phenotypically designated as ESBL producer.[23]

Genotypic analysis of antimicrobial resistance

Salmonella Typhi exhibits antimicrobial resistance due to the acquisition of a variety of genetic components localized either on plasmid or bacterial chromosome, or mutations in some genes. A number of genes responsible for conferring resistance to bacteria have been identified and reported. In this study, for genotypic analysis of antimicrobial resistance, the most commonly reported drug resistance genes for each group of antimicrobials were selected and PCR was performed targeting *gyrA*, *gyrB*, *parC*, *parE*, *qnrB*, *qnrD*, and *aac(6′)-lb-cr* genes for fluoroquinolones, $bla_{TEM,}$, bla_{SHV-1}, bla_{CTX-M1}, $bla_{CTX-M15}$, and $bla_{CTM-M14}$ genes for cephalosporins; *VIM*, *NDM*, and *GES*, genes for carbapenems; and *mphA*, *ereA*, *mefA*, *mphB, msrA*, *ermA*, and *ermB* genes for azithromycin. Respective primers are given in Supplementary Table S1.

Generally, 25 μL of DNA amplification mixture contained 2.5 μL of 10 × Taq Buffer, 1.5 μL of 25 mM $MgCl_2$, 1 μL of 10 pmol of each primer, 0.75 μL of 2.5 mM dNTP, 0.2 μL of Taq polymerase, 5 μL (2–10 ng) of template, and distilled water to make up the volume of 25 μL.

PCR conditions were as follows: initial denaturation at 95°C for 5 minutes and 30 cycles of denaturation at 95°C for 1 minutes, annealing temperature as mentioned in Supplementary Table S1 for each primer set for 1 minute and extension at 72°C for1 minute, with a final extension step at 72°C for 10 minutes using SuperCycler SC-200 (Kyratec, Korea). A non template control was included in each run. The PCR products were analyzed by gel electrophoresis on 1.5% agarose stained with ethidium bromide (Et-Br) under UV transilluminator.

Sequence-based confirmation of drug resistance genes

Amplified products of the targeted drug resistance genes were purified using the PCR purification kit (Thermo Scientific GeneJET PCR Purification Kit, Cat No. K0701) following the manufacturer's instructions and sent for commercial DNA sequencing to Macrogen (Republic of Korea). The nucleotide sequences obtained were blasted on NCBI for confirmation of drug resistance genes and mutations were identified for genes responsible for resistance.

Phenotypic detection of efflux activity by Et-Br cart wheel method

Efflux pumps are considered among one of the possible resistance mechanisms of bacteria against antimicrobials. Efflux pump activity can be phenotypically detected by using Et-Br-agar cart wheel method[24] to undermine the resistance mechanisms involved in the antimicrobial resistant isolates.

In this procedure, two sets of tryptone soy agar (TSA) plates were made with the addition of Et-Br having different concentrations in both the sets. One set contained 1.5 mg/L Et-Br, whereas the other set contained 2.5 mg/L of Et-Br. After preparation of TSA plates, bacterial cultures, including test isolates and positive control, whose turbidity was equal to 0.5 McFarland standard, were taken and streaked from middle to the edge of TSA plate as a single straight line. The plates were then incubated for 16 hours at 37°C. After incubation, the plates were examined under gel documentation system.

Confirmation by carbonyl cyanide m-chlorophenyl hydrazine method

Carbonyl cyanide m-chlorophenyl hydrazine (CCCP) method was used for the confirmation of efflux pump activity. In this procedure, Mueller–Hinton agar plates were prepared. Two representative antibiotics (ciprofloxacin and ceftazidime), one from each group of fluoroquinolones and cephalosporins were chosen. Similarly, representative isolates from resistant and susceptible groups were chosen for this procedure. Bacterial cultures whose turbidity was equal to 0.5 McFarland standard was swabbed on Mueller–Hinton agar plates. Antibiotics were placed in duplicates on these plates. Ten microliters of CCCP whose concentration was 20 mg/L was dispensed on one of the antibiotics on the plate, which worked as efflux inhibitor. The plates were then incubated for 16 hours at 37°C. After incubation, zones of inhibition were measured. A difference of ≥5 mm in zone diameters between discs with and without CCCP was taken as positive for efflux pump-mediated resistance.[25]

Genotypic detection of efflux pump genes

Efflux-mediated drug resistance and its distribution among isolates of *Salmonella* is significant to completely understand the processes that happens in a process of natural selection for resistance of antibiotics.[25] For the identification of the most common efflux pump genes, PCR was performed, using the protocol mentioned in the previous section. These include *armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, *npmA*, *qnrB*, *qnrD*, and *aac(6′)-lb-cr*. Respective primers and their annealing temperatures are given in Supplementary Table S2.

Identification and subtyping of H58 haplotype in Salmonella *Typhi isolates*

H58 haplotype is globally associated with multidrug strains of *Salmonella* Typhi. To discriminate the *Salmonella* Typhi isolates in this study, the detection and subtyping of H58 haplotypes was performed by using the protocol reported by Murgia et al.[16] Briefly, as the first step for screening for H58 haplotype, the primers used for the detection of H58 and non-H58 strains were H58 F (5′-GCA GGCAAAATCGAAATCAG-3′) and H58 R (5′-CAAACC GTTGAATCGGAAGT-3′) with an amplification product of 107 bp.

In the second step, the subtyping of H58 *Salmonella* Typhi was performed by PCR-RFLP only on the isolates, which belonged to H58 haplotype and were positive by first PCR. Three SNPs for RFLP analysis (in this study named H58/Ia, H58/Ib, H58/II) were detected to divide H58 *Salmonella* Typhi strains in three groups corresponding to lineage I, lineage II, and node of the haplo group. Three sets of primers were used to amplify the regions (Supplementary Table S3). PCR conditions were same as reported for H58 primers with 62°C annealing temperature.

Statistical analysis

Data were analyzed using SPSS software. The chi-square test value and 95% confidence interval were found for the comparison of drugs and resistance gene profiles of antimicrobials to check any significant differences between two batches of the collected isolates. p-Value was calculated through chi-square test and was considered significant if <0.05.

Results

Isolation and identification of Salmonella *Typhi*

Forty-five isolates of *Salmonella* Typhi were recovered by culturing the blood samples of suspected typhoid patients admitted to Allied Hospital, Faisalabad; Basic Health Unit, Jaranwala; and Children Hospital, Faisalabad, Pakistan, and confirmed by PCR. All of 31 *Salmonella* Typhi cultures from the stocks were also confirmed as *Salmonella* Typhi by PCR.

Antimicrobial susceptibility profile

Isolates revived from the NIBGE culture stocks (2005–2008) were found mostly susceptible to the used drugs (Fig. 1). All (100%) of these isolates showed resistance against nalidixic acid, 30% were found resistant to pefloxacin, and 6.4% were resistant to ceftazidime. Only one isolate was resistant to azithromycin, whereas 61.2% of the isolates showed intermediate resistance toward ciprofloxacin as mentioned in Table 1.

Recent isolates (2018) exhibited a significant increase in resistance patterns as compared with previous isolates. Resistance to cefotaxime, cefixime, ceftazidime, cefepime, cefoperazone, and ceftriaxone was observed in 25%, 20%, 16%, 13%, 11%, and 2% of the isolates, respectively. Notably, resistance to azithromycin was seen in 75% of these isolates. The resistance against azithromycin and ceftriaxone was contrary to the extensively drug-resistant (XDR) *Salmonella* Typhi clone reported from Sindh indicating the regional differences in bacterial drug resistance behavior. Pefloxacin was ineffective against 86% of the isolates. An overall increase in the resistance was also observed toward fluoroquinolones as the isolates resistant to ciprofloxacin, ofloxacin, levofloxacin, and aztreonam were found as 45%, 35%, 31%, and 20%, respectively. Like the previous isolates (2005–2008), all of the 2018 isolates showed 100% resistance to nalidixic acid (Table 1).

The important finding is that a considerable number of these isolates demonstrated varying degrees of intermediate resistance against different drugs indicating their potential to attain complete resistance. The intermediate resistance against cefepime, ceftazidime, levofloxacin, ciprofloxacin, cefoperazone, cefixime, cefotaxime, aztreonam, ofloxacin, and ceftriaxone, was detected as 71%, 55%, 53%, 53%, 51%, 28%, 27%, 27%, 17.7%, and 13%, respectively.

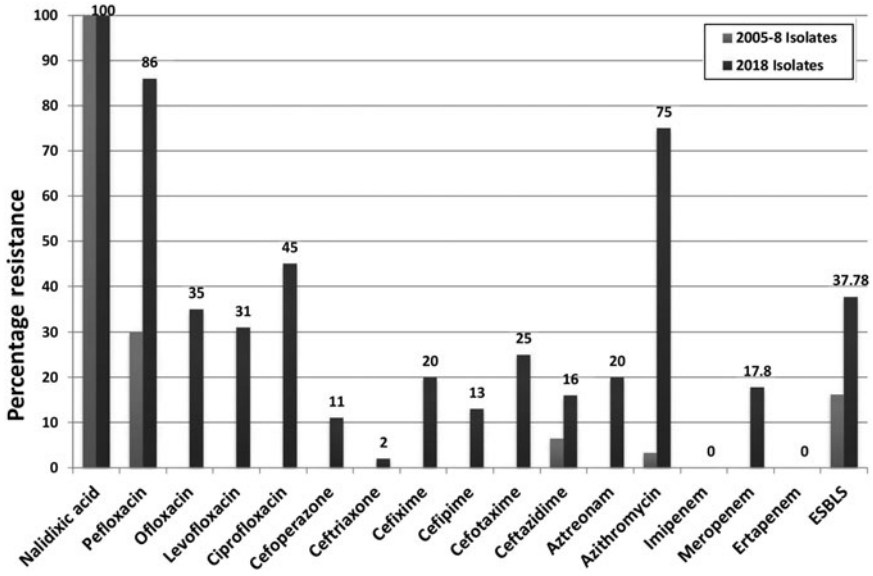

FIG. 1. Graphical presentation of comparison of antimicrobial resistance profile of *Salmonella* Typhi; 2005–2008 isolates and 2018 isolates.

Detection of carbapenem resistance

Some of the isolates also showed intermediate resistance toward carbapenems. That is, meropenem, imipenem, and ertapenem with figures of 42.2%, 20%, and 4% of the isolates. Complete resistance to meropenem was also observed in 17.8% of isolates (Table 1). None of the isolates demonstrated complete resistance toward imipenem and ertapenem.

Phenotypic detection of ESBL producers

Twenty-one (47%) *Salmonella* Typhi isolates were detected as ESBL producers indicated by extended zone of inhibition produced by antibiotic+clavulanic acid (a β-lactamase inhibitor) using combination disc method. Out of these, 16 isolates were detected for ceftazidime alone and with clavulanic acid; 3 for cefpodoxime alone and with clavulanic acid; and 3 cefotaxime alone and with clavulanic acid each (Supplementary Table S4).

Overall, 2.3% of the isolates were found to exhibit XDR phenotype, ESBL producers, and carbapenem resistant simultaneously, whereas 6.7% were carbapenem resistant as well as ESBL producer (Supplementary Fig. S1).

Determination of MIC for fluoroquinolones and cephalosporins

MIC values were checked for the four antimicrobial agents that belonged to two main groups of antimicrobials (fluoroquinolones and cephalosporins), which are mainly used for the treatment of typhoid fever.

A total of 55.6% of isolates were intermediate resistant to ciprofloxacin with MICs from 0.12 to 0.5 μg/mL, and 44.4% of isolates were susceptible to ciprofloxacin with MICs from 0.008 to 0.03 μg/mL.

Twenty-two percent of isolates were intermediately resistant to levofloxacin with MICs from 0.25 to 1 μg/mL, and 77.8% of isolates were susceptible to levofloxacin with MICs from 0.03 to 0.12 μg/mL (Table 2).

Around 28% of isolates exhibited resistance to ceftriaxone MICs greater than 32 μg/mL, whereas 28% of isolates have demonstrated intermediate resistance to ceftriaxone with MIC of 2 μg/mL, and 43% of the isolates were susceptible to ceftriaxone with MICs from 0.06 to 0.25 μg/mL.

Around 44% of isolates were resistant to cefotaxime with MIC greater than 32 μg/mL, and 56% of isolates were susceptible to cefotaxime with MICs from 0.03 to 1 μg/mL (Table 2).

Genotypic analysis of drug resistance

For resistance, bla_{TEM-1} gene was detected in 84% of the new isolates, whereas figure for the old isolates was 35.4%. The bla_{SHV-1} was also targeted and amplification was seen in 68.8% of the new isolates (Supplementary Fig. S6). Notably, this gene was not detected in any of the old isolates. The bla_{CTX-M1}, $bla_{CTM-M14,}$ and $bla_{CTX-M15}$ were also targeted but no specific amplification was seen in any of the isolates.

For quinolone resistance, chromosomal genes, *gyrA*, *gyrB*, *parC*, and *parE*, were targeted. The detection of *gyrA* and *gyrB* genes were detected in 84% and 91% of the new isolates, whereas these genes were detected in 74% and 87% of the old isolates, respectively. However, no amplification was detected for *parC* and *parE* genes in the old isolates, whereas these genes were detected in 84% and 82.2% of the new isolates. Similarly, no amplification was seen for *aac(6′)-lb-cr* gene in the old isolates, but it was detected in 44.4% of the new isolates (Supplementary Fig. S5).

Table 1. Comparison of Phenotypic Antimicrobial Drug Resistance Profiling of Fluoroquinolones, Cephalosporins, Azithromycin, and Carbapenems by Disc Diffusion Assay in 2005–2008 and 2018 Isolates

Name of drug/group of drug	*Isolates from 2005 to 2008*			*Isolates from 2018*			p
	Sensitive	*Intermediate*	*Resistant*	*Sensitive*	*Intermediate*	*Resistant*	
Fluoroquinolones							
Nalidixic acid	0 (0%)	0 (0%)	31 (100%)	0 (0%)	0 (0%)	45 (100%)	
Pefloxacin	21 (70%)	0 (0%)	10 (30%)	6 (14%)	0 (0%)	39 (86%)	<0.0001[a]
Ofloxacin	31 (100%)	0 (0%)	0 (0%)	21 (47.3%)	8 (17.7%)	16 (35%)	0.0002[a]
Levofloxacin	31 (100%)	0 (0%)	0 (0%)	7 (16%)	24 (53%)	14 (31%)	0.0007[a]
Ciprofloxacin	12 (38.7%)	19 (61.2%)	0 (0%)	1 (2%)	24 (53%)	20 (45%)	<0.0001[a]
Cephalosporins							
Cefoperazone	31 (100%)	0 (0%)	0 (0%)	17 (38%)	23 (51%)	5 (11%)	0.0578
Ceftriaxone	31 (100%)	0 (0%)	0 (0%)	38 (85%)	6 (13%)	1 (2%)	0.4314
Cefixime	31 (100%)	0 (0%)	0 (0%)	24 (53%)	12 (27%)	9 (20%)	0.0084[a]
Cefepime	31 (100%)	0 (0%)	0 (0%)	7 (16%)	32 (71%)	6 (13%)	0.0379[a]
Cefotaxime	31 (100%)	0 (0%)	0 (0%)	22 (51%)	12 (27%)	11 (25%)	0.0027[a]
Ceftazidime	29 (93.6%)	0 (0%)	2 (6.4%)	13 (29%)	25 (55%)	7 (16%)	0.2100
Aztreonam	31 (100%)	0 (0%)	0 (0%)	24 (53%)	12 (27%)	9 (20%)	0.0084[a]
Azithromycin							
Azithromycin	30 (96.7%)	0 (0%)	1 (3.22%)	11 (25%)	0 (0%)	34 (75%)	<0.0001[a]
Carbapenems							
Imipenem	31 (100%)	0 (0%)	0 (0%)	36 (80%)	9 (20%)	0 (0%)	
Meropenem	31 (100%)	0 (0%)	0 (0%)	18 (40%)	19 (42.2%)	8 (17.8%)	0.0136[a]
Ertapenem	31 (100%)	0 (0%)	0 (0%)	43 (96%)	2 (4%)	0 (0%)	

[a]Significant difference is shown in the increase of drug resistance over the past decade.

Table 2. Minimum Inhibitory Concentration of *Salmonella* Typhi isolates by *E*-test Strip Method

	Percentage of Salmonella *Typhi isolates for each antimicrobial*			
MIC value, μg/mL	*Ciprofloxacin*	*Levofloxacin*	*Ceftriaxone*	*Cefotaxime*
>32[a]	0	0	29%	44%
16	0	0	0	0
8	0	0	0	0
4	0	0	0	0
2	0	0	29%	0
1	0	0	0	11%
0.5	0	0	0	0
0.25	22%	22%	14%	0
0.12	33%	22%	14%	11%
0.06	0	33%	14%	22%
0.03	22.2%	22%	0	11%
0.015	0	0	0	0
0.008	22.2%	0	0	0
0.004	0	0	0	0
0.002	0	0	0	0

[a]No zone was observed and MIC was considered >the highest value on the strip (values may be round up).
MIC, minimum inhibitory concentration.

However, no amplification was observed for *qnrA* and *qnrS* genes that are associated with plasmid-mediated resistance. Two more genes that are associated with plasmid-mediated resistance, *qnrB* and *qnrD*, were detected in 6.7% and 2.2% of the new isolates, respectively.

For carbapenem resistance, *NDM* (New Delhi metallo-lactamase), *GES*, and *VIM* (Verona integron-encoded metallo-β-lactamase) genes were targeted. None of these genes was detected in any isolate of the old stock. However, these genes were detected in 33.3%, 66.7%, and 75% of the new isolates, respectively (Fig. 2). Ten isolates (21%) were found coharboring VIM and GES genes. The sequences of these genes were submitted in NCBI GenBank with accession numbers MK816302 and MK816303.

For azithromycin resistance, *msrA*, *mefA*, and *mphA* genes were detected. No amplification was detected for these genes in the old isolates, whereas, these genes were detected in 29%, 27%, and 16% of the new isolates (Supplementary Fig. S7). However, no amplification was observed for *mphB*, *ermA*, *ermB*, and *ereA* genes in any of the isolates.

Statistical analysis

Phenotypically, fluoroquinolones, carbapenems, azithromycin, and some of the cephalosporins exhibited significant difference in the year 2018 as compared with 2005. Genotypically, two genes of fluoroquinolones presented significant difference, whereas cephalosporins, carbapenems, and azithromycin also showed significant difference in the year 2018 as compared with 2005 as correlated with the phenotypic results. Both phenotypic and genotypic results were analyzed by chi-square test, and p-value <0.05 indicated that there is significant difference in the emergence of drug resistance over that last decade (Tables 1 and 3).

Sequencing of drug resistance genes

Purified PCR products for drug resistance genes were sequenced by Macrogen. The deduced nucleotide sequences were BLAST searched against GenBank database of NCBI and were found to have 97–99% homologies with *gyrA* gene (Accession FR716806.1), *gyrB* gene (Accession KY611579.1), *ParC* gene (Accession AB071987.1), *ParE* gene (Accession AM283477.1), bla_{TEM} gene (Accession CP100733.1), *aac(6′)-Ib-cr* gene (Accession AY348316.1), *VIM* (Accession FR695890.1), *qnrB* (Accession AL513382.1), *qnrD* (Accession KJ685893.1), and *GES* gene (Accession MH251972.1), respectively. The percentage homologies are given in Supplementary Table S5.

Phenotypic detection of efflux activity by Et-Br cart wheel method

The isolates were found to have an efflux system by the indication of fluorescence. Isolates that showed no fluorescence were chosen as having active efflux system, whereas the isolates that showed fluorescence were chosen as having no efflux system. Our drug-resistant representative isolates showed no fluorescence thus designated to have an active efflux system (Supplementary Fig. S2).

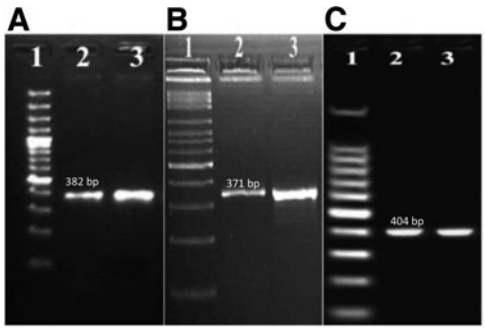

FIG. 2. PCR-based identification of carbapenem resistance genes. **Lane A1, B1**: 100 bp plus DNA Ladder (Cat No. NL1407; Vivantis). **Lane C1**: 100 bp DNA Ladder (Cat No. G2101; Promega Fisher Scientific). **Lane A2, A3**: Amplified product of *VIM* gene. **Lane B2, B3**: Amplified product of *GES* gene. **Lane C2, C3**: Amplified product of *NDM* gene.

Table 3. Comparison of Genotypic Antimicrobial Resistance Profile in 2005–2008 and 2018 isolates

Name of drug/group of drug	*Corresponding genes*	*Isolates from 2005 to 2008*		*Isolates from 2018*		p
		Positive	*Negative*	*Positive*	*Negative*	
Fluoroquinolones						
	gyrA	23 (74%)	8 (26%)	38 (84%)	7 (16%)	0.2880
	gyrB	27 (87%)	4 (13%)	41 (91%)	4 (9%)	0.5807
	parC	0 (0%)	31 (100%)	38 (84%)	7 (16%)	<0.0001[a]
	parE	0 (0%)	31 (100%)	37 (82.2%)	8 (17.8%)	<0.0001[a]
	qnrB	0 (0%)	31 (100%)	1 (2.2%)	44 (97.8%)	0.4089
	qnrD	0 (0%)	31 (100%)	3 (6.7%)	42 (93.3%)	0.1440
	aac(6′)-lb-cr	0 (0%)	31 (100%)	20 (44.4%)	25 (55.6%)	<0.0001[a]
Cephalosporins						
	bla_{TEM-1}	11 (35.4%)	20 (64.6%)	38 (84%)	7 (16%)	<0.0001[a]
	bla_{SHV-1}	0 (0%)	31 (100%)	31 (68.8%)	14 (31.2%)	<0.0001[a]
Azithromycin						
	mphA	0 (0%)	31 (100%)	7 (16%)	8 (84%)	0.0201[a]
	mefA	0 (0%)	31 (100%)	12 (27%)	33 (73%)	0.0017[a]
	msrA	0 (0%)	31 (100%)	13 (29%)	32 (71%)	0.0011[a]
Carbapenems						
	NDM	0 (0%)	31 (100%)	15 (33.3%)	30 (66.7%)	0.0004[a]
	GES	0 (0%)	31 (100%)	30 (66.7%)	15 (33.3%)	<0.0001[a]
	VIM	0 (0%)	31 (100%)	34 (75%)	11 (25%)	<0.0001[a]

[a]The prevalence of resistance genes has been increased significantly in the last decade.

Confirmation by CCCP method

All the isolates that were chosen to have active efflux system showed inhibition of their efflux activity by showing the difference of ≥5 mm in zones of inhibition against our representative antibiotics from each group with and without CCCP. The results were further confirmed by a duplicate run (Supplementary Fig. S3).

Genotypic detection of efflux pump genes

For the detection of efflux pump genes, *armA*, *npmA*, *qnrB*, *qnrD*, and *aac(6′)-lb-cr* showed amplification with our drug-resistant representative isolates. No amplification was observed with *rmtA*, *rmtB*, *rmtC*, and *rmtD* (Supplementary Fig. S4).

Identification and subtyping of H58 Salmonella *Typhi*

For the identification of H58 *Salmonella* Typhi strains, PCR was conducted on 76 strains (old and new isolates) of unknown haplotype, and was detected in 36 strains with the amplification product of 107 bp. All these 36 strains belonged to 2018 isolates, and none of the 2005–2008 isolates were positive for H58 haplotyping PCR. Thirty-six strains were then tested to detect the three mutations by PCR-RFLP to subtype H58 strains belonging to lineage I, lineage II, and node of H58. The mutation H58/Ia was accurately observed in all strains of lineage I and not in those of lineage II and node. The second mutation, H58/Ib, was not detected in any of the lineages and node. Finally, the mutation H58/II was detected in strains of lineage II and not in those of lineage I and node. Both approaches for detection and subtyping of H58 *Salmonella* Typhi were thus found 100% concordant. Furthermore, the detection limit of each PCR was 0.06 ng of DNA, demonstrating the high sensitivity of the developed assays.

Analysis of Salmonella *Typhi strains of unknown haplotype*

The PCR for H58 identification was used to screen 76 *Salmonella* Typhi strains of unknown haplotype, showing that 47.3% (36/76) of the strains were H58. The subtyping results revealed that 16.7% (6/36) of H58 strains belonged to lineage I, in particular to one of the haplotypes H58B, H62, or H64, as they carried the mutation H58/Ia and not the mutations H58/Ib or H58/II (Fig. 3 and Table 4). Instead, 19.4% (7/36) of H58 strains belonged to lineage II and consequently to one of the haplotypes H58G, H58I, or H58J, as they carried only the mutation H58/II. Finally, the remaining 63.9% (23/36) belonged to the node (H58A) or to H65, as they did not carry any of the tested mutations.

Discussion

The traditional antityphoidal drugs have turned out to be noneffective against the emerging drug-resistant strains of *Salmonella* Typhi.[26,27] The primary treatment for typhoid nowadays are the cephalosporins and fluoroquinolones, individually or in combination. In any case, resistance is developing against these medications progressively, which is extremely alarming.[28,29] Not surprisingly, third- and fourth-generation cephalosporins have given heterogenous outcomes. A significant number of isolates were found to be resistant to the cefepime (13%), ceftazidime (25%), cefixime (20%), cefoperazone (11%), and cefotaxime (16%), yet ceftriaxone was extremely effective as only one (2%) isolate was found to be resistant against this drug in the current study. About 75% of the newly collected isolates were found resistant to azithromycin. Our finding related to ceftriaxone and azithromycin resistance are contrary to the resistance profile of XDR clone reported from Sindh in 2018, which was resistant to ceftriaxone but being susceptible to azithromycin, the later was recommended as the drug of choice for XDR *Salmonella* Typhi.[12]

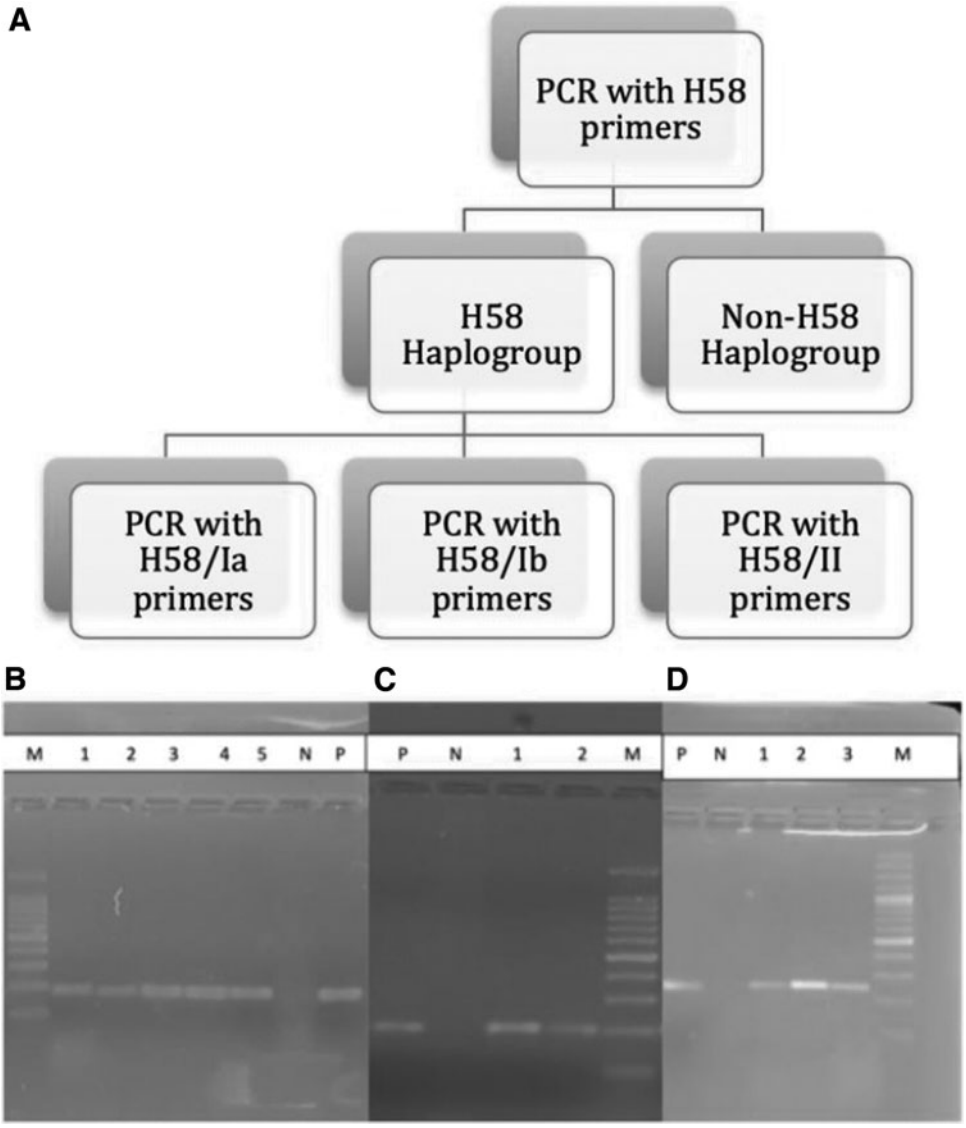

FIG. 3. **(A)** Schematic representation of the protocol to identify and subdivide H58 *Salmonella* Typhi strains. **(B)** H58/Ia with a product size of 176 bp. *Lane M*: DNA ladder of 100 bp (Solis Biodyne—07-11-0000S). *Lane 1–5*: *Salmonella* Typhi showing amplified product of haplotype H58/Ia gene of size 176 bp. *Lane P*, *N*: Negative control and positive control, respectively. **(C)** H58/Ib with a product size of 205 bp. *Lane M*: DNA ladder of 100 bp (Solis Biodyne—07-11-0000S). *Lane 1, 2*: *Salmonella* Typhi showing amplified product of haplotype H58/Ib gene of size 205 bp. *Lane N*, *P*: Negative control and positive control, respectively. **(D)** H58/II with a product size of 234 bp. *Lane M*: DNA ladder of 100 bp (Solis Biodyne—07-11-0000S). *Lane 1–3*: *Salmonella* Typhi showing amplified of haplotype H58/II gene of size 234 bp. *Lane P*, *N*: Negative control and positive control, respectively.

However, notable resistance was found against first-generation fluoroquinolone, including nalidixic acid (100%), but third- and fourth-generation fluoroquinolones gave heterogeneous outcomes. A large number of isolates were resistant to pefloxacin (86%), but other fluoroquinolones, such as ciprofloxacin, levofloxacin, and ofloxacin, gave better results as the resistance levels were estimated as 45%, 31%, and 35%, respectively. These findings are consistent with a previous report of Afzal et al.[30]

In Asian countries, few incidents of *Salmonella* Typhi resistant to β-lactam antimicrobials due to the production of ESBL have been encountered. An isolate that harbored bla_{SHV} gene was detected in 2008 in the Philippines.[31] In 2013, a study conducted in India distinguished five MDR isolates of *Salmonella* Typhi that exhibited positive results for bla_{TEM-1}, yet none harbored bla_{SHV} and bla_{CTX-M}.[32] In our study, we have identified the *Salmonella* Typhi isolates positive for bla_{TEM-1} and bla_{SHV}, however, none of our isolates showed positive results for bla_{CTX-M}. The difference in the genotypic and phenotypic data may be due to the presence of underexpressed genes in intermediately resistant isolates, or may be due to some other genes not targeted in this study.

CRE has emerged as a noteworthy medical risk in North America.[33]A carbapenemase gene encoding NDM, VIM, and GES was detected in 33.3%, 75%, and 66.7% of our new isolates of *Salmonella* Typhi. These genes were previously reported in Enterobacteriaceae members other than *Salmonella* Typhi, but these are reported probably first time in *Salmonella* Typhi in the present study. Less percentage of *Salmonella* Typhi isolates was phenotypically resistant to carbapenems as compared with genotyping results, which may be due to a significant percentage of intermediate resistance to carbapenems indicating the potential for development of complete resistance. Carbapenemase gene *NDM-1* was first recognized in *Klebsiella pneumonia* and *Escherichia coli* strains isolated in 2008 from a patient in Sweden after inhabiting in a New Delhi healing facility.[34] The major root of VIM-type Enterobacteriaceae is Greece, where it is predominating in *K. pneumonia* and *E. coli*.[35] The co-occurrence of VIM and GES genes in *Salmonella* Typhi isolates is a matter of serious concern as the emergence of XDR isolates of *Salmonella* Typhi has already worsened the treatment situation.

Table 4. *Salmonella* Typhi Strains of Unknown Haplotype Analyzed in This Study and Results of H58 Typing

N° of Salmonella *Typhi strains*	*H58 haplogroup*	*H58 lineage/ predicted haplotype by RFLP*
4	+	II/H58G, H58I, H58J
5	+	Node/H58A, H65
7	+	Node/H58A, H65
8	+	Node/H58A, H65
9	+	II/H58G, H58I, H58J
11	+	II/H58G, H58I, H58J
12	+	II/H58G, H58I, H58J
13	+	Node/H58A, H65
17	+	Node/H58A, H65
19	+	I/H58B, H62, H64
20	+	Node/H58A, H65
21	+	I/H58B, H62, H64
23	+	Node/H58A, H65
25	+	Node/H58A, H65
26	+	Node/H58A, H65
27	+	II/H58G, H58I, H58J
33	+	I/H58B, H62, H64
34	+	II/H58G, H58I, H58J
35	+	Node/H58A, H65
36	+	Node/H58A, H65
37	+	Node/H58A, H65
39	+	Node/H58A, H65
42	+	I/H58B, H62, H64
43	+	Node/H58A, H65
47	+	Node/H58A, H65
48	+	Node/H58A, H65
49	+	I/H58B, H62, H64
50	+	Node/H58A, H65
52	+	Node/H58A, H65
51	+	Node/H58A, H65
56	+	Node/H58A, H65
57	+	II/H58G, H58I, H58J
61	+	Node/H58A, H65
63	+	I/H58B, H62, H64
64	+	Node/H58A, H65
66	+	Node/H58A, H65

The rise of ESBL-producing *Salmonella* is an after effect of selective pressure forced by the abuse of broad-spectrum antimicrobials, for example, third- and fourth-generation fluoroquinolones and cephalosporins.[36] Simultaneously, the emergence of first ever carbapenem-resistant isolates can also be attributed to inadequate dose exposure to carbapenems. Expanded pervasiveness of these resistant *Salmonella* will prompt failures of the present treatment. Persistent observation of the susceptibility profiles and enhancing the directed measures for infection will be helpful to control the spread of the disease with resistant isolates.

There are numerous mechanisms involved in the acquisition of antimicrobial resistance by pathogenic bacteria and a significant role is played by efflux pumps, which expel the antimicrobials out of the bacterial cell. An enormous number of chemically different substrates as well as antibiotics can make their way to create multidrug efflux pumps. Et-Br cart wheel method was used by us to detect active efflux system in our representative resistant isolates. The main advantage of this procedure is the evaluation of efflux activity by using a diverse range of efflux pump substrate that is, Et-Br, a substrate pumped out by most of the reported efflux systems.[37] A gene that results in the aminoglycoside efflux by encoding methyltransferase is *armA*.[38] Among targeted efflux pump genes we found *armA* gene in most of our drug-resistant representative isolates, however, in the previous studies, *mdtK* (84.46%) was found along with the *golS* gene, which is basically multidrug efflux pump promoter gene in most of *Salmonella* serovars.[39]

Other efflux pump genes that is, *npmA*, *qnrB*, *qnrD*, and *aac(6′)-lb-cr* also showed promising results with our drug-resistant representative isolates. Previously, the presence of *qnrD* and *aac(6′)-lb-cr* were reported in efflux pump studies on different *Salmonella* serovars, whereas *npmA* and *qnrB* were not found in previously reported *Salmonella* serovars.[40] Multiple factors are involved to create the difference between genotypic resistance and phenotypic resistance. These various factors might be no or low expression of gene or might be due to expression of other resistance genes.

As mentioned above, the efflux pumps' expression may be due to various contributing factors of MDR in different *Salmonella* serovars. In the present study, the efflux activity of all drug resistance representative isolates was examined by Et-Br cart wheel method. In Enterobacteriaceae, Et-Br is deliberated as the most usual substrate of efflux pumps.[41] For the most efficient and quickest detection of multiple drug-resistant bacteria associated with efflux pumps, Et-Br cartwheel procedure has been utilized. To evaluate the essential efflux activity in various microorganisms, such as *K. pneumoniae*, *Staphylococcus aureus*, *E. coli*, and *Pseudomonas aeruginosa*, Et-Br method has been used.[41]

In the present study, our drug-resistant representative isolates had exhibited noticeable efflux activity at 1 and 1.5 μg/mL of Et-Br. However, the occurrence of efflux pump activity might not be always linked with antimicrobial resistance. Besides antimicrobial resistance, it is possible that the efflux pumps are physiologically involved in the expulsion of different metabolites that are harmful to the cells. However, the activity of efflux pumps may show a significant role in the increase of MDR among various microorganisms.

The typing of drug-resistant bacterial strains is useful to identify their clonal spread. Besides investigating the drug resistance mechanisms, the H58 haplotyping of the strains under study was also carried out. H58 haplotype of *Salmonella* Typhi is globally associated with the MDR character. Previous study indicated a phylogeographical distribution of the H58 *Salmonella* Typhi, emphasizing the significance of discrimination among the population.[42] We confirmed the diffusion of haplogroup with 47.4% of H58. Furthermore, we also found that some of our H58 isolates belonged to H58B, H62, or H64 (lineage I); some belonged to H58G, H58I, or H58J strains (lineage II). Finally, H58A (node) or H65 strains were also found in our isolates. These outcomes are in agreement as previously reported.[16]

In conclusion, it is clearly evident from comparison of 2005–2008 and 2018 isolates that emergence of drug resistance is increased considerably over the period of 10 to 13 years. We can state that the clinical isolates of *Salmonella* Typhi characterized from Faisalabad

district, are demonstrating expanding fluoroquinolone resistance as shown by the increment in effective dose of ciprofloxacin. Anyway, third- and fourth-generation fluoroquinolones like ofloxacin and levofloxacin are still exceptionally successful. Among third-generation cephalosporins, ceftriaxone indicated promising outcomes, which is contrary to the XDR clone reported from Sindh highlighting the importance of data collection from each endemic region independently. The notable finding is emergence of carbapenem resistance in *Salmonella* Typhi rendering the clinicians to give a serious consideration for selection of antimicrobials as this may be a sequel of the exposure of bacteria to this most effective group of antibiotics.

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Authors' Contributions

Y.S. planned and designed the project; Q.A. performed and analyzed experiments and wrote the article; M.T. and A.A. supported in the phenotypic characterization; A.S. supported in collection of samples; ABA and M.W. provided guidance in statistical analysis for the article; A.A. helped in phenotypic analysis and article writing; A.H. and M.I. edited and proof read the article; Y.S. supervised the project and finalized the article.

Ethics Approval

The study was approved by the Institutional Medical Ethics Committee of National Institute for Biotechnology and Genetic Engineering College, Pakistan Institute of Engineering and Applied Sciences (NIBGE-C, PIEAS) Faisalabad, Pakistan (ID No. 25092017).

Availability of Data and Materials

All materials are available by the corresponding author.

Disclosure Statement

No competing financial interests exist.

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Supplementary Material

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3
Supplementary Figure S4
Supplementary Figure S5
Supplementary Figure S6
Supplementary Figure S7
Supplementary Table S1
Supplementary Table S2
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Supplementary Table S5

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Address correspondence to:
Yasra Sarwar, DVM, MPhil, PhD (Biotechnology)
National Institute for Biotechnology
and Genetic Engineering College
Pakistan Institute of Engineering
and Applied Sciences (NIBGE-C, PIEAS)
Faisalabad 38000
Pakistan

E-mail: yasrasarwar@yahoo.com