Original Article

Distribution of Ciprofloxacin- and Azithromycin-Resistant Genes among *Salmonella* Typhi Isolated from Human Blood

Abstract

Context: *Salmonella* Typhi has developed resistance to different groups of antibiotics. **Aims:** The purpose of the present study was to assess the distribution of ciprofloxacin- and azithromycin-resistant genes among *Salmonella* Typhi isolated from human blood. **Settings and Design:** This cross-sectional study was conducted in the Department of Microbiology of a tertiary care hospital in Bangladesh from July 2019–June 2020. **Subjects and Methods:** Clinically suspected enteric fever patients, irrespective of age and gender, who attended the laboratory of the Department of Microbiology and outpatient department of Medicine of tertiary care hospital. Blood culture and sensitivity tests were done. The positive growth of *Salmonella* Typhi was identified by Gram staining, colony morphology, and biochemical test. Then, *Salmonella* Typhi was identified by using *Salmonella*-specific antisera. Final identification was made by using *16s rRNA* by polymerase chain reaction (PCR). PCR was also done to detect quinolone and azithromycin resistance genes. **Results:** A total number of 83 samples yielded positive cultures, of which 50 isolated organisms were identified as *Salmonella* species; however, among these isolates, *Salmonella* Typhi was detected in 40 (48.2%) isolates. Among 12 ciprofloxacin-resistant isolates, 8 (66.67%) were positive for the *gyrA* gene, 1 (8.33%) was positive for the *qnrB* gene and *qnrS* gene, 2 (16.67%) were positive for *aac* (6′)-*Ib-cr*. Among 12 azithromycin-resistant isolates, 2 (16.66%) were positive for *mphA* and *mefA* genes, respectively. **Conclusion:** In conclusion, the *gyrA*, *aac* (6′)-*Ib-cr*, *mphA,* and *mefA* genes are found for the first time in tertiary care hospitals from the quinolones and azithromycin-resistant *Salmonella* Typhi.

Keywords: *Azithromycin, ciprofloxacin, polymerase chain reaction, resistant genes, Salmonella Typhi*

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Introduction

Enteric fever is caused by *Salmonella enterica* serovar Typhi and Paratyphi A, B, and C.[1] *Salmonella* Typhi, *Salmonella* Paratyphi A, *Salmonella* Paratyphi B, and *Salmonella* Paratyphi C are referred to collectively as typhoidal *Salmonella,* whereas other serovars are grouped as nontyphoidal *Salmonella*.[2] Typhoidal *Salmonella* strains are human host-restricted organisms that cause typhoid fever and paratyphoid fever, together referred to as enteric fever.[3]

Salmonella is serologically positive for lipopolysaccharide antigens O9 and O12, protein flagellar antigen H, and capsular polysaccharide antigen Vi.[4] The Vi capsular antigen is largely restricted to *Salmonella* Typhi, although it is shared by some strains of *Salmonella* Paratyphi C. Vi-negative strains of *Salmonella* Typhi are less infectious and less virulent than Vi-positive strains.[5]

The current increase in fluoroquinolone resistance to *Salmonella* Typhi has raised concerns due to the limited treatment options available in enteric fever.[6] Resistance to quinolone and fluoroquinolones occurs due to mutation within the DNA gyrase (topoisomerase II) and topoisomerase IV genes. It is often associated with overexpression of the efflux pump, decrease expression of outer membrane protein, and the presence of plasmid-encoded qnr genes.[7,8] The qnr gene encodes a pentapeptide repeat protein that protects DNA gyrase against inhibition by quinolone and fluoroquinolones.[9,10]

Azithromycin is used to treat typhoid fever.[11] Azithromycin is an azalide antimicrobial agent that is equivalent

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or superior to chloramphenicol, fluoroquinolones, and extended-spectrum cephalosporins for the management of uncomplicated enteric fever proven in clinical trials.[12] Resistance to this antibiotic has been reported in India and other countries.[13] Mechanisms of azithromycin resistance include the mutations in target genes or efflux pumps and the presence of specific resistance genes such as *mphA*, *mphB*, *mefA*, *mefB*, *ereA,* and *ermA* genes.[14] The purpose of the present study was to assess the distribution of ciprofloxacin and azithromycin-resistant genes among *Salmonella* Typhi isolated from human blood.

Subjects and Methods

After obtaining approval from the institutional ethical committee, this cross-sectional study was conducted in the Department of Microbiology of a tertiary care hospital in Bangladesh from July 2019 to June 2020. Clinically suspected enteric fever patients, irrespective of age and gender, who attended the laboratory of the Department of Microbiology and outpatient department of Medicine of tertiary care hospital in Bangladesh for blood culture and sensitivity test were included in this study. Patients or legal guardians of the patients who did not give consent were excluded from the study.

Identification of *Salmonella* spp.

Blood was collected for blood culture in the standard procedure for the isolation of *Salmonella* species.[15] Trypticase soya broth was used for primary blood culture then subculture was done on blood agar and MacConkey agar media. The identification was made by biochemical tests; after inoculation, they were aerobically incubated at 37°C for 24 h in aerobic incubator.[16] *Salmonella*-specific antisera for determination of the O antigen of *Salmonella* Typhi (Mast™ Diagnostic, UK) was used.

Antimicrobial susceptibility testing

Antimicrobial susceptibility was done by Kirby–Bauer modified disc diffusion technique, and antibiotic disks were collected from commercial sources (Oxoid Ltd, UK). The zone of inhibition was interpreted according to the Clinical and Laboratory Standards Institute. Ampicillin (10 µg), chloramphenicol (30 µg), sulfamethoxazole/trimethoprime (25 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), cefixime (5 µg), ceftriaxone (30 µg), Cefepime (30 µg), azithromycin (15 µg), amoxicillin/clavulanic acid (30 µg), piperacillin/tazobactam (110 µg), and imipenam (10 µg) were used. *Escherichia coli* ATCC 25922 was used as control strain to assess the performance of the method.[16] Within 30 min of placement of antibiotic discs, inoculated plates were incubated aerobically at 37° C for overnight.

Detection of multidrug resistant *Salmonella*

Detection of multidrug-resistant (MDR) *Salmonella* strain was performed. *Salmonella* strains that were resistant to all three first-line anti-typhoidal antimicrobial agents, namely ampicillin, chloramphenicol, and trimethoprim sulfamethoxazole were detected as MDR organisms.

Determination of minimum inhibitory concentration of ciprofloxacin and azithromycin

MIC of ciprofloxacin and azithromycin were done by agar dilution method. The Agar dilution method was used to determine the susceptibility of ciprofloxacin and azithromycin. The bacterial suspension of 0.5 McFarland turbidity standard was prepared. As 0.5 McFarland turbidity standard contains 1.5×10^8 CFU/ml, 10 times dilution (1 ml test inoculums compared to turbidity standard added with 9 ml of normal saline) of test inoculums was done to achieve 1.5×10^7 CFU/ml. To obtain 1.5×10^4 CFU/ml on the agar surface, 1 µl of 10 times diluted inoculum were placed on the Mueller–Hinton agar plate. The plate was then incubated aerobically at 37° C overnight.[16] Different concentrations of ciprofloxacin and azithromycin were prepared and impregnated in 50 ml Mueller–Hinton agar media. Bacterial inoculums were applied onto the agar surface, and the plates were incubated at 37°C overnight. The lowest concentration of antibiotic-impregnated Mueller–Hinton agar showing no visible growth on agar media was considered MIC of that drug for that strain of bacteria.[17] *E. coli* ATCC 25922 was used as the control organism.[16]

Molecular methods

Polymerase chain reaction (PCR) was done to detect *Salmonella* Typhi, quinolone, and azithromycin resistance genes.[18] To prepare bacterial pellets, a loop full of 5–6 bacterial colonies were subcultured into Mueller–Hinton agar media at 37°C for 24 h. A loop full of bacterial colonies was inoculated into a falcon tube containing trypticase soya broth. After incubating at 37°C overnight,

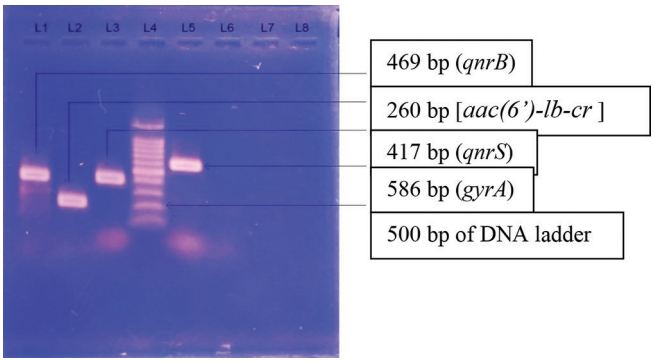

Figure 1: Photograph of gel electrophoresis of amplified DNA of 469 bp for *qnrB* gene (lane 1), amplified DNA of 260 bp for *aac (6')-lb-cr* gene (lane 2), amplified DNA of 417 bp for *qnrS* gene (lane 3), hundred bp DNA ladder (lane 4), amplified DNA of 586 bp for *gyrA* gene (lane 5), negative control without DNA (TE buffer) (lane 6), negative control *Escherichia coli* ATCC 25922 (lane 7), negative sample (lane 8). Table 10: had shown distribution of azithromycin resistance genes among azithromycin resistant *Salmonella* Typhi detected by PCR. Among 12 azithromycin resistant isolates, 2 (16.66%) were positive for *mphA* and *mefA* genes respectively. No *mphB, ereA, ermA, ermB* were detected in any isolates

the falcon tubes were centrifuged at 4000 rpm for 10 min, after which the supernatant was discarded. A small amount of sterile trypticase soya broth was added into falcon tubes with pellets and mixed evenly. Then, an equal amount of bacterial suspension was placed into 2–3 microcentrifuge tubes. The microcentrifuged tubes were then centrifuged at 4000 g for 10 min, and the supernatant was discarded. The microcentrifuged tubes containing bacterial pellets were kept at −20°C as pellets until DNA extraction. Bacterial DNA was extracted by the boiling method.[18] Genes were detected by PCR using the primers as shown in Tables 1 and 2.

PCR assays were performed in a DNA thermal cycler. PCR reaction consisted of preheat at 94°C for 10 min, followed by 36 cycles of (denaturation at 94°C for 30 s, annealing at 52°C for 40 s, and elongation at 72°C for 1 min), followed by final extension at 72°C for 10 min. Then, the product was held at 4°C. After amplification, products were processed for gel documentation or kept at −20°C till tested.

Table 1: Azithromycin resistance gene

Genes (Nguyen *et al*., 2009)			
Genes		**Sequence (5′ to 3′)**	**Amplicon**
mph (A)	F	GTGAGGAGGAGCTTCGCGAG	403
	R	TGCCGCAGGACTCGGAGGTC	
mph (B)	F	GATATTAAACAAG TAATCAGAATAG	494
	R	GCTCTTACTGCATCCATACG	
erm (A)	F	TCTAAAAAGCATGTAAAAGAAA	533
	R	CGATACTTTTTGTAGTCCTTC	
erm (B)	F	GAAAAAGTACTCAACCAAATA	639
	R	AATTTAAGTACCGTTACT	
ere (A)	F	GCCGGTGCTCATGAACTTGAG	420
	R	CGACTCTATTCGATCAGAGGC	
mef (A)	F	AGTATCATTAATCACTAGTGC	345
	R	TTCTTCTGGTACTAAAAGTGG	

Statistical analysis

Data were analyzed using Microsoft Office Excel (2013) software (Microsoft, Redmond, WA, USA).

Results

A total number of 83 (25.69%) samples yielded positive cultures, of which 50 isolated organisms were identified as *Salmonella* species. Furthermore, *Salmonella* Typhi was detected in 40 (48.2%) isolates in out of 50 isolates [Table 3].

The identification of *Salmonella* Typhi by biochemical test and PCR was done in this study. Among 50 bacteriologically diagnosed typhoid fever cases, 40 (80.0%) were positive for *Salmonella* Typhi by biochemical characteristics and PCR, respectively [Table 4].

The antibiotic susceptibility pattern of *Salmonella* Typhi isolated from patients with enteric fever was recorded. Among the 40 isolated *Salmonella* Typhi, all were sensitive

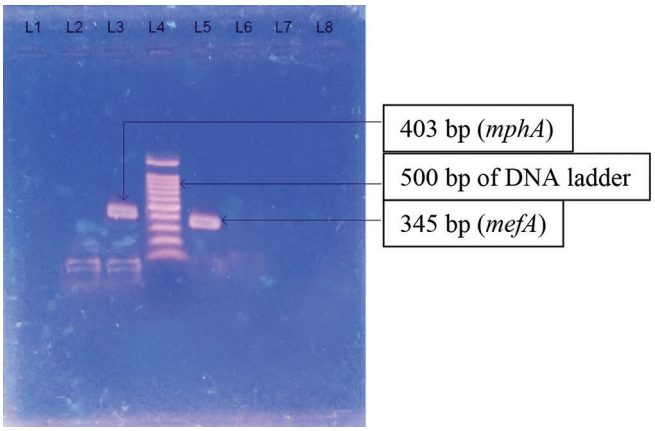

Figure 2: Photograph of gel electrophoresis of negative control without DNA (TE buffer) (lane 1), negative control *Escherichia coli* ATCC 25922 (lane 2), amplified DNA of 403 bp for *mphA* gene (lane 3), hundred bp DNA ladder (lane 4), amplified DNA of 345 bp for *mefA* gene (lane 5), negative sample (lane 6), blank (lane 7), blank (lane 8)

Table 2: Quinolone resistance genes

Gene		Sequence (5′ to 3′)	Size (bp)	Reference
qnrA	F	ATTTCTCACGCCAGGATTTG	516	Robicsek *et al*., 2006
	R	GATCGGCAAAGGTTAGGTCA		
qnrB	F	GATCGTGAAAGCCAGAAAGG	469	Robicsek *et al*., 2006
	R	ACGATGCCTGGTAGTTGTCC		
qnrC	F	GGGTTGTACATTTATTGAATC	447	Chen *et al*., 2012
	R	TCCACTTTACGAGGTTCT		
qnrD	F	CGAGATCAATTTACGGGGAATA	581	Cavaco *et al*., 2009
	R	AACAAGCTGAAGCGCCTG		
qnrS	F	ACGACATTCGTCAACTGCAA	417	Robicsek *et al*., 2006
	R	TAAATTGGCACCCTGTAGGC		
aac (6′)-Ib-cr	F	TTGGAAGCGGGGACGGAM	260	Wareham *et al*., 2010
	R	ACACGGCTGGACCATA		
gyrA	F	CGTCGCGTACTTTACGCCATGAACG	586	Dasgupta *et al*., 2018
	R	ATACCTTGCCGCGACCGGTACGG		

to cefixime, ceftriaxone, cefepime, and imipenem. All *Salmonella* were resistant to nalidixic acid. However, 86% were resistant to ampicillin and 54% were resistant to chloramphenicol and sulfamethoxazole/trimethoprim, respectively [Table 5].

The MDR strain among isolated *Salmonella* species was detected. Among 50 isolated *Salmonella* species, 9 (18.0%) isolates were MDR *Salmonella* strains and 41 (82.0%) were non-MDR *Salmonella* strains [Table 6].

Table 7 demonstrates the MIC of ciprofloxacin among ciprofloxacin-resistant *Salmonella* Typhi by agar dilution method. Out of 12 ciprofloxacin-resistant *Salmonella* Typhi, one (8.3%) had MIC of 0.48 μg/ml, 3 (25%) had MIC of 1 μg/ml, 2 (16.7%) had MIC of 2 μg/ml, 3 (25%) had MIC of 4 μg/ml, and 3 (25%) had MIC of 8 μg/ml.

Table 8 demonstrates the MIC of azithromycin among azithromycin-resistant *Salmonella* Typhi by agar dilution method. Out of 12 azithromycin-resistant *Salmonella* Typhi, 5 (41.67%) had MIC of 128 μg/ml, 4 (33.33%) had MIC of 64 μg/ml, and 3 (25%) had MIC of 32 μg/ml.

Table 9 demonstrates the distribution of quinolone resistance genes among ciprofloxacin-resistant *Salmonella* Typhi detected by PCR. Among 12 ciprofloxacin-resistant isolates, 8 (66.67%) were positive for the *gyrA* gene, 1 (8.33%) was positive for the *qnrB* gene and *qnrS* gene, and 2 (16.67%) were positive for *aac* (6′)-*Ib-cr*. No *qnrA*, *qnrC*, and *qnrD* genes were detected in any isolates.

Table 3: Organisms isolated from blood culture positive samples (*n*=83)

Isolated organism	Frequency (%)
Salmonella Typhi	40 (48.2)
Others	43 (51.8)
Total	83 (100.0)

Table 4: Identification of *Salmonella* Typhi by biochemical test and polymerase chain reaction by using *16S rRNA* (*n*=40)

Identification	*Salmonella* Typhi, *n* (%)	Other *Salmonella* spp., *n* (%)
Biochemically	40 (80.0)	10 (20.0)
PCR	40 (80.0)	10 (20.0)

PCR: Polymerase chain reaction

Table 5: Antibiotic susceptibility pattern of *Salmonella* Typhi isolated from enteric fever patients (*n*=40)

Antimicrobial agents	Sensitive, *n* (%)	Resistant, *n* (%)
Ampicillin	5 (12.5)	35 (87.5)
Chloramphenicol	19 (47.5)	21 (52.5)
Sulfamethoxazole/ trimethoprim	19 (47.5)	21 (52.5)
Piperacillin/tazobactam	37 (92.5)	3 (7.5)
Imipenem	40 (100)	0
Ceftriaxone	40 (100)	0
Cefexime	40 (100)	0
Cefepime	40 (100)	0
Amoxycillin/clavulanic acid	33 (82.5)	7 (17.5)
Ciprofloxacin	28 (70)	12 (30)
Azithromycin	28 (70)	12 (30)
Nalidixic acid	0	40 (100)

Table 6: Distribution of multidrug-resistant strains among isolated *Salmonella* spp. (*n*=40)

Resistance	*Salmonella* Typhi, *n* (%)
MDR	7 (17.5)
Non-MDR	33 (82.5)
Total	40 (100.0)

MDR: Multidrug resistant

Discussion

Antibiotic is the main therapeutic option for the treatment of enteric fever, and the mortality rate may reach up to 30% in the absence of effective antibiotic therapy.[15] This study was designed for the distribution of ciprofloxacin and azithromycin-resistant genes among *Salmonella* Typhi isolated from human blood.

In the present study, among 323 enteric fever suspected cases, a total of 83 (25.69%) were culture positive. Among them, 50 (15.47%) were positive for *Salmonella* species, which was confirmed by biochemical tests and specific antisera and 33 (10.21%) were other organisms. Among 50 culture-positive *Salmonella* species, 40 (80%) isolates were *Salmonella* Typhi and 10 (20%) were *Salmonella* Paratyphi. In a study by Akter *et al*.,[19] the *Salmonella* Typhi was 77.68%, whereas *Salmonella* Paratyphi was 22.32%. Saha[20] reported that *Salmonella* Typhi and *Salmonella* Paratyphi ratio was 4:1. *Salmonella* Typhi was found in 48.19% of samples which was almost similar to the study of Dahhan *et al*.[21] who found 44.5% *Salmonella* Typhi among the culture-positive sample. The findings of these studies were consistent with the present study.

Enteric fever, caused by the MDR strain, has become a significant cause of morbidity and mortality over recent years.[7] In the present study, 7 (17.5%) MDR *Salmonella* Typhi strains and 2 (20%) MDR *Salmonella* Paratyphi strains were detected. A study by Naser[22] in DMC found 11.11% MDR *Salmonella* Typhi strain. In surveillance held in Bangladesh (2005–2013), Saha[20] reported that 15.92% were MDR *Salmonella* Typhi. Khanam *et al*.[23] reported that 13.0% were MDR *Salmonella* Typhi strains among the adult study population. Aljanaby and Medhat[24] from Iraq reported that 43.58% were MDR *Salmonella* Typhi strains.

The present study was carried out to detect the *gyrA* gene and other plasmid-mediated quinolone resistance genes

Table 7: Minimum inhibitory concentration of ciprofloxacin-resistant *Salmonella* Typhi (*n*=12)

MIC of ciprofloxacin (µg/ml)	*Salmonella* Typhi, *n* (%)
≥8	3 (25.0)
4	3 (25.0)
2	2 (16.7)
1	3 (25.0)
0.48	1 (8.3)
0.24	0
0.12	0
≤0.06	0
Total	12 (100.0)

CLSI (2020) breakpoint for MIC of ciprofloxacin for *Salmonella*; Sensitive ≤0.06 µg/mL; Intermediate 0.12–0.5 µg/mL; Resistant ≥1 µg/ml. MIC: Minimum inhibitory concentration; CLSI: Clinical and Laboratory Standards Institute

Table 8: Minimum inhibitory concentration of azithromycin-resistant *Salmonella* Typhi (*n*=12)

MIC of azithromycin (µg/ml)	*Salmonella* Typhi, *n* (%)
≥256	0
128	5 (41.7)
64	4 (33.3)
32	3 (25.0)
16	0
8	0
4	0
≤2	0
Total	12 (100.0)

CLSI (2020) breakpoint for MIC of azithromycin for *Salmonella*; Sensitive ≤16 µg/ml; resistant ≥32 µg/ml. MIC: Minimum inhibitory concentration; CLSI: Clinical and Laboratory Standards Institute

such as *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, and aac (6′)-*Ib-cr*. Among 12 ciprofloxacin-resistant isolates, 8 (66.67%) were positive for *gyrA* genes, 1 (8.33%) was positive for *qnrB* gene, 1 (8.33%) was positive for qnrS gene, 2 (16.67%) were positive for *aac* (6′)-*Ib-cr*. No *qnrA*, *qnrC*, *qnrD* genes were detected in any isolates which was shown in [Figure 1]. Suman *et al*.[25] reported that all of the isolated *Salmonella* Typhi and *Salmonella* Paratyphi were *gyrA* gene positive. Gomes *et al*.[14] from Ghana reported that no *qnrA* or *qnrB* genes were detected, but two isolates were found to harbor *qnrS*-resistant gene. The study by Naser[22] reported that 3.70% were positive for *qnrS* and 7.41% were positive for *qnrB* gene, which was close to the present study. The identified *gyrA* (66.67%), *aac* (6′)-*Ib-cr* (16.67%) in this study, were the first detected quinolone resistance genes in *Salmonella* Typhi isolates. Quinolone resistance genes are capable of horizontal transfer, thereby accelerating the spread of this resistance mechanism among various clinical pathogens.

In the present study, among 12 azithromycin-resistant isolates, 2 (16.67%) were positive for mphA genes and 2 (16.67%) were positive for *mefA* genes. No *mphB*, *ereA*, *ermA*, *ermB* were detected in any isolates which was shown in [Figure 2]. The identified *mphA* (16.67%) and *mefA* (16.67%) in this study, were the first detected azithromycin resistance genes in *Salmonella* Typhi isolate in a tertiary care hospital in Bangladesh. Previously, *Salmonella* strains resistant to azithromycin have also been found in other countries.[12] In this study, azithromycin resistance genes were absent in 66.7% azithromycin resistant *Salmonella* Typhi isolates, which might be due to the possibility of other varieties of genes for azithromycin efflux pumps that enhance efflux of drug and mutation in *rplD* or *rplV* genes.

Table 9: Distribution of quinolone resistance genes among ciprofloxacin (*n*=12) resistant *Salmonella* Typhi detected by the polymerase chain reaction

Gene	Frequency (%)
gyrA	8 (66.7)
qnrC	0
qnrD	0
qnrS	1 (8.3)
qnrA	0
qnrB	1 (8.3)
aac (6′)-*Ib-cr*	2 (16.7)
Total	12 (100.0)

Table 10: Distribution of azithromycin resistance genes among azithromycin (*n*=12) resistant *Salmonella* Typhi detected by the polymerase chain reaction

Gene	Frequency (%)
mphA	2 (16.67)
mphB	0
ereA	0
ermA	0
ermB	0
mefA	2 (16.67)
Total	4 (33.34)

Salmonella Typhi is a human-restricted pathogen and the leading cause of enteric fever worldwide, which causes the highest mortality and morbidity in developing countries. The antibiotic resistance pattern of *Salmonella* Typhi is changing over time. In this study, ciprofloxacin- and azithromycin-resistant genes were found. It may necessitate to modify the treatment option for enteric fever.

Conclusion

Quinolone resistance genes such as *qnrB*, *qnrS*, *gyrA*, and *aac* (6′)-*Ib-cr* were detected and azithromycin resistance genes such as *mphA* and *mefA* were also found. The *gyrA*, *aac* (6′)-*Ib-cr*, *mphA*, and *mefA* were not detected previously in this institute, and these were the first time detected resistant genes of *Salmonella* Typhi in tertiary care hospital. Therefore, it was very clear from this result that the resistant pattern of *Salmonella* Typhi is changing over

time. Further large-scale studies should be conducted to get the real scenario.

Ethical statement

The study was approved by the institutional Ethics Committee Of Dhaka Medical College (Approval No: ERC-DMC/ECC/2019/401(R).

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Nil.

Conflicts of interest

There are no conflicts of interest.

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