Detection of *Salmonella typhi* Isolates and Ceftriaxone Strains Harbouring *CTX-M-15* Gene

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ABSTRACT

Objective: To determine $_{bla}$*CTX-M-15(Cefotaxime-Munich*) gene amongst the extensively drug resistant (XDR) *Salmonella typhi* (*S. typhi*) isolates by quantitative Polymerase Chain Reaction (qPCR).

Study Design: Observational, cross-sectional study.

Place and Duration of the Study: PNS Shifa Hospital and Bahria University of Health Sciences (BUHS), from January to June 2022.

Methodology: All the patients clinically suspected of enteric fever, whose blood culture specimens yielded growth of *S. typhi* were included in this study. These samples were confirmed by serotyping and biochemical reactions. The ceftriaxone resistance was evaluated by antibiotic susceptibility test according to CLSI 2020 guidelines, whereas $_{bla}$*CTX-M-15* gene was detected by (PCR) using gene-specific primers.

Results: Out of 149 *S. typhi* isolates, 87.2% were confirmed XDR *S. typhi* resistant to ceftriaxone (CRO). Among these, 83.9% harboured $_{bla}$*CTX-M-15* gene.

Conclusion: There was a very high frequency of XDR *S. typhi* harbouring $_{bla}$*CTX-M-15* in Karachi, Pakistan.

Key Words: $_{bla}$*CTX-M-15*, *Salmonella typhi, Third generation cephalosporin, Typhoid fever, Extensively drug resistant*.

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INTRODUCTION

An unprecedented and indiscriminate use of 3rd generation antibiotics over the 1st line of drugs has promoted the emergence of antimicrobial-resistant strains of *S. typhi*. The first case of *S. typhi* resistant to ceftriaxone was reported in an 11-month boy from Bangladesh in 1999.[1] These strains which were resistant to first-line antibiotics, including chloramphenicol, ampicillin, trimethoprim-sulfamethoxazole, fluoroquinolones and ceftriaxone are considered as Extensively drug resistant (XDR) *S. typhi*. In 2016, the world's largest outbreak of XDR *S. typhi* was reported from the Province of Sindh, Pakistan, affecting more than 800 people.[2] As per a recent survey carried out by the Field Epidemiology and Laboratory Training Programme, a total of 22,354 typhoid cases were reported from November 2016 to February 2020 in Pakistan. Out of these, 17000 XDR were reported from Sindh, 12,708 from Karachi and 4,892 from different districts of Sindh.[3]

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The molecular and whole-genome sequencing of *S. typhi* indicates that ceftriaxone resistance is due to the elaboration of extended spectrum β lactamases (ESBL) enzyme, which is encoded by $_{bla}$*CTX-M* genes.[4] The ESBL has a wide range of activity against the most commonly prescribed β lactam antibiotics, readily degrading penicillin and cephalosporins like monbactam, but inhibited by sulbactam, tazobactam, and clavulanic acid.[5]

The most prominent and common $_{bla}$*CTX-M* enzyme in humans, environment, and animals are *CTX-M14* (cluster 9), *CTX-M-3,* and *CTX-M 15* (cluster 1). These variants have increased activity against ceftazidime, a drug which is not inhibited by other *CTX-M* enzymes. The allelic variants of cluster 1 differ from each other only by one amino acid variation, e.g., *CTX-M-15* has glycine instead of aspartate (Asp240) at the terminal β-loop. This substitution at β-loop makes the enzyme more accepting of larger ceftazidime molecules, thus causing increasing hydrolytic activity against the ceftazidime.[6] *CTX-M-15* was initially reported only in *Escherichia coli* (*E.coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*) only, which later became dominant in *S. typhi* as well. This acquisition of *CTX-M-15* by *S. typhi* is dreadful, especially in countries where typhoid is endemic. As of the last 2 years, more than 10,000 cases of XDR *S. typhi* have been reported from Hyderabad and Karachi alone.[7] This increasing *S. typhi* resistance to ceftriaxone calls for an urgent need for preventive measures to control the situation before the last resort antibiotics become

impervious as well. Therefore, the objective of this study was to evaluate the frequency of XDR *S.typhi* harbouring *CTX-M-15*.

METHODOLOGY

This cross-sectional study was conducted at PNS Shifa Hospital and BUHS Karachi after an approval from the Ethical Review Committee of BUHS under the reference number (ERC 82/ 2021). The sample size was calculated using OpenEpi, version 3. The duration of this study was from January to June 2022.

After taking consent from the patients, blood samples with the suspicion of enteric fever irrespective to age and gender were collected and processed. All the samples were then incubated in automated BACTEC™, blood culture system (Biomerieux). The majority of clinically significant organisms yielded growth within 3 days of incubation. Afterwards, positive cultures were inoculated on 5% sheep blood agar and MacConkey agar. Plates were incubated for 24 hours at 36°C and then were checked for bacterial growth. *S. typhi* growth were identified by their growth characteristics, colony morphology, and biochemical profile i.e. *S. typhi* had non-haemolytic mucoid colonies of 2-3 mm on blood agar whereas there were non-lactose fermenting colonies on MacConkey agar. These colonies were oxidase negative and Serologically O9 antisera (Typhi O9 antisera SSI Diag-nostica's *Salmonella* Sero-Quick Group kit). Antimicrobial susceptibility test was then performed by the Kirby-Bauer disc diffusion method on Muller-Hinton agar (MH agar). The MH agar was then incubated for 24 hours. The XDR isolates were identified and preserved in a 1.5 ml sterile eppendrof containing Brain Heart infusion (BHI) culture media, which is an enriched non-selective media. These tubes were then incubated again for 24 hours, and after a short spin, preserved at -80°C. All XDR *S. typhi* isolates were included in this study whereas MDR *S. typhi* isolates were excluded.

All frozen samples were kept at room temperature to thaw before the DNA extraction. Then, Qiagen DNA mini extraction kit, Germany (Cat # 51304) was used for extracting *S. typhi* DNA followed by DNA quantification using Quantiflour, dsDNA system Promega, USA (Cat#: E2670). Once the required amount of DNA was extracted, detection of *CTX-M-15* gene was conducted on Rotor-Gene Q – QIAGEN using QuantiFast SYBR Green PCR Kit (400) (QIAGEN, Hilden, Germany) with primers as used in one of the previous study.[8] A 25 µl reaction mix was prepared which composed of 12.5 µl of quantiFast SYBR green master mix, 2.5 µl of 1um concentration forward and reverse *CTX-M-15* primer, and 2.5 µl of RNAase free water. Then 5 µl sample was dispensed in each of the PCR tubes containing the master mix amplified according to the following thermal protocol of initial activation at 95°C for 10 minutes, followed by 40 cycles each of denaturation at 95°C for 30 seconds, annealing at 58°C for 1 minute, primer extension at 72°C for one minute followed by one cycle of thermal extension at 72°C for 7 minutes and melt curve at 52-95°C for one minute. The amplified products were then analysed using 1.5% agarose gel for 45 minutes. All samples were then visualised on gel doc and matched with the bands of positive and negative control samples of *S. typhi* harbouring *CTX-M-15*.

Statistical analysis was done on SPSS version 25.0. The mean and standard deviation values were presented for age. The frequency and percentages were calculated for all categorical variables. Independent sample t-test was applied between age and ceftriaxone (CRO). Chi-square test was applied to see the significance between two categorical variables. The p-value ≤0.05 was considered to be statistically significant.

RESULTS

During the six months of the study time 149 blood samples positive for S. typhi were reported from PNS Shifa Hospital, Karachi. Out of these, 64 (42.95%) were females whereas 85 (57%) were males.

Out of 149 clinical isolates positive for *S. typhi*, 130 (87.24%) exhibited resistance to ceftriaxone as XDR, and only 20 (13.4%) were identified as ceftriaxone sensitive strains. Only XDR strains were further processed for the detection of *CTX-M-15* gene. Ceftriaxone resistance was observed in 75 (57.69%) males and 54 (41.53%) females. In this study, CRO resistance among indoor patients was 79.2%, whereas in outdoor patients, it was 20.7%. Age-distribution indicated that 85 (57%) were less than 10 years, 34 (22.8%) were between 10-20 years, and 30 (20.1%) were above 20 years. The mean age was 13.87 years among the CRO resistant whereas 4.95 years among sensitive clinical isolates. Both Levene's Test for Equality of Variances and t-test for Equality of Means (independent t-test) showed a significant value of 0.00.

The PCR products of all the samples were run on agarose gel for detection of the amplified fragment. The amplicons of 108 samples out of 130 were within the expected size for specific genes as seen in Figure 1. The prevalence of *CTX-M-15* among the XDR isolates and its prevalence among specific age groups are indicated in Table I and II.

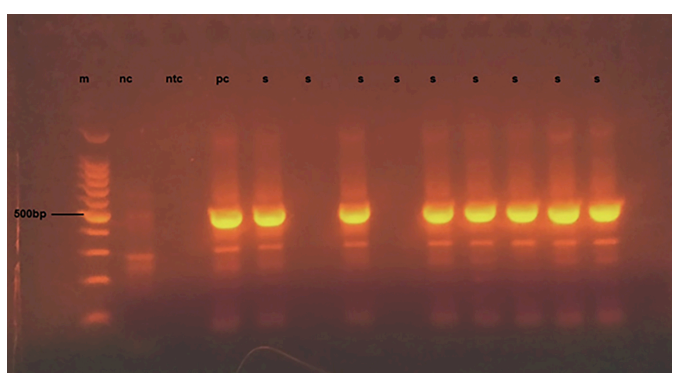

Figure 1: Gel electrophoresis indicating positive sample bands matching with PC (positive control). M-100bp ladder, NTC (non-template control, NC (negative control), PS (positive control), NS (negative sample).

Table I: Prevalence of *CTX-M-15* with specific age group. p-value of 0.50 was considered statistically significant (Chi-square test).

			Age groups			Total	p-value
			<10 years	10-20 years	>20 years		
CTX-M-15	Yes	Count	55	28	25	108	0.050
		% within Age_Groups	64.7%	82.4%	83.3%	72.5%	
	No	Count	30	6	5	41	
		% within Age_Groups	35.3%	17.6%	16.7%	27.5%	
Total		Count	85	34	30	149	
		% within Age_Groups	100.0%	100.0%	100.0%	100.0%	

Table II: Prevalence of *CTX-M-15* gene in *XDR S. typhi*. p-value of <0.00 was considered statistically significant (Chi-square test).

			Res	p-value
CTXM15	Yes	Count	108	<0.00
		% within CRO	83.9%	
	No	Count	22	
		% within CRO	17.1%	
Total		Count	130	
		% within CRO	100.0%	

DISCUSSION

The resistance to 3rd generation cephalosporin is mediated by elaboration of the *CTX-M* family, especially *CTX-M-15*. In recent years other ESBL enzymes especially carbapenemases have captured researchers' attention but *CTX-M-15* remains the most prominent enzyme amongst others and is referred to as prototype in the development and spread of antibiotic resistance. These enzymes have achieved uncontrolled pandemic status by co-harbouring other resistant elements as well as highly transferable plasmids, transposons, and mobile genetic elements. This dynamic genome favours persistence, which under constant selective chemical pressure, enables the bacterium to continuously evolve. It is, therefore, important to continuously report its prevalence to activate active control and stewardship programmes in order to restrict its spread and improvise treatment plans.

To the best of authors' knowledge, this is the first study not only from Pakistan but worldwide that reports a high frequency of XDR *S. typhi* harbouring *CTX-M-15* gene over a short period of six months. In the early years after the 2016 XDR *S. typhi* endemic, the number of such strains from different regions of Pakistan indicated an incidence between 25-70% of such strains.[9-12] This growing incidence is in accordance with several publications thereafter, which quantify that XDR *S. typhi* strains are on the rise, and if this pattern ensues, it might reach 100% in the near future.[13,14]

Children are the most vulnerable population that are widely exposed and affected by *S. typhi*. In this study, the prevalence of XDR *S. typhi* was found to be 57% in children less than 10 years. The study is in agreement with other studies from across Pakistan and globally.[15,16] On the other hand, the literature repeatedly specifies that typhoid fever is highly prevalent amongst males.[17,18] This can be explained by the fact that the male intestine predominantly has pro-inflammatory cytokine response (e.g., interleukin-6, tumour necrosis factor alpha, and macrophage inflammatory protein-2). On the contrary, females' immune response is chiefly anti-inflammatory (e.g., interleukin-10), implying that the female intestine is resilient and more resistant to bacteria as compared to males. Another plausible explanation for this predominance may be attributed to outdoor exposure of males and poor Water, Sanitation, and Hygienic (WASH) practices among them.[19]

The variance in the epidemiology of XDR *S. typhi* is observed worldwide and may be attributed to demographics, socio-economic status, and practice of WASH.[21] Its spread to Italy, USA, Spain, China, Australia, and England had also been reported. According to the CDC report, XDR *S. typhi* cases in the USA from 2018 to 2021 have been 71, amongst which 69 had a recent travel history from Pakistan (Centre of Disease Control and Prevention, 2021). Approximately, similar numbers were reported from a retrospective study conducted in England.[20-23] On the other hand, in a densely populated country like India, which has the same climate, demographics, poverty, and healthcare as Pakistan, there are very few reports regarding XDR *S. typhi*. This prevalence might not indicate a true trend of AMR as clinically patients do not respond to third generation cephalosporins in India. India might not be reporting XDR *S. typhi* due to a lack of proper law enforcement of national surveillance, financial constraints, and ambiguous healthcare policies.[22]

Although XDR *S. typhi* is prevalent in Pakistan, very few studies have characterised it on a molecular basis. A study from Punjab reported that among 34.4% of XDR, only 0.5% were positive for *CTX-M-15* genes.[23] In an overview from 2019–2020, populations in the setting of Lahore, Pakistan, Kim *et al*. reported 45 XDR isolates out of which 18 were selected for molecular characterisation. They found that *CTX-M-15* gene resides in IncY plasmid which co-harbours with qnr (fluoroquinolone-resistance) genes with numerous

recent point mutations.[12,24] In the present study, it was found that 83.9% of 130 XDR *S. typhi* isolates were positive for the *CTX-M-15* gene and only 22 did not express the *CTX-M-15* gene. It can be assumed that these 22 isolates were XDR by conventional methods, demonstrating resistance to 3rd generation cephalosporin, which might have been attributed to the presence of an AmpC gene. These are chromosomal genes which have recently been captured on the plasmid and are responsible for co-resistance phenomena in Enterobacteriaceae.[24] The main limitation of this study was the inability to determine other ESBL genes and *CTX-M* variants.

CONCLUSION

This study reported an alarming high frequency of XDR *S. typhi,* i.e. 87.24%. Among these XDR isolates, 83.9% were harbouring *CTX-M-15* gene. These circulating XDR *S. typhi* pose a serious public health threat as they could transmit and proliferate not only in humans but in the environment as well. Therefore, the awareness of antimicrobials consumption and its usage in all sectors should be raised. Likewise, detection tests for ESBLs in suspected isolates should be introduced in medical laboratories to facilitate rapid detection and proper treatment, discouraging unprecedented use of antibiotics.

ETHICAL APPROVAL:

This study was conducted at PNS Shifa Hospital and BUHS, Karachi after the approval from Ethical Review Committee of BUHS under the reference number (ERC 82/ 2021).

PATIENTS' CONSENT:

Informed, written consents were obtained from the patients participating in this study.

COMPETING INTEREST:

There was no conflict of interest declared by the authors.

AUTHORS' CONTRIBUTION:

RS: Conception of the study, collection and interpretation of data for the work, drafting and revising it critically for important intellectual content.
YT: Supervision and critical revision for important intellectual content.
LS: Conception of the study, clinical supervision and critical revision for important intellectual content.
FA: Supervision and analysis of molecular work.
SB: Literature review and layout.
All authors approved the final version of the manuscript to be published.

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