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Original Article

Microbiology Section

Detection of *bla*$_{CTX-M}$ Extended Spectrum Betalactamase Producing Salmonella enterica Serotype Typhi in a Tertiary Care Centre

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ABSTRACT

Introduction: Infections caused by *Salmonella* are an important public health threat in tropical and subtropical countries. Due to the emergence of resistance to ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole (multidrug resistant salmonellae) in the late 1980s, fluoroquinolones and extended spectrum cephalosporins became the drugs of choice. Resistance to cefotaxime and ceftriaxone due to the production of Extended Spectrum Beta Lactamase (ESBL) and reduced susceptibility to ciprofloxacin have emerged resulting in treatment failure. The Cefotaximase (CTX-M) type ESBLs are the most widespread beta lactamase among Enterobacteriaceae including salmonellae.

Aim: To detect the presence of *bla*$_{CTX-M}$ in salmonellae causing human infections. Detection of *qnr* genes to identify the coexistence of *bla*$_{CTX-M}$ and *qnr* gene.

Materials and Methods: The study included 103 consecutive, non-repetitive salmonellae isolated from clinical specimens obtained from July 2015- June 2016 which were identified up to species level by conventional/automated methods .Susceptibility to various classes of antimicrobial agents was determined by disc diffusion method. Minimum Inhibitory Concentration (MIC) to cefotaxime and ceftriaxone was determined by agar dilution method. The results were interpreted in accordance with Clinical & Laboratory Standard Institute (CLSI) (guidelines 2015. Detection of the ESBL phenotype was performed by the combined disk method. Polymerase Chain Reaction (PCR) amplification of all isolates was performed using group specific primers to characterize the presence of *bla*$_{CTX-M,}$ *qnrA* ,*qnrB* and *qnrS*.

Result: Of the 103 study isolates two isolates of *Salmonella typhi* were resistant to cefotaxime and ceftriaxone and had a MIC of 128µg/ml. PCR amplification and sequencing detected the presence of *bla*$_{CTX-M-15}$ in these two isolates. These two isolates exhibited resistance to ciprofloxacin in vitro but *qnr* gene was not detected in these isolates.

Conclusion: Resistance to third generation cephalosporins among salmonellae is a cause for concern as it may lead to treatment failure. It is imperative to continuously monitor the susceptibility pattern as enteric fever is endemic in India.

Keywords: Enteric fever, Multidrug resistance, Third generation cephalosprorins

INTRODUCTION

Infections caused by *Salmonella* are an important public health threat in tropical and subtropical countries. In the past, ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole were the antibiotics used for the treatment of enteric fever [1]. Due to the emergence of multidrug resistant among salmonellae in the late 1980s, fluoroquinolones namely ciprofloxacin and extended spectrum cephalosporins such as ceftriaxone and cefotaxime became the drugs of choice [1]. More recently azithromycin has been found to be effective in the treatment of enteric fever [2,3]. Among *Salmonella typhi* resistance to cefotaxime and ceftriaxone due to the production of ESBL and reduced susceptibility to ciprofloxacin resulting in treatment failure is being reported from many countries [4].

The ESBLs reported in salmonellae includes *TEM, SHV, PER* and *CTX-M* [5]. Among Enterobacteriaceae including salmonellae, the Cefotaximase (*CTX-M*) type ESBLs are the most widespread betalactamase. The earliest *CTX-M1* type Cefotaximase was isolated in 1989 in Germany [6]. They belong to Ambler class A and are mainly plasmid borne. They hydrolyse cefotaxime more effectively than ceftazidime or ceftriaxone. These enzymes have 40% identity with the *TEM* or *SHV* type betalactamase. The *CTX-M* enzyme consists of 40 types [7].

Seven *CTX-M* beta lactamases (*CTX-M* 2,3,4,5,6,7,9,15) in *Salmonella enterica* have been reported from South America, Europe and Mediterranean countries. Of these, *CTX-M* 5,9,15 have been reported predominantly in the non-typhoidal strains [5]. However, *CTX-M* in *Salmonella enterica* serotype *typhi* is rarely described from Indian isolates [8]. This study was therefore undertaken to detect the presence of *bla*$_{CTX-M}$ in salmonellae causing human infections. Fluoroquinolone resistance occurs either due to mutation in the quinolone resistance determining region of *gyrA*, *gyrB*, *parC* and *parE* or due to plasmid mediated resistance of *qnr* type [9]. Since, it has been found that the cephalosporin resistance mediated by ESBL often coexists with *qnr* genes that encodes for ciprofloxacin resistance, detection of *qnr* genes was also done [4]. Thus this study was done to detect *bla*$_{CTX-M}$ in salmonellae that cause human infections and to identify the coexistence of *bla*$_{CTX-M}$ and *qnr* gene.

MATERIALS AND METHODS

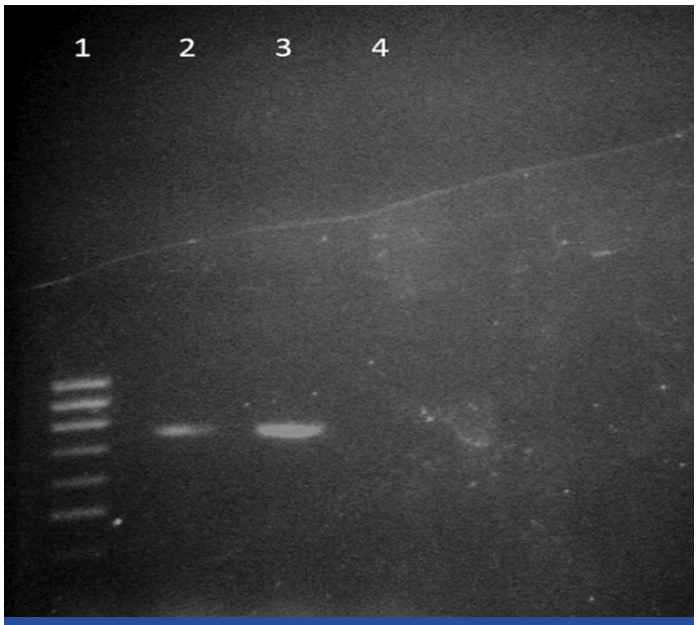
This prospective period study was conducted at Sri Ramachandra Medical College and Research Institute after obtaining Institutional ethics committee approval. The study was conducted form July 2015 to June 2016.

Inclusion Criteria

All isolates of *Salmonella* from clinical samples with a relevant clinical history.

Gene	Primer Sequence 5'- 3'	Product size
bla_{CTX-M}	*CTX-M*-F: CGCTGTTGTTAGGAAGTGTG *CTX -M*-R: GGCTGGGTGAAGTAAGTGAC	754bp
qnrA	*qnrA*- F: ATTTCTCACGCCAGGATTTG *qnrA*- R: GATCGGCAAAGGTTAGGTCA	516-bp
qnrB	*qnrB*- F: GATCGTGAAAGCCAGAAAGG *qnrB*- R: ACGATGCCTGGTAGTTGTCC	469-bp
qnrS	*qnrS*- F: ACGACATTCGTCAACTGCAA *qnrS*- R : TAAATTGGCACCCTGTAGGC	417-bp

[Table/Fig-1]: Primers used in the study.
F- Forward primer.
R- Reverse primer.



[Table/Fig-2]: PCR for $bla_{CTX-M\ 15}$.
Lanes 1: Molecular mass marker (100bp DNA ladder); Lane 2: Positive control (Previous positive bla_{CTX-M} was confirmed by gene sequencing); Lane 3: $bla_{CTX-M15}$ positive (amplicon size- 754 bp); Lane 4: Negative control - *E.coli* ATCC® 25992™

Exclusion Criteria

Duplicate isolates from the same patient were excluded.

Bacterial Isolates

One hundred and three *Salmonella* isolates from clinical specimens such as blood (94), stool (7), cerebrospinal fluid (1) and pus from splenic abscess (1) collected were included in the study. All the isolates were identified up to species level by conventional / automated methods using VITEK-2 system (Vitek2 GN -card; BioMerieux, Brussels, Belgium). The isolates were serotyped on the basis of agglutination with somatic O, phase I flagellar H antigens by the slide agglutination tests with antisera (King Institute, Chennai) as specified by the Kauffmann- White scheme.

Antimicrobial Susceptibility Testing

By the disk diffusion antimicrobial susceptibility was determined on Mueller -Hinton agar and interpreted according to the guidelines of the CLSI 2015. The antibiotics tested were ciprofloxacin (5 µg), ceftriaxone (30 µg) and cefotaxime (30 µg), chloramphenicol (30 µg), trimethoprim–sulfamethoxazole (1.25/23.75 µg), ampicillin (10µg), azithromycin (15 µg). MIC to cefotaxime and ceftriaxone was determined by agar dilution method with the range tested being 0.008-128µg/ml and interpreted in accordance with CLSI guidelines 2015 [10].

Detection of the ESBL phenotype was performed by the combined disk method using cefotaxime (30 µg) and ceftazidime (30 µg), based on the inhibitory effect of clavulanic acid [5,7]. *Escherichia coli* (ATCC® 25992™) (negative control) and *Klebsiella pneumoniae* (ATCC® 700603™)(positive control) were used as controls. The zone diameter difference of >5 mm for either antimicrobial agent tested in combination with clavulanic acid versus the zone diameter of the agent when tested alone, was considered indicative of production of ESBL

Polymerase Chain Reaction (PCR) amplification

All isolates were subjected to PCR using group specific primers to characterise the bla_{CTX-M} [10]. Co-existence of *qnrA, qnrB* and *qnrS* were also looked for [11]. The primers used in the study are shown in [Table/Fig-1].

Extraction of DNA: DNA was extracted from the study isolates by boiling method. A single colony was inoculated into 1.5ml of Luria Bertani broth and incubated overnight. This was centrifuged at 10,000 rpm for 10 minutes. The pellets were suspended in 500µl of distilled water and lysed by heating at 100ºC for 10 minutes and centrifuged for 1 minute. The supernatant was utilized as a template for amplification.

PCR conditions for bla_{CTX-M}: 2 µl of the supernatant was mixed in the 23 µl of the master mix, which contained 0.1 µl of Taq polymerase (Takara Bio Inc.) in 2.5 µl of 10x Taq polymerase buffer, 0.5 µl of dNTP ((Takara Bio Inc.), 1 µl of primer and 18.9 µl Milli Q. Amplification reactions were performed under the following conditions: initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds with an extension at 72°C for 50 seconds, and a final extension for one cycle at 72°C for 5 minutes. The PCR product was then run on a 1.5 % agarose gel for detection of the amplified fragment [11].

PCR conditions for *qnrA, qnrB and qnrS*: *A* 2 µl of the supernatant was mixed in the 23 µl of the master mix, which contained 0.1 µ l of Taq polymerase (TaKaRa Bio Inc.) in 2.5 µl of 10x Taq polymerase buffer, 0.5 µl of dNTP (TaKaRa Bio Inc.), 1 µl of primer and 18.9 µl Miili Q.

Amplification reactions were performed under the following conditions: initial denaturation at 94°C for 5 minutes, followed by 32 cycles of denaturation at 94°C for 45 seconds, annealing at 53°C for 45 seconds with an extension at 72°C for 60 seconds, and a final extension for one cycle at 72°C for 5 minutes. The PCR product was then run on a 1.5 % agarose gel for detection of the amplified fragment [12].

DNA Sequencing

The DNA of bla_{CTX-M} positive isolates was extracted with PureLink Genomic DNA Kit (Invitrogen), according to the user manual provided with the kit and subjected to automated DNA sequencing (ABI 3100, Genetic Analyser, Applied Biosystems, Foster city, CA, USA). The aligned sequences were analysed with the Bioedit sequence program and similarities searches for the nucleotide sequences were performed with the BLAST program.

Clinical History

The clinical history of the patients with ESBL producing *Salmonella* infection was collected retrospectively from the medical records department. The data collected were the chief complaints during admission, course in the hospital stay, treatment given and the outcome.

RESULTS

Of the 103 study isolates, the majority were *Salmonella typhi* (68), followed by *Salmonella paratyphi A* (26). The others serotypes were *Salmonella typhimurium* (7) and *Salmonella enteritidis* (2).

The antimicrobial susceptibility profile of the 103 *Salmonella* isolates was ampicillin 87.4% (90/103), chloramphenicol 96.1% (99/103), cotrimoxazole 96.1% (99/103) ciprofloxacin 91.2%(94/103), ceftriaxone 98.1%(101/103), cefotaxime 98.1%(101/103) and azithromycin (100%).

MIC_{90} to both cefotaxime and ceftriaxone was 1 µg/ml. Two isolates of *Salmonella typhi* which were isolated from blood culture were resistant to cefotaxime and ceftriaxone and had a MIC of 128µg/ml. PCR amplification and sequencing detected the presence of $bla_{CTX-M-15}$ in these isolates. PCR for $bla_{CTX-M-15}$ is shown in [Table/Fig-2]. *qnr* was not detected in any of the study isolates.

DISCUSSION

Enteric fever is endemic in India with *Salmonella enterica var Typhi (S. typhi*) and *Salmonella enterica var Paratyphi A (S. paratyphi A)* being the major causative agents [1]. These pathogens are transmitted by the faeco-oral route in regions with poor standards of hygiene and sanitation accounting for high morbidity and mortality. Antibiotic therapy constitutes the mainstay of management of enteric fever. Failure to treat an infection properly leads to prolonged illness, complications and development of carrier state. Fluoroquinolones and third-generation cephalosporins were used for the treatment of salmonellae infections after the emergence of MDR Salmonellae (resistant to ampicillin, chloramphenicol and co-trimoxazole) [9,13,14]. This lead to the occurrence of reduced susceptibility to ciprofloxacin. Resistance to third-generation cephalosporins among salmonellae though low at present (1%) is also emerging in India [3].

Plasmid-mediated quinolone resistance is mediated by *qnr* genes which encode a protein that protects DNA gyrase from ciprofloxacin and by *aac(6')-Ib-cr*, an aminoglycoside modifying enzyme with activity against ciprofloxacin. Plasmids bearing *qnr* or *aac(6')-Ib-cr* may also carry an extended spectrum cephalosporin resistance gene, which results in the development of resistance to both fluoroquinolone and cephalosporin and thereby limiting the therapeutic options available for the management of invasive salmonellosis [4,15]. In this study the *CTX-M* bearing salmonellae did not harbor the *qnr* genes, though they exhibited resistance to ciprofloxacin in vitro. Hence, the presence of other quinolone resistance mediators like *gyr A*, *gyr B* and *par C* should be looked for.

In countries like Germany, Philippines and Kuwait *CTX-M-15* and *SHV-12* has been described in typhoidal Salmonellae which showed high-level resistance to ceftriaxone. *S. typhi* producing ACC-1 *AmpC* β- lactamase has been reported from India [4,16]. Though ESBL producing Non typhoidal *Salmonella* have been cited worldwide, their occurrence in serovar *Typhi* and *Paratyphi A* is not common [9,15,17].

The incidence of *CTX-M-15* among enteric fever producing salmonellae is not alarming as evident in this study, but the potential for their widespread dissemination may result in treatment failures. In many tropical countries including the Indian subcontinent, the widespread availability and uncontrolled use of antibiotics can lay the foundation for rapid dissemination [18].

Since, the bla_{CTX-M} is located on plasmids in conjunction with mobile genetic elements such as IS *Ecpl*, they disseminate rapidly. Gene transfer experiments in many studies have been performed to localize their position on plasmids. But non-transferrable bla_{CTX-M} has also been described suggesting their possible chromosomal location. There is a reservoir of ESBLs and *CTX-M* genes among *Escherichia coli* and *Klebsiella pneumoniae* which constitutes a huge risk factor for spread of resistance to other pathogenic Enterobacteriaceae [19]. Additionally, use of third generation cephalosporins to treat enteric fever and other infections caused by *Salmonella* species may the increase the incidence of *CTX-M* producing salmonellae. Decreased susceptibility to ciprofloxacin among *Salmonella* species has also contributed to the increased use and subsequent development of resistance to cephalosporins among salmonellae. The continued surveillance of cephalosporin resistant *Salmonella* in combination with prudent use of these agents both in animals and humans is crucial for limiting the spread of *CTX-M* producing salmonellae [19].

There are 2 reports of occurrence of *CTX-M15* type ESBL in *Salmonella enterica*. This includes two Indian patients with *Salmonella typhi* and 2 others with *paratyphi C* and *paratyphi A*. All were travellers returning from India to UAE and Japan respectively [17,20].

The occurrence of $bla_{CTX-M15}$ in conjunction with quinolone resistance is alarming because it further limits the therapeutic options available for typhoid fever especially in endemic countries such as India [15]. In the past *CTX-M15* ESBLs have been found exclusively in *Escherichia coli* and *Klebsiella* species. *Salmonella* strains can acquire the gene encoding for this enzyme from *Escherichia coli* and *Klebsiella* species in the community. This speculation is supported by the fact that the mobile genetic element IS*Ecpl* is responsible for mobilization of the *bla* genes and it is identified upstream of several bla_{CTX-M} genes [17] .

Azithromycin is presently being used for the management of uncomplicated typhoid fever and associated with a prompt resolution of clinical symptoms and low prevalence of relapse and convalescent fecal carriage [12]. However, azithromycin treatment failure in a patient with invasive salmonellae infection has been described [21].

CONCLUSION

To conclude, enteric fever producing salmonellae harbor the *CTX-M* gene thus making cefotaxime ineffective for treatment. This also implies that use of other third generation cephalosporins for treatment may result in failure of therapy. Though the incidence of *CTX-M* in this study is not alarming, the potential for rapid dissemination due to the location of the genetic elements and the huge reservoir encountered in Enterobactericeae is a cause for concern.

LIMITATION

In present study, sample size was too small to conclude any statistically significant results. Also, further studies on Salmonella harboring TEM, SHV and PER genes needs to be carried out as these genes also confers resistance to third generation cephalosporins.

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