Research Article

Detection of multidrug resistance and associated genes among *Salmonella* species from enteric fever cases

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Abstract

Background*: Salmonella* spp. has rapidly gained resistance to ampicillin, chloramphenicol, co-trimoxazole, and tetracycline (ACCoT) which necessitated the use of fluoroquinolones and cephalosporins. However, there are reports on emergence of fluoroquinolone-resistant *Salmonella* isolates in various parts of Asia and low resistance to third-generation cephalosporins. The incidence of multi-drug resistant (MDR) *Salmonella enterica* serovar Typhi (*S.* Typhi) in India increased in the 1990s. After a decade, studies showed reduced MDR percentage. Hence this study was performed to detect antibiotic resistance patterns and associated genes among clinical isolates of *Salmonella*.

Methods*:* A total of 171 clinical isolates of *Salmonella* isolates collected between 2011 to 2016 from a tertiary care hospital in Chennai, India were included. Antibiotic susceptibility testing and screening for extended-spectrum β-lactamase production was performed. Genes encoding resistance to β-lactam antibiotics (bla_{TEM}, bla_{SHV}, and $bla_{\text{CTX-M}}$), chloramphenicol (*cat*, *cmlA*, and *floR*), co-trimoxazole (*sul1*, *sul2*, *sul3*, and *dfr*), and tetracycline (*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), and *tet*(G)) were detected using PCR and nucleotide sequencing analysis.

Results*:* The majority of the isolates were susceptible to ACCoT antibiotics, cephalosporins, and carbapenems. Most of the isolates were resistant to nalidixic acid and 1.7% were sensitive to ciprofloxacin. All the isolates (n=171) were negative for ESBL. Multidrug resistance was seen in 4.1% isolates and all the MDR *Salmonella* isolates were found to contain $bla_{\text{TEM-1}}$(TEM-1-type β-lactamase), *cat*, *dfrA17*, *sul1*, and *tet*(B) genes.

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Conclusion: We have observed a low MDR prevalence and reduced susceptibility to the fluoroquinolone, ciprofloxacin. This changing susceptibility pattern of *Salmonella* species over time warrants continuous monitoring and judicious use of antibiotics to prevent the emergence of resistant strains.

Keywords: Beta-lactamases, enteric fever, fluoroquinolones, multidrug resistance, Salmonella species

Introduction

In the 1990s, the treatment of choice for infections due to *Salmonella* spp. included chloramphenicol, ampicillin, co-trimoxazole (trimethoprim-sulfamethoxazole) or tetracycline (ACCoT). However, *Salmonella* has rapidly gained resistance to these antibiotics which necessitated the use of a quinolone (nalidixic acid), fluoroquinolones, such as ciprofloxacin and cephalosporins as the drugs of choice for the treatment of enteric fever. There are reports on the emergence of fluoroquinolone-resistant isolates in various parts of Asia. In 2001-2005, *S.* Typhi resistance to fluoroquinolones was 10%, which increased to 66% during 2011-2015 and there have been a few reports of resistance to third-generation cephalosporins throughout both time periods.[1]

Chloramphenicol resistance is mediated by the plasmid encoded chloramphenicol acetyltransferases (CAT)[2], by a non-enzymatic chloramphenicol resistance gene *cmlA*[3] or due to chloramphenicol/florfenicol exporter encoded by a plasmid-borne gene *floR*.[4]

In clinical enterobacterial isolates, resistance to the β-lactam drug, ampicillin, occurred most commonly due to plasmid encoded TEM- and SHV-β-lactamases. In Gram-negative enteric bacteria, trimethoprim-insensitive dihydrofolate reductase (DHFR) encoded by different *dfr* genes and drug resistant variants of dihydropteroate synthase (DHPS) encoded by plasmid-borne *sul* genes are responsible for trimethoprim and sulfamethoxazole resistance.[5] Most of the *dfr* genes have been found on transposons, plasmids and reside as gene cassettes within variable parts of integrons which assist in the spread of trimethoprim resistance.[1]

In Gram-negative microorganisms, tetracycline resistance is mainly due to efflux proteins expressed by *tet* genes.[6] Localization of *tet* genes on mobile genetic structures such as plasmids, transposons, and integrons might accelerate the spread of tetracycline resistance among bacteria.[1]

The first multidrug-resistant (MDR) strains (i.e. *Salmonella* strains resistant to first line antibiotics such as ampicillin, chloramphenicol, co-trimoxazole and tetracycline) of *S.* Typhi emerged in Southeast Asia in 1987.[7] During the 1990s, there was an increased incidence of MDR *S.* Typhi (MDRST) in India. After a decade, reports from India showed a decrease in MDR percentage as low as 5%.[1]

This study was performed to detect the pattern of drug resistance, β-lactamase production and genes associated with resistance to ampicillin, chloramphenicol, co-trimoxazole, tetracycline in *Salmonella* species from enteric fever cases in Chennai, India.

Methods

A total of 171 consecutive, non-duplicate *Salmonella* isolates from blood samples of patients attending tertiary care hospitals in Chennai during 2011 to 2016 were included in this study. Standard biochemical procedures were followed for characterization and identification of the isolates.[8] *Salmonella* isolates were serotyped using specific antisera procured from the King Institute of Preventive Medicine and Research, Chennai, India.

Kirby–Bauer disk-diffusion method was performed to detect antibiotic susceptibility of the isolates against 12 antibiotics as per CLSI 2017 guidelines.[9] The following antibiotics were tested: ampicillin (10µg), chloramphenicol (30µg), co-trimoxazole (1.25/23.75µg), tetracycline (30µg), cefotaxime (30µg), ceftazidime (30µg), ceftriaxone (30µg), cefixime (5µg), cefepime (30µg), nalidixic acid (30µg), ciprofloxacin (5µg) and imipenem (10µg). Antibiotic discs were obtained from Hi-Media Laboratories. The MICs of ampicillin, chloramphenicol, and tetracycline (Hi-Media Laboratories) were determined by the agar dilution method as per CLSI 2017 guidelines.[9] *Escherichia coli* ATCC 25922 was used as the control strain.

Detection of β-lactamase-encoding genes and nucleotide sequencing analysis

Combination disc method was used for screening of extended-spectrum β-lactamase (ESBL) production among the isolates.[9] Detection of β-lactamase encoding genes bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$ was carried out in MDR *Salmonella* isolates using polymerase chain reaction (PCR), and nucleotide sequencing analysis was performed using an ABI 3730XL DNA Analyser (Applied Biosystems).[10]

Detection of genes encoding resistance to chloramphenicol, co-trimoxazole and tetracycline and DNA sequencing

Genes encoding resistance to chloramphenicol (*cat*, *cmlA* and *floR*), co-trimoxazole (*sul1*, *sul2*, *sul3* and *dfr*) and tetracycline (*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E) and *tet*(G)) were detected in MDR *Salmonella* isolates by PCR using published primer sets with a boiled suspension of bacterial cells as the DNA template (Table 1). Amplification of target DNA was carried out in a thermal cycler (Eppendorf, Germany) in a reaction mixture volume of 25 µl containing 1.5 U *Taq* DNA polymerase (New England BioLabs) in the reaction buffer provided by the manufacturer containing 1.5 mM $MgCl_2$,200µM dNTPs, 0.2 µM selected primer and 2 µl DNA template. PCR products were separated by agarose (1.5 %) gel electrophoresis. The amplified product was sequenced using an ABI 3730XL DNA Analyser (Applied Biosystems, USA). Nucleotide sequences were analysed by searching GenBank using BLAST (http://www.ncbi.nlm.nih.gov/blast/).

Table 1: Primers used in this study.

Gene(s)	Oligonucleotide sequence (5′ to 3′)	Annealing temperature (°C)	Amplicon size (bp)	Reference
bla_{TEM}	F: ATGAGTATTCAACATTTCCG	58	867	[11]
	R: CTGACAGTTACCAATGCTTA			
bla_{SHV}	F: GGTTATGCGTTATATTCGCC	60	867	
	R: TTAGCGTTGCCAGTGCTC			
bla_{CTX-M}	F: ATGTGCAGYACCAGTAARGT	50	593	
	R: TGGGTRAARTARGTSACCAGA			
Cat	F: AAGTTGGCAGCATTCACCCG	61	573	[12]
	R: TCGTGGTATTCACTCCAGAGCG			
cmlA	F: TGTCATTTACGGCATACTCG	55	455	[13]
	R: ATCAGGCATCCCATTCCCAT			
floR	F: CACGTTGAGCCTCTATAT	55	868	
	R: ATGCAGAAGTAGAACGCG			
sul1	F: TGGTGACGGTGTTCGGCATTC	63	789	
	R: GCCAGGGTITCCGAGAAGGTG			
sul2	F: CGGCATCGTCAACATAACC	50	722	
	R: GTGTGCGGATGAAGTCAG			
sul3	F: CATTCTAGAAAACAGTCGTAGTTCG	51	990	
	R: CATCTGCAGCTAACCTAGGGCTTTGGA			
dfrA1, dfrA5, dfrA15, dfrA15b, dfrA16, dfrA16b	F: GTGAAACTATCAATGG	55	474	[14]
	R: TTAACCCTTTTGCCAGATTT			
dfrA14, dfrA6	F: GAGCAGCTICTITTIAAAGC	60	393	
	R: TTAGCCCTTTIICCAATTTT			
dfrA7, dfrA17	F: TTGAAAATTTCATTGATTG	55	474	
	R: TTAGCCTTTTTTCCAAATCT			
dfrB1, dfrB2, dfrB3	F: GATCACGTGCGCAAGAAATC	60	141	
	R: AAGCGCAGCCACAGGATAAAT			
dfrA12, dfrA13	F: GGTGSGCAGAAGATTTTTCGC	60	319	
	R: TGGGAAGAAGGCGTCACCCTC			
tet(A)	F: GTAATTCTGAGCACTGTCGC	62	917	[15]
	R: CTGCCTGGACAACATTGCTT			
tet(B)	F: CTCAGTATTCCAAGCCTTTG	57	396	
	R: ACTCCCCTGAGCTTGAGGGG			
tet(C)	F: GGTTGAAGGCTCTCAAGGGC	62	589	
	R: CCTCTTGCGGGAATCGTCC			
tet(G)	F: GCAGCGAAAGCGTATTTGCG	60	680	
	R: TCCGAAAGCTGTCCAAGCAT			
tet(D)	F: GCTGGTGATTACACTGCTGG	60	477	[16]
	R: AGTATTGCCGCAATGACAAA			
tet(E)	F: CACTGTGATGATGGCACTGG	60	468	
	R: GCCTGTAACGAAAGTTGACC			

Results

Of the 171 isolates recovered from blood cultures, 76.6% (131/171) were *Salmonella* Typhi, 22.2% (38/171) were *Salmonella* Paratyphi A, and 1.2% (2/171) were *Salmonella* Paratyphi B. Antibiotic susceptibility patterns of *S.* Typhi and *S.* Paratyphi A isolates are shown in Table 2. The *S.* Paratyphi B isolates were sensitive to all the antibiotics tested, except one isolate which was resistant to nalidixic acid and intermediate resistant to ciprofloxacin.

Table 2: Antibiotic resistance pattern of the *S.* Typhi and *S.* Paratyphi A isolates

Antibiotics	***S.* Typhi (n = 131)**		***S.* Paratyphi A (n=38)**		**Total (n= 169)**	
	n	%	n	%	n	%
Ampicillin	6	4.6	1	2.6	7	4.1
Chloramphenicol	6	4.6	1	2.6	7	4.1
Co-trimoxazole	6	4.6	1	2.6	7	4.1
Tetracycline	6	4.6	1	2.6	7	4.1
Cefotaxime	0	0	0	0	0	0
Ceftazidime	0	0	0	0	0	0
Ceftriaxone	0	0	0	0	0	0
Cefepime	0	0	0	0	0	0
Cefixime	0	0	0	0	0	0
Nalidixic acid	126	96.2	38	100	164	97
Ciprofloxacin	6	4.6	3	7.9	9	5.3
Imipenem	0	0	0	0	0	0

Of the 38 *S.* Paratyphi A isolates, one was resistant to ACCoT antibiotics, three were resistant to ciprofloxacin and all were sensitive to the cephalosporins and imipenem. Minimum Inhibitory Concentration (MIC) of 131 *S.* Typhi isolates showed sensitivity to ampicillin (≤8 µg/ml) in 124 (94.6%), and chloramphenicol (≤8µg/ml) and tetracycline (≤4 µg/ml) in 125 (95.4%). Of the 38 *S.* Paratyphi A isolates, 37 (97.4%) were sensitive to ampicillin (≤8µg/ml), chloramphenicol (≤ 8µg/ml) and tetracycline (≤4 µg/ml).

Among the 171 *Salmonella* isolates tested, 163 (95.3%) were sensitive to ampicillin and 164 (95.9%) were sensitive to chloramphenicol, co-trimoxazole and tetracycline. Multidrug resistance was seen in 7/171 (4.1%) isolates, in which six isolates were serotype *S.* Typhi and one was *S.* Paratyphi A. All the *Salmonella* isolates (n=171) were susceptible to the cephalosporins (cefotaxime, ceftazidime, ceftriaxone, and cefepime), except one isolate, which showed intermediate resistance to cefixime. The majority of the isolates were resistant to nalidixic acid (96.5%) and 1.7% were sensitive, 5.3% were resistant and 93% were intermediate resistant to ciprofloxacin. However, 100% (171/171) sensitivity was observed for the carbapenem antibiotic, imipenem.

All the isolates were negative for ESBL production by the combination disc method. TEM-type β-lactamase (bla_{TEM}) was detected in 7/171 (4.1%) isolates by PCR. Nucleotide sequencing analysis revealed that the seven isolates carried the $bla_{\text{TEM-1}}$ gene (Accession number KF551994) but not bla_{SHV} and $bla_{\text{CTX-M}}$ genes. The isolates showed resistance to ampicillin but were sensitive to the cephalosporins.

Chloramphenicol resistance encoding gene *cat* was detected in 7/171 (4.1%) *Salmonella* isolates which showed resistance to chloramphenicol. None of the isolates were positive for *cmlA* and *floR* genes. The seven co-trimoxazole resistant *Salmonella* isolates were positive for *dfr* and *sul1* genes. Nucleotide sequence analysis revealed that the seven *dfr* positive isolates carried the *dfrA17* gene (GenBank accession number MK348062) and none of the resistant isolates (n = 7) were positive for *sul2*, *sul3*, and other *dfr* genes tested in this study. *Salmonella* isolates which showed resistance to tetracycline (7/171) were positive for *tet*(B) gene and negative for *tet*(A), *tet*(C), *tet*(D), *tet*(E) and *tet*(G) genes.

Discussion

Enteric fever caused by *Salmonella* species is a major systemic infection in developing countries, including India. Most of the isolates were sensitive to ACCoT drugs (ampicillin, chloramphenicol, co-trimoxazole and tetracycline), cephalosporins and carbapenems. The seven multidrug-resistant isolates were resistant to nalidixic acid and of intermediate resistance to ciprofloxacin. All the MDR *Salmonella* isolates were sensitive to the cephalosporins tested in this study.

Mutations in genes encoding DNA gyrase (*gyrA* and/or *gyrB*) and topoisomerase IV (*parC* and/or *parE*) were reported to confer resistance to nalidixic acid and reduced susceptibility to fluoroquinolones. Mutation analysis in quinolone resistance determining regions of *gyrA*/*gyrB* and *parC*/*parE* were not analysed in this study. Several studies have documented that there is a steady increase in fluoroquinolone resistant *Salmonella* strains in India.[1] Nalidixic acid resistant *Salmonella* isolates have been reported to show decreased fluoroquinolone susceptibility *in-vivo*, which affects the treatment outcome. This led to a revision of ciprofloxacin breakpoints in CLSI guidelines 2012,[1] and we have interpreted our results based on CLSI 2017 guidelines. As per the revised CLSI guidelines, 1.7% of our study isolates were sensitive to ciprofloxacin with the majority in the intermediate range.

Chloramphenicol has been considered as a treatment of choice for enteric fever from 1948 until the 1970s, when widespread resistance occurred. The common mechanism of resistance to chloramphenicol in bacteria is its enzymatic inactivation by acetylation via acetyltransferases encoded by the *cat* gene,[2] which was detected in the seven chloramphenicol resistant *Salmonella* isolates in our study.

Ampicillin and co-trimoxazole were introduced in the treatment of infection caused by chloramphenicol resistant *Salmonella* strains. Ampicillin resistance in *Salmonella* is mainly due to the production of β-lactamase. We have detected $bla_{\text{TEM-1}}$ gene which encodes TEM-1 β-lactamase in our isolates. The TEM-1 β-lactamase has been shown to hydrolyze only penicillins and early cephalosporins, but the resistance range of TEM-1descendants may extend to broad-spectrum (3rd and 4thgeneration) cephalosporins.[17]

The combination of trimethoprim-sulfamethoxazole (co-trimoxazole) has been shown to have a synergistic effect and affects folic acid synthesis in bacteria. Most trimethoprim–

sulfamethoxazole resistance genes reside within integrons (horizontally transferable genetic elements) which play an important role in their dissemination.[5] The plasmid borne *dfrA17* gene, which encodes trimethoprim insensitive dihydrofolate reductase was detected in seven co-trimoxazole resistant isolates. However, to the best of our knowledge there are no reports on dihydrofolate reductase (DfrA17) producing typhoidal *Salmonella* from India. The seven co-trimoxazole resistant isolates in our study which were positive for *dfr* gene, also carried the sulfamethoxazole resistant gene *sul1*. In *Salmonella*, tetracycline resistance is mainly associated with *tet*(A), *tet*(B), *tet*(C), *tet*(D) or *tet*(G) genes.[1] Only *tet*(B) gene was detected among tetracycline resistant isolates in our study.

Multidrug-resistance in *Salmonella* due to transferable plasmids was reported worldwide in the late 1980s. A study on enteric fever in five Asian countries (India, China, Indonesia, Pakistan, and Vietnam) revealed that the prevalence of MDRST ranged from 7-65%.[18] The National *Salmonella* Phage Typing Centre in New Delhi conducted a study from 1990 to 1992 across India and reported that about 64% of *S*. Typhi were multidrug resistant. The study also reported that a high percentage of MDR isolates (71.5%) were observed in Central India, followed by North India (62%) and South India (58.2%).[19] In comparison with previous studies, which reported more than 50% MDR *Salmonella*,[18,19] we have observed a low MDR prevalence (7/171, 4.1%) among our study isolates, which is in agreement with a multicentre study report (less than 5% MDR) from India.[20] A decreasing trend has been reported in the isolation of MDRST strains in India.[1]

Conclusion

Over the last decade there has been a notable decline in MDR and re-emergence of *Salmonella* strains susceptible to ampicillin, chloramphenicol and co-trimoxazole. Several studies have also suggested the reuse of these first line antibiotics in the treatment of enteric fever. We have observed a decrease in susceptibility to ciprofloxacin, which requires MIC testing for susceptibility, along with stringent antibiotic policies to prevent the selection of existing resistant sub populations. This changing susceptibility pattern of *Salmonella* species over time warrants continuous monitoring and judicious use of antibiotics to prevent the emergence of resistant strains.

Declarations

Acknowledgement: None to acknowledge
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Author contributions: SE: Conceptualization, Methodology, Investigation, Formal analysis, Writing – Original draft preparation. GM: Investigation, Resources. EMS: Resources. ST: Investigation. SS: Supervision, Writing – Reviewing and Editing.

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