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Review article

Isolation, Identification and Antimicrobial Susceptibility testing of clinical strains of *Salmonella enterica*

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Abstract

Enteric fever, a gastrointestinal disease, triggered by *Salmonella enterica* infection is considered to be most prevalent and common infectious disease across the globe. The therapeutic regime to cater the *Salmonella* infection employs administration of antibiotic therapy. However, due to unwarranted exploitation of antibiotics to cater the infection has led to the emergence of resistance genes in *S. enterica* as a result of mutations in their chromosomal genes. In the current era, the complexity of MDR strains have resulted the need of thorough investigation of MDR clinical strains of *Salmonella enterica*. In line to this, the present study was based upon isolation and identification of *S. enterica* from stool samples of patients suffering from typhoid fever and analysis of drug resistance pattern by antimicrobial susceptibility testing (AST). In the present study, 280 stool samples from patients with enteric fever were collected from Sawai madhopur district (Rajasthan) during July 2022 to January 2024. The isolation of *Salmonella sp.* was carried out using enrichment and selective media viz. Bismuth Sulphite Agar (BSA) and Xylose Lysine Deoxycholate Agar (XLD) followed by biochemical characterization and AST studies by the Kirby Bauer disc diffusion method based on Clinical and Laboratory Standards Institute (CLSI) guidelines. Out of 280 stool samples tested, 268 clinical isolates of *S. enterica* was isolated and identified by various biochemical tests. In the AST studies, maximum clinical isolates exhibited resistance to Co-trimoxazole, 3rd Generation Cephalosporin (Cefixime), Carbapenems (Imipenem and Meropenem) and Penicillin's

(Amoxycillin/Potassium Clavulanate, Piperacillin/Tazobactam and Ticacillin/Clavulanate). The present study also reports isolation of 2 MDR strains of *S. enterica* whose further identification of genome sequencing studies can provide additional insights over presence of resistance genes to help aid in identification of novel antibiotics/targets to cater the *Salmonella* infection.

INTRODUCTION

Enteric fever is recognized as significant public health problem, specially in developing countries where inadequate sanitation and limited access to effective treatment contributes to its persistence (Akbar et al., 2015). It is mainly caused by *Salmonella enterica* and Salmonella bongori, which are transmitted through the fecal-oral route (Andino and Hanning, 2015). The spread of Salmonella infection is commonly associated with contaminated food sources such as poultry, dairy, and other animal products, leading to substantial economic losses in the food industry (Petri et al., 2008; Ehuwa et al., 2021). According to the World Health Organization (WHO), approximately 16 to 17 million typhoid cases are reported annualy across the globe, resulting in approximately 600,000 deaths (Ayuti et al., 2024). The asperity of a Salmonella eruption is majorly affected by bacterial strain characteristics, serovar distribution, environmental factors, and host susceptibility. Given the rising consumption of poultry products, the risk of Salmonella-induced foodborne infections continues to be a critical concern, particularly during seasonal transitions and the monsoon period from June to August (Omwandho & Kubota, 2010; Rahman et al., 2014). The Center for Disease Control and Prevention (CDC) has also highlighted multidrug-resistant *S. enterica* infections as a growing global health crisis due to the limited availability of effective antimicrobial agents (Bosch et al., 2016).

A major challenge in combating *S. enterica* infection is the emergence of antibiotic-resistant strains due to the prolonged and indiscriminate use of conventional antimicrobial agents. Resistance to first-line antibiotics such as ampicillin, chloramphenicol, amoxicillin, and trimethoprim-sulfamethoxazole was first reported in the late 1980s, necessitating the adoption of fluoroquinolones as an alternative treatment (Rowe et al., 1997; Rabsch et al., 2001). However, widespread fluoroquinolone resistance-attributed to genetic mutations in DNA gyrase and topoisomerase-IV (gyrA, gyrB, parC, and parE genes) has shifted the focus on third-generation cephalosporins (e.g., ceftriaxone, cefotaxime, ceftazidime, cefixime) and macrolides such as azithromycin (Galimand et al., 2005; Capoor et al., 2007). Despite ongoing research efforts, comprehensive data on the antimicrobial susceptibility profile of *S. enterica* remains insufficient (Eng et al., 2015). The lack of robust surveillance data and scientific studies on the evolving antimicrobial resistance patterns of *S. enterica* underscores the need for continuous monitoring and research efforts to develop targeted therapeutic strategies. The present study was

undertaken to ascertain the antimicrobial susceptibility pattern of clinical *Salmonella enterica* strains recovered from patients diagnosed with enteric fever in the Sawai Madhopur district, Rajasthan, India. The findings of the work will furnish valuable insights into the actual antibiotic resistance trends of clinical *S. enterica* strains, aiding in the development of effective therapeutic strategies for improved treatment outcomes.

MATERIALS AND METHODS

Patient selection criteria and Sample Collection

A total of 280 stool samples from patients with high fever (>100.4 F), a positive widal test (typhi "O" >1:40, typhi "H" >1:40, paratyphi "AH" >1:20 and paratyphi "BH" >1:20) along with symptoms like headache, stomach pain, weakness etc were included in this study. The samples were collected in sterilized containers from diiferent hospitals of Sawai Madhopur district during the period of July 2022 to January 2024. Prior to the sample collection, written consent was obtained from the respective hospitals with Reference No. RX-2022-017, 2022/1168, RX-55-217-2022, CNH/22/103 permitting the utilization of stool samples for research purposes while ensuring patient anonymity and confidentiality. The collected stool samples were transported to Soni Immunology Microbiology & Research Lab, Sawai Madhopur, Rajasthan for further isolation of Salmonella sp. and susceptibility testing.

Isolation of Salmonella sp.

For the isolation of Salmonella sp. and minimize the risk of the growth of other microbial species, a combination of selective enrichment and selective plating technique was employed as per the International Organization of Standards (ISO) (ISO-6579:2017). Briefly describing, 0.5-1.0 g of stool sample was transferred in 5 ml Selenite F broth for enrichment followed by incubation at 37°C for 24 hours. From the selenite broth enriched culture, 10 µl of loop full culture was streaked over selective Bismuth Sulphite Agar (BSA, Hi-Media) and Xylose Lysine Deoxycholate Agar (XLD, Hi-Media) plates followed by incubation at 37°C for 24-48 hours (Tosisa et al., 2020). The plates were observed for the appearance of colonies and their morphological characteristics. The colonies from BSA and XLD agar plates were picked up and streaked over nutrient agar plates for further biochemical identification.

Biochemical Identification

The suspected colonies were subjected to Gram's Staining by employing Gram Stain kit (Himedia) as per the instructions of manufacturer. The stained smears were observed under the FM-200 Microscope, Weswox, India for the presence of Gram-negative bacilli. The isolates were further identified by employing catalase test, oxidase test and IMViC group of biochemical assays (Hossain et al. 2006). The identified multi drug resistant *S. enterica* strain was re-confirmed by HiSalmonellaTM Identification Kit-KB011 (Hi-media) as per the manufacturer instructions (Table 1)

Table 1: Representing Biochemical tests for bacterial identification

Name of Biochemical test	Procedure	Positive Result	Reference
Methyl Red (MR) Test	1. Inoculate MR broth with bacterial cultures under study and incubate at 37°C for 24 hours. 2. Add a few drops of methyl red indicator.	Red color indicates a positive result.	Douglas et al. (1998), OIE (2000)
Voges-Proskauer (VP) Test	1. Inoculate VP broth with bacterial cultures under study and incubate at 37°C for 24 hours. 2. Add 3 drops of creatine solution, 2 drops of α-naphthol, and 3 drops of KOH.	Pink or red color indicates a positive result.	Douglas et al. (1998), OIE (2000)
Triple Sugar Iron (TSI) Test	1. Streak and stab the bacterial colony into TSI agar slant and incubate at 37°C for 24 hours. 2. Observe color changes in the slant and butt.	Yellow color change indicates a positive result.	Douglas et al. (1998), OIE (2000)
Simmons Citrate Test	1. Streak bacterial culture on Simmons Citrate agar slant. 2. Incubate at 37°C for 24 hours.	Blue color indicates a positive result.	Rahman et al. (2019)
Motility Indole Urea (MIU) Test	1. Streak bacterial colony on MIU agar slant. 2. Incubate at 37°C for 24 hours. 3. Add Kovacs reagent for the indole test.	Pink slant indicates urease-positive; red-cherry ring for indole test.	Rahman et al. (2019)

Antibiotics susceptibility Test

susceptibility of Salmonella isolates following Clinical and Laboratory Standards Institute (CLSI) guidelines. Colonies of *S. enterica* isolated from BSA agar were spread evenly over the entire surface of Mueller Hinton agar (MHA) plates with the help of a swab stick. A panel of antibiotics was placed on lawn culture of the test organism and incubated at 370C for 15 hours or overnight (Wayne 2011). The antibiotic panel of PBL Bio-Disc includes, amikacin (30 mcg), amoxicillin/clavulanic acid 20/10 mcg), ampicillin/sulbactam (10/10 mcg), azithromycin (15 mcg), cefixime (5 mcg), cefotaxime (30 mcg), cefpodoxime (10 mcg), ceftazidime (30 mcg), ceftriaxone (30 mcg), ciprofloxacin (5 mcg), chloramphenicol (30 mcg), co-trimoxazole (1.25/23.75 mcg), gentamicin (10 mcg), imipenem (10 mcg), kanamycin (5 mcg), levofloxacin (5 mcg), meropenem (10 mcg), nalidixic acid (30 mcg), nitrofurantoin (300 mcg), ofloxacin (5 mcg), piperacillin/tazobactam (100/10 mcg), streptomycin (10 mcg), tetracycline (30 mcg), ticarcillin/clavulanic acid (75/10 mcg), cefdinir (5 mcg), aztreonam (30 mcg), tobramycin (10 mcg). The diameter of the zone of inhibition (ZOI) after incubation was measured and the Zone of

The Kirby-Bauer disk diffusion method was employed to determine the antimicrobial

Inhibition (ZOI) for each antibiotic was compared to a table based on CLSI guidelines to indicate whether it was 'Resistant', 'Intermediate' or 'Susceptible'. *S. enterica* isolates resistant to three or more antibiotics were classified as multidrug resistant (MDR) isolates (Wayne, 2011; Rahman et al., 2019).

RESULTS

Of a total of 280 stool specimens processed, 268 (95.7%) stool specimens were positive for *S. enterica* growth via appearance of black color and silver color colonies over BSA and XLD agar plate respectively (Figure 1). The identity of these isolates were ascertained by series of biochemical tests followed by confirmation with HiSalmonella identification kit wherein the isolates was found to exhibit positive reaction in MR, TSI, Simmon Citrate test and negative reaction in VP, Urea and indole test as per the characteristics of Salmonella sp. In the confirmatory analysis by HiSalmonella Identification Kit, the isolates were found to exhibit positive reaction among 9 test parameters including MR, Urease, H2S production, citrate utilization, lysine utilization, ONPG, Lactose, Arabinose and Maltose (Table 2).

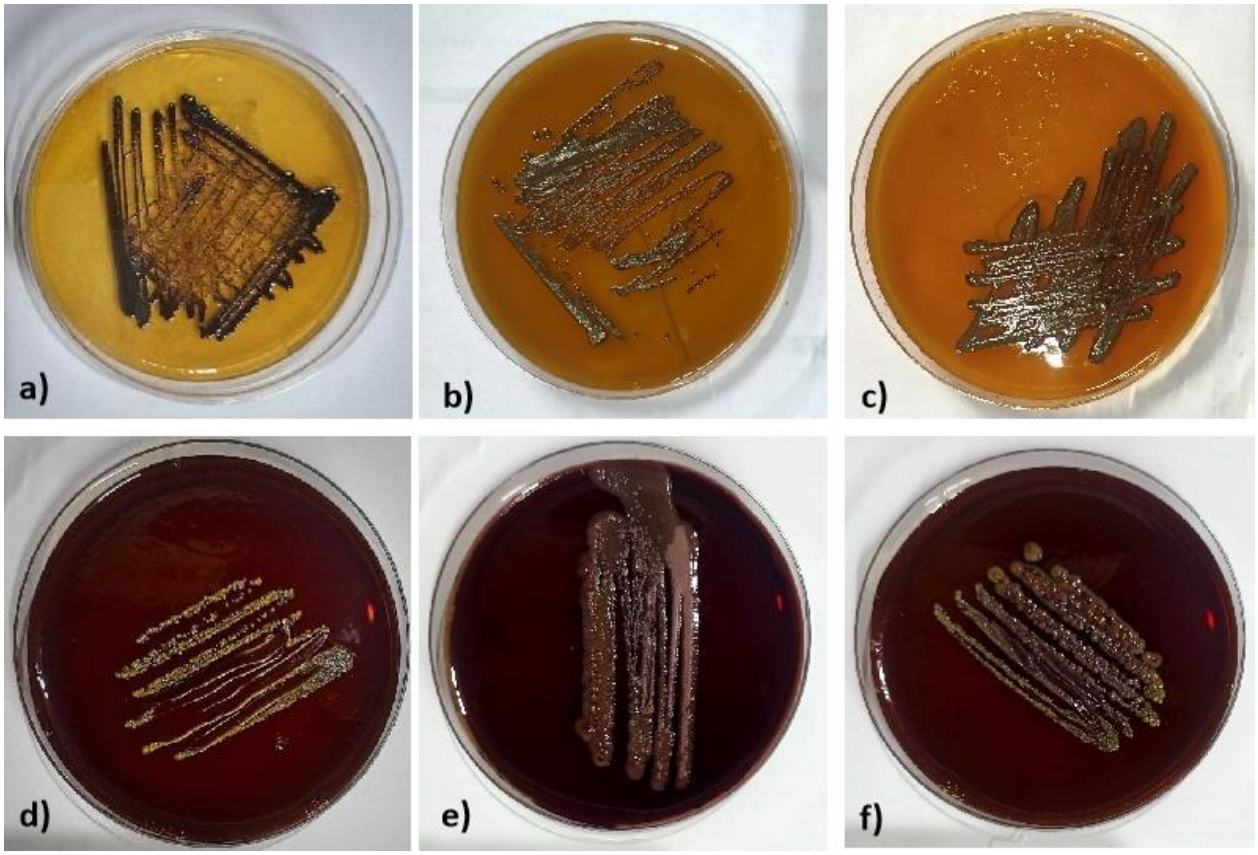

Figure 1: Growth of clinical Salmonella enterica clinical isolates on selective media. (a–c) show isolates grown on Bismuth Sulfite Agar (BSA), while (d–f) show the corresponding isolates on Xylose Lysine Deoxycholate (XLD) agar. (a) and (d): Clinical isolate no. 12, (b) and (e): Clinical isolate no. 35, (c) and (f): Clinical isolate no. 110. Each pair demonstrates colony morphology on both BSA and XLD media, aiding in preliminary identification and differentiation of Salmonella species.

Table 2: Representing HiSalmonellaTM Identification kit KB011 interpretation and results

No.	Test name	Procedure of test	Original color	Test result	Negative result
1	Methyl Red	2 drops of Methyl red indicator	Colorless	Red	Positive
2	Voges Proskauer	2 drops of Baritt reagent A and B	Light Yellow	Slight copper	Negative
3	Urease	-----	Orangish Yellow	Pink	Positive
4	H2S production	-----	Orangish Yellow	Brown	Positive
5	Citrate utilization	-----	Green	Blue	Positive
6	Lysine utilization	-----	Light Purple	Voilet	Positive
7	ONPG	-----	Colorless	Light Yellow	Positive

8	Lactose	-----	Red	Yellow	Positive
9	Arabinose	-----	Red	Yellow	Positive
10	Maltose	-----	Red	Yellow	Positive
11	Sorbitol	-----	Red	Pink	Negative
12	Dulcitol	-----	Red	Pink	Negative

In the KB disc diffusion assay, the antibiotic susceptibility pattern of clinical *S. enterica* strains displayed very interesting results. The isolated *S. enterica* strains were found to be resistant to broad class of antiobiotics viz. penicillin (amoxicillin/clavulanic acid, piperacillin/tazobactam and ticarcillin/clavulanic acid), carbapenem (meropenem), cephalosporins (cefixime, cefdinir and cefpodoxime), fluoroquinolones (ciprofloxacin, levofloxacin and nitrofurantoin), aminoglycoside (tobramycin) and macrolides (azithromycin) (Figure 2).

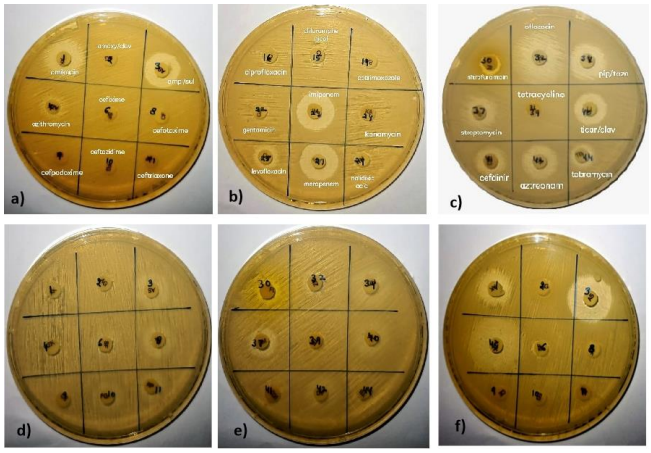

Figure 2: Antibiotic susceptibility testing of clinical Salmonella enterica isolates using the Kirby-Bauer disc diffusion method. Platess (a–c): Clinical isolate no. 35, Plates (d–e): Clinical isolate no. 12, Plate (f): Clinical isolate no. 110. Zones of inhibition were recorded to determine the sensitivity or resistance of isolates against a panel of antibiotics.

Further, the antibiotic susceptibility pattern of all *S. enterica* isolates under study displayed maximum resistance towards cefdinir (98.5 %) followed by cefpodoxime and tobramycin with value of 93.6%. However, nitrofurantoin also displayed resistance among 90.3% isolates and amoxicillin/clavulanic acid among 69.0% isolates (Table 3). On the other hand, of all the isolates under study, 92.6 % isolates were found to be susceptible for ampicillin/sulbactam and 87.3 % isolates for chloramphenicol and tetracycline. while the lowest rates of susceptibility were observed for ceftazidime (62.3%), cotrimoxazole (68.7%). Furthermore, two isolates were found to exhibit multidrug resistance in AST studies.

Table-3: Representing antibiotic susceptibility index against S. enterica (n=number of specimens)

Antibiotic's name	n Resistant %	n Intermediate %	n Sensitive %
Amikacin	35 (13.0%)	150 (55.9%)	83 (31.1%)
Amoxicillin/Clavulanic acid	185 (69.0%)	33 (12.3%)	50 (18.7%)
Ampicillin/Sulbactam	02 (0.7%)	18 (6.7%)	248 (92.6%)
Azithromycin	133 (49.7%)	83 (30.9%)	52 (19.4%)
Cefixime	83 (30.9%)	102 (38.2%)	83 (30.9%)

Cefotaxime	33 (12.3%)	217 (81.0%)	18 (6.7%)
Cefpodoxime	251 (93.6%)	12 (4.5%)	05 (1.9%)
Ceftazidime	31 (11.6%)	70 (26.1%)	167 (62.3%)
Ceftriaxone	34 (12.7%)	201 (75.0%)	33 (12.3%)
Ciprofloxacin	83 (31.0%)	51 (19.0%)	134 (50.0%)
Chloramphenicol	01 (0.4%)	33 (12.3%)	234 (87.3%)
Co-trimoxazole	68 (25.4%)	16 (5.9%)	184 (68.7%)
Gentamicin	17 (6.3%)	148 (55.2%)	103 (38.5%)
Tobramycin	251 (93.6%)	00 (00%)	17 (6.4%)
Imipenem	03 (1.1%)	102 (38.0%)	163 (60.9%)
Kanamycin	78 (29.1%)	138 (51.5%)	52 (19.4%)
Levofloxacin	115 (42.9%)	50 (18.6%)	103 (38.5%)
Meropenem	33 (12.3%)	101 (37.7%)	134 (50.0%)
Nalidixic acid	31 (11.5%)	75 (28.0%)	162 (60.5%)
Nitrofurantoin	242 (90.3%)	07 (2.6%)	19 (7.1%)
Ofloxacin	69 (25.7%)	17 (6.3%)	182 (68.0%)
Piperacillin/Tazobactam	66 (24.6%)	147 (54.8%)	55 (20.6%)
Streptomycin	05 (1.8%)	146 (54.5%)	117 (43.7%)
Tetracycline	12 (4.5%)	22 (8.2%)	234 (87.3%)
Ticarcillin/Clavulanic acid	09 (3.3%)	162 (60.4%)	97 (36.3%)
Cefdinir	264 (98.5%)	04 (1.5%)	00 (00%)
Aztreonam	26 (9.7%)	234 (87.3%)	08 (3.0%)

CONCLUSION AND FUTURE PERSPECTIVE

Several epidemiological studies have highlighted the rising incidence of *Salmonella enterica* globally. The increasing antimicrobial resistance of Salmonella underscores the need for prudent antibiotic use, continuous surveillance, and controlled treatment strategies to prevent multidrug resistance. Our findings indicate a growing pattern of antibiotic resistance in *S. enterica* in Rajasthan which may contribute to the persistence of typhoid fever through food contamination and public health challanges. This underscores the need for prudent antibiotic use, continuous surveillance, and evidence-based treatment strategies to mitigate the risk of multidrug resistance (MDR). Further research is essential to characterize the molecular mechanisms of resistance and the epidemiological patterns of MDR strains in this region. Whole-genome sequencing can also aid in uncovering the genetic basis of resistance and its transmission dynamics. Additionally, implementing improved

hygiene practices, enhanced food safety regulations, and stringent antibiotic stewardship programs will be crucial in controlling the spread of typhoid fever.

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