

Rapid detection of ceftriaxone resistance in clinical isolates of extensively drug resistant *Salmonella enterica serovar Typhi*

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Abstract

Objective: To see the efficacy of rapid colour test for the sensitivity of ceftriaxone against clinical isolates of *salmonella typhi*.

Methods: The cross-sectional validation study was conducted at the Department of Microbiology, Pakistan Navy Ship Shifa Hospital, Karachi, from Nov 2018 to April 2019, and comprised clinical isolates of *salmonella typhi* that were obtained from five different hospitals in Karachi and Hyderabad. The isolates were tested using the rapid colour test. All the isolates were also tested using the conventional disc diffusion method and minimum inhibitory concentrations on the Vitek-2 version 8.01.

Results: Of the 97 isolates, 83(85.5%) were ceftriaxone-resistant and 14(14.4%) were ceftriaxone-sensitive. Sensitivity and specificity of the rapid colour test were 100% when compared with the results of the other methods. All the results were readable within 2 hours on the colour test.

Conclusion: The colour test was found to be a rapid, accurate and inexpensive tool to screen for ceftriaxone resistance in typhoid-endemic areas.

Keywords: Ceftriaxone, Drug resistance, Meropenem, *Salmonella*, Susceptibility testing, Typhoid fever.

(JPMA 71: 1639; 2021) DOI: <https://doi.org/10.47391/JPMA.1204>

Introduction

Typhoid fever is a constant public health challenge in developing countries usually transmitted through consumption of contaminated food and water. It is caused by bacterium *salmonella enterica serovar typhi* (*S. typhi*). Humans are the only hosts for *S. typhi* and this gram-negative bacterium is transmitted through the faeco-oral route.¹ According to recent estimates, around 21 million cases of typhoid occur annually with approximate 222,000 deaths.² In Pakistan, it is the most common disease in children aged <15 years, with very high infection rates in cities like Karachi and Hyderabad.³ Residents of Sindh and Punjab provinces are at highest risk of developing this disease.⁴

After the emergence of resistance to fluoroquinolones, parenteral ceftriaxone / cefotaxime or oral cefixime injectable third-generation cephalosporin has remained the drug of choice for empirical treatment of typhoid fever, especially in the southeast Asia region. Multidrug resistant (MDR) strains of *S. typhi* are a public health concern in Pakistan, especially in Karachi.⁵ The first case of *S. typhi* resistant to ceftriaxone was reported from Bangladesh in 1999.⁶ In Pakistan, ceftriaxone remained the drug of choice for complicated enteric fever cases till November, 2016, when an outbreak of ceftriaxone-resistant typhoid cases was reported in Sindh; predominantly from Hyderabad and

Karachi.^{7,8} A total of 8405 enteric fever cases were reported from different cities of the province from November 2016 to Dec 30, 2018. Out of these, 5418 were infected with ceftriaxone-resistant *S. typhi* and this outbreak is still not over. Strains resistant to first-line drugs, including ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole, fluoroquinolones and ceftriaxone, are considered extensively drug resistant (XDR) *S. typhi* strains.

It was confirmed by whole-genome sequencing of XDR *S. typhi* strains that these harbour a plasmid encoding resistance genes, including the beta lactamase cefotaxime (*bla*CTX-M-15) extended spectrum beta-lactamase (ESBL) and quinolone resistance gene (*qnrS*). It was suggested that an MDR H58 clone acquired an ESBL plasmid from an *escherichia* (*E.*) *coli* or an enteric bacterial donor.⁹⁻¹¹ For most of XDR typhoid cases, the only oral antibiotic available with known efficacy is azithromycin, which is a broad-spectrum macrolide antibiotic used to treat patients with suspected uncomplicated typhoid fever. Patients with severe or complicated typhoid fever are treated with injection meropenem. Other potential treatment options include imipenem-cilastatin combination, tigecycline, piperacillin-tazobactam, ceftazidime-avibactam, fosfomycin, and colistin. However, there is limited experience and evidence on their efficacy in XDR typhoid cases. Their cost would also be a limiting factor for their use as routine drugs to treat typhoid fever cases in developing countries.^{12,13}

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Due to this public health alarming situation, the National Institute of Health (NIH), Pakistan, also published an advisory for the prevention and treatment of XDR typhoid cases in 2018.¹⁴ Some hospitals in Pakistan have also published local guidelines for empiric treatment of typhoid fever. Due to high prevalence of XDR typhoid cases in Sindh, especially in complicated cases, ceftriaxone cannot be used for enteric fever empirically, while widespread injudicious use of azithromycin and meropenem can lead to the development of resistance to these antibiotics as well. Strains resistant to azithromycin have already been seen in south Asia.¹⁴ Routine *S. typhi* antimicrobial susceptibility testing methods take at least 48-72 hours.¹⁵ This can lead to injudicious use of both meropenem and ceftriaxone in patients requiring admission. There is a need to develop a rapid and cost-effective method for rapid detection of ceftriaxone resistance in XDR typhoid endemic areas. The current study was planned to develop a rapid colour test to check ceftriaxone resistance in clinical isolates of XDR *S. typhi*.

Materials and Methods

The cross-sectional validation study was conducted at the Department of Microbiology and Immunology, Pakistan Navy Ship (PNS) Shifa Hospital, Karachi. After approval from the institutional ethics review committee, the sample size was calculated using the World Health Organisation (WHO) calculator applying the formula: $N = z^2 p(1-p)/d^2$; where z = confidence interval (CI), p = prevalence and d = precision.¹⁶

Clinical isolates of *S. typhi* and one ATCC 25922 *E. coli* strain was evaluated. These isolates were collected from five different hospitals in the two major cities of Karachi and Hyderabad. All the isolates were sub-cultured on 5% sheep blood agar and sensitivity to ceftriaxone was performed by disc diffusion method, as recommended by the Clinical and Laboratory Standards Institute (CLSI) in 2019.¹⁷ Minimum inhibitory concentrations (MICs) were determined by ceftriaxone E-test (MIC range 0.016-256ug/ml, Oxoid, UK) and Vitek-2 system (updated 8.01 version, Biomerieux, France). The Vitek-2 system uses breakpoints according to CLSI 2016 version (≥ 4 resistant for ceftriaxone). The results of these MICs are shown in technical appendix table 1.¹⁸

The rapid ceftriaxone sensitivity test utilises two reagents; stock solution of ceftriaxone, and rapid ceftriaxone sensitivity solution. For stock solution of ceftriaxone, 1g salt ceftriaxone sodium powder (Sigma-Aldrich) was diluted in distilled water to make a final concentration of 1mg/ml. Further, 250ml of rapid ceftriaxone sensitivity solution was prepared by adding fixed concentrations of Mueller Hinton Broth Cation Adjusted (MHB-CA), phenol red (Daejung, Korea) and 10% anhydrous dextrose (Daejung, Korea). The

final potential of hydrogen (pH) of the solution was 6.7. For rapid ceftriaxone test, suspension of test organisms was made from freshly grown colonies on 5% sheep blood agar to obtain a final inoculum of 3.0-3.5 MacFarland turbidity (9.0×10^8 CFU/ml).

A pilot study was performed in sterile tubes on 25 isolates of *S. typhi*; 12 XDR and 13 ceftriaxone-sensitive strains. The results were read at 1, 1.5, 2 and 3 hours (Figure 1). The results were readable at 1.5h, but all the test tubes produced best appreciable results at 2h. These test isolates were not included in the main study.

The rapid ceftriaxone test was conducted in a 96-well polystyrene microtiter plate (sterile, round-bottom, Thermo Fisher Scientific, United States). For the isolates, 0.2ml ceftriaxone from the stock solution was added in 19.8ml MHB solution to make a concentration of 10ug/ml. From this solution, 150ul was dispensed in the respective microtitre wells. Besides, 50ul of the bacterial suspension was added in the respective wells so that the final concentration of ceftriaxone in each well was 7.5ug/ml and the final concentration of bacteria was 108 CFU/ml. After

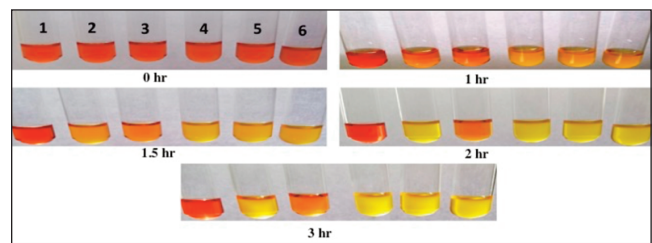


Figure-1: Pilot study showing rapid ceftriaxone test reactions in tubes at 0, 1, 1.5, 2 and 3 hours. Orange / slight colour change indicates sensitive strains, and yellow colour, resistant. 1) ATCC 25922 *Escherichia (E.) coli* sensitive strain. 2) A known extensively drug-resistant (XDR) *salmonella (S.) typhi* isolate 3) Paratyphi A strain. 4-6) XDR *S. Typhi* isolates.

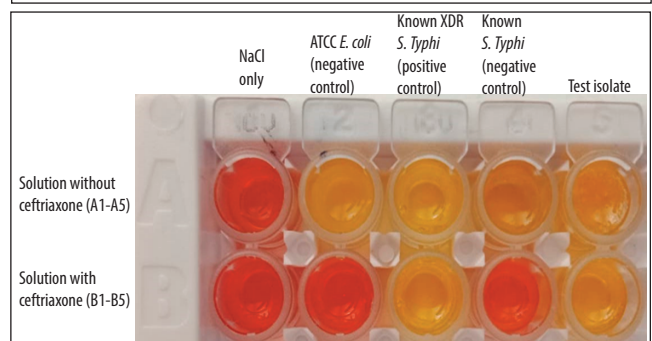


Figure-2: Rapid ceftriaxone test on microtiter plate at 2-hour incubation (representative results). Row A: well A1 containing only sodium chloride (NaCl) to check colour change due to saline. Wells A2-A5 have solution without ceftriaxone. Yellow colour indicates growth in all these wells in the absence of ceftriaxone. Row B: Wells from B1-B5 contained solution with ceftriaxone. The isolate in well B3 and B5 showed colour change (extensively drug-resistant [XDR] salmonella typhi).

inoculation, the tray was gently kept in an incubator at $35\pm 2^{\circ}\text{C}$ without being sealed for free flow of oxygen. The tray was read at 15 minutes for any spontaneous change in colour and then at 1, 1.5, 2 and 3 hours. No change in orange colour or slight change in colour was considered negative, meaning ceftriaxone-sensitive, while change in colour from orange to yellow was considered positive, meaning ceftriaxone-resistant, that is growth of organisms in the presence of the drug (Figure 2).

A 2x2 table was made to calculate sensitivity and specificity of the rapid colour test while keeping the Vitek-2 system as the gold standard. Sensitivity was defined as the ability of a method to detect true ceftriaxone resistance in an isolate, while specificity meant ability of a method to detect true ceftriaxone sensitive isolate.

Results

Of the 97 isolates, 83(85.5%) were ceftriaxone-resistant and 14(14.4%) were ceftriaxone-sensitive. On rapid colour test, all the isolates (100%) showed concordance with the results of the other methods.

Out of total 82 ceftriaxone-resistant clinical isolates, excluding one positive control *S. typhi*, 79(96.3%) gave appreciable reaction (from orange to yellow) at 1.5 h while

2(2.4%) produced reaction at 2 h. At 3 h, the yellow colour change became deeper. There was only 1(7%) ceftriaxone-sensitive isolate which showed slight change in colour at 2 h. The isolate was retested by colour method with no colour change on repetition. There was only 1(1.2%) ceftriaxone-resistant isolate which showed variations in MICs >12 on E-test and >8 on Vitek, but these MICs were in the resistant range.

Out of 82 XDR clinical isolates, 72(87.8%) had MICs >256 on E-test and >64 on Vitek-2. Among these 72 isolates, 50(69.4%) were from Karachi. Sensitivity and specificity of the rapid colour test was 100% when compared with Vitek 2 results (Table 2).

Discussion

To the best of our knowledge, the current study is the first on colour test for rapid detection of ceftriaxone resistance against clinical isolates of XDR *S. typhi*. A study was done based on the principle of organism metabolism and subsequent colour change due to change in pH was conducted using the Nordmann and Poirel (NP) test.¹⁹ The study evaluated rapid detection of polymyxin resistance against various isolates of enterobacteriaceae. Sensitivity and specificity of rapid NP test was 99.3% and 95.4% respectively when compared with the standard broth

micro-dilution method.¹⁸ Subsequently, more studies were published based on the rapid NP colour test for polymyxin resistance against various enterobacteriaceae isolates.^{20,21}

Ceftriaxone has been used as drug of choice for the treatment of MDR isolates of *S. typhi*, especially in typhoid-endemic countries. However, the outbreak of XDR *S. typhi* in Sindh has questioned the use of this drug empirically. Most of the confirmed cases of XDR typhoid are treated with injection meropenem in combination with oral azithromycin. Use of meropenem empirically can also lead to the development of resistance against this last-resort

Table-1: Rapid colour test results for ceftriaxone resistance against various clinical isolates of salmonella typhi.

S/No	Number/isolates	Species	City and Hospital	Disc diffusion result	E-test MICs	Vitek-2 MICs	Rapid color test
1	S1-S24	<i>S. Typhi</i>	Kci, PNS Shifa	R	>256	>64	+
2	S25-S26	<i>S. Typhi</i>	Kci, PNS Shifa	R	>32	>32	+
3	S27-S29	<i>S. Typhi</i>	Kci, PNS Shifa	S	0.25	<1	-
4	S30-S31	<i>S. Typhi</i>	Kci, PNS Shifa	S	0.50	<1	-
5	S32-S35	<i>S. Typhi</i>	Kci, PNS Shifa	S	0.125	<1	-
6	S36-S37	<i>S. Typhi</i>	Kci, PNS Shifa	S	0.064	<1	-
7	S-38	<i>S. Typhi</i>	Kci, PNS Shifa	S	0.19	<1	-
8	S-39	<i>S. Typhi</i>	Kci, PNS Shifa	S	0.094	<1	-
9	In1-In21	<i>S. Typhi</i>	Kci, Indus Hosp	R	>256	>64	+
10	In22-In27	<i>S. Typhi</i>	Kci, Indus Hosp	R	>32	>32	+
11	L1-L12	<i>S. Typhi</i>	Hyd, LUMHS	R	>256	>64	+
12	H1-H10	<i>S. Typhi</i>	Hyd, CMH	R	>256	>64	+
13	M1-M5	<i>S. Typhi</i>	Kci, CMH Malir	R	>256	>64	+
14	M6-M7	<i>S. Typhi</i>	Kci, CMH Malir	R	>32	>32	+
15	STC-1 (Positive control)	<i>S. Typhi</i>	PNS Shifa Institutional control	R	>256	>64	+
16	STC-2 (Negative control)	<i>S. Typhi</i>	PNS Shifa Institutional control	S	0.064	<1	-
17	ATCC 25922 (Negative control)	<i>E. coli</i>	United States of America	S	0.023	<1	-

R: Resistant; S: Susceptible; +: Positive colour means resistant; -: Negative colour means susceptible. Kci: Karachi; Hyd: Hyderabad; LUMHS: Liaquat University of Medical and Health Sciences; PNS: Pakistan Naval Ship.

Table-2: Comparison of results of rapid ceftriaxone test with Vitek 2 results (n=98).

Antibiotic Ceftriaxone	Rapid ceftriaxone test	Vitek 2		Performance of Rapid ceftriaxone test				
		R	S	Sensitivity	Specificity	PPV	NPV	DA
	Positive	83	0	100 %	100%	100%	100%	100%
	Negative	0	15					

PPV: Positive predictive value; NPV: Negative predictive value, DA: Diagnostic accuracy.

antibiotic. The Sanford guide of antimicrobial therapy has recommended injection meropenem usage for complicated or severe infections in XDR endemic areas.^{22,23}

The rapid colour test for ceftriaxone resistance is easy to perform and does not require any sophisticated equipment or expertise. The test is performed on colonies grown on non-inhibitory media, such as sheep blood agar. In routine practice, this test can be performed using simple sterile test tubes, and results can be achieved and communicated in just 2 hours. In the current study, there was only one isolate, which produced a weak colour change, but, on repeat testing, there was no colour change (negative test). A weak colour change should be taken as negative.²⁰

The current study has two limitations. First, broth micro-dilution method was not performed for ceftriaxone resistance though resistance was counter-checked by two separate methods as per CLSI guidelines.¹⁷ Second, the mechanism of ceftriaxone resistance of these isolates was not checked at the molecular level.

The ongoing outbreak of XDR *S. typhi* is so far limited to Sindh and all efforts are being made to control its spread. The colour test has utility in areas facing the threat of XDR typhoid, but it is believed that these strains may spread to other areas as already cases of XDR *S. typhi* have been reported in the United Kingdom and the United States possibly owing to people travelling from Pakistan.²⁴

Conclusion

The colour test was found to be a rapid, accurate and inexpensive tool to screen for ceftriaxone resistance in typhoid-endemic areas. The use of this test might contribute to an early initiation of appropriate antibiotic therapy.

Acknowledgements: We are thankful to Dr Nazia Khurshid (Indus Hospital Karachi), Dr Abeera Ahmed (CMH Malir), Dr Muhammad Abid Farooque (CMH Hyderabad) and Mr Aftab Durrani (Liaquat University of Medical and Health Sciences Hyderabad) for providing us clinical isolates for this study.

Disclaimer: None.

Conflict of interest: None.

Source of Funding: None.

References

- Das JK, Hasan R, Zafar A, Ahmed I, Ikram A, Nizamuddin S, et al. Trends, associations, and antimicrobial resistance of *Salmonella typhi* and paratyphi in Pakistan. *Am J Trop Med Hyg* 2018; 99: 48-54.
- Narcisa M, Giovanni S, Bianca MA, Andrea C, Clementina C, Marianna M, et al. Travel related typhoid fever: Narrative review of the scientific literature. *Int J Environ Res Public Health* 2020; 17: 615.
- Yousafzai MT, Qamar FN, Shakoor S, Saleem K, Lohana H, Karim S, et al. Ceftriaxone-resistant *Salmonella Typhi* Outbreak in Hyderabad City of Sindh, Pakistan: High Time for the Introduction of Typhoid Conjugate Vaccine. *Clin Infect Dis* 2019; 68: S16-21.
- Antillón M, Warren JL, Crawford FW, Weinberger DM, Kürüm E, Pak GD, et al. The burden of typhoid fever in low-and middle-income countries: a meta-regression approach. *PLoS Neglected Trop Dis* 2017; 11: e0005376.
- Aziz S, Malik L. Emergence of multi resistant enteric infection in a paediatric unit of Karachi, Pakistan. *J Pak Med Assoc* 2018; 68: 1848-50.
- Saha SK, Talukder SY, Islam M, Saha S. A highly ceftriaxone-resistant *Salmonella Typhi* in Bangladesh. *Pediatr Infect Dis J* 1999; 18: 387.
- Qamar FN, Yousafzai MT, Khalid M, Kazi AM, Lohana H, Karim S, et al. Outbreak investigation of ceftriaxone-resistant *Salmonella enterica* serotype Typhi and its risk factors among the general population in Hyderabad, Pakistan: a matched case-control study. *Lancet Infect Dis* 2018; 18:1368-76.
- Yang YA, Chong A, Song J. Why Is Eradicating Typhoid Fever So Challenging: Implications for Vaccine and Therapeutic Design. *Vaccines (Basel)* 2018; 6: 45.
- Kuang D, Zhang J, Xu X, Shi W, Yang X, Su X, et al. Increase in Ceftriaxone Resistance and Widespread Extended-Spectrum β -Lactamases Genes Among *Salmonella enterica* from Human and Nonhuman Sources. *Foodborne Pathog Dis* 2018; 15: 770-5.
- Djehgout B, Saha S, Sajib MS, Tanmoy AM, Islam M, Kay GL, et al. Ceftriaxone-resistant *Salmonella Typhi* carries an IncI1-ST31 plasmid encoding CTX-M-15. *J Med Microbiol* 2018; 67: 620-7.
- Tanmoy AM, Westeel E, De Bruyne K, Goris J, Rajoharison A, Sajib MS, et al. *Salmonella enterica* serovar Typhi in Bangladesh: Exploration of Genomic Diversity and Antimicrobial Resistance. *mBio* 2018; 9: 2112-8.
- Levine MM, Simon R. The Gathering Storm: Is Untreatable Typhoid Fever on the Way? *mBio* 2018; 9: e00482-18.
- Parry CM, Ribeiro I, Walia K, Rupali P, Baker S, Basnyat B. Multidrug resistant enteric fever in South Asia: unmet medical needs and opportunities. *BMJ* 2019; 364: k5322.
- National Institute of Health Pakistan. Advisory for the treatment and prevention of XDR typhoid. Pakistan. No.F.1-31/Misc/FEDSD/2018. [Online] [Cited 2019 April 22]. Available from: URL: <https://www.nih.org.pk/wp-content/uploads/2019/02/Advisory-for-Typhoid-5-oct.pdf>.
- Saha SK, Darmstadt GL, Baqui AH, Hanif M, Ruhulamin M, Santosham M, et al. Rapid identification and antibiotic susceptibility testing of *Salmonella enterica* serovar Typhi isolated from blood: implications for therapy. *J Clin Microbiol* 2001; 39: 3583-5.
- Mohamad AP, Mohsen V, Mitra R. Sample size calculation in medical studies. *Gastroenterol Hepatol Bed Bench* 2013; 6: 14-7.
- Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. 29th ed. Document M100. CLSI, Wayne, PA; 2019.
- Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing 26th ed. Document M100-S26. CLSI, Wayne, PA; 2019.
- Nordmann P, Jzyol A, Poirel L. Rapid detection of polymyxin resistance in Enterobacteriaceae. *Emerg Infect Dis* 2016; 22: 1038-43.
- Simar S, Sibley D, Ashcraft D, Pankey G. Evaluation of rapid polymyxin NP test for polymyxin B resistance detection using *Enterobacter cloacae* and *Enterobacter aerogenes* isolates. *J Clin Microbiol* 2017; 55: 3016-20.

21. Malli E, Florou Z, Tsilipounidaki K, Voulgaridi I, Stefos A, Xitsas S, et al. Evaluation of rapid polymyxin NP test to detect colistin-resistant *Klebsiella pneumoniae* isolated in a tertiary Greek hospital. *J Microbiol Methods* 2018; 153: 35-9.
 22. Black D, Chambers HF, Eliopoulos G, Freedman D, Gilbert DN, Kim K, editors. *The Sanford guide to antimicrobial therapy*. 48th ed. Sperryville, VA: Antimicrobial Therapy, Inc; 2019.
 23. Ryan ET, Andrews J. Treatment and prevention of enteric (typhoid and paratyphoid fever). Post TW. Ed. *Up-to-date*. Waltham, MA: Up-to-date Inc. [Online] [Cited 2019 April 22]. Available from: URL: <http://www.uptodate.com>.
 24. Chatham-Stephens K, Medalla F, Hughes M, Appiah GD, Aubert RD, Caidi H, et al. Emergence of Extensively Drug-Resistant *Salmonella* Typhi Infections Among Travelers to or from Pakistan - United States, 2016-2018. *MMWR Morb Mortal Wkly Rep* 2019; 68: 11-3.
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