AZITHROMYCIN SUSCEPTIBILITY TESTING FOR SALMONELLA ENTERICA ISOLATES: COMPARING DISK DIFFUSION RESULTS WITH MIC GRADIENT STRIPS

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ABSTRACT

Background: Enteric fever remains an imperative public health problem in developing countries. After the emergence of cephalosporin resistance in Salmonella enterica subsp. enterica serovar Typhi, azithromycin is increasingly being used for oral treatment of enteric fever. Reports of sporadic azithromycin resistance have been reported across the country, additionally, misuse of azithromycin during the COVID-19 pandemic has concerns regarding emerging azithromycin resistance. This study evaluated the reliability of the disc diffusion method as a screening test for detecting azithromycin resistance by comparing it with the minimum inhibitory concentrations (MIC) gradient strip results, in 231 typhoidal salmonellae.

Material and Methods: This prospective study was conducted in the Section of microbiology of the Shaukat Khanum Memorial Cancer Hospital and Research Centre, Lahore from March 2022 to March 2023. Isolates recovered from blood cultures of patients, suffering from enteric fever were selected. Azithromycin susceptibility testing was performed both by disk diffusion and as well as gradient strips and their results were compared.

Results: Among typhoidal salmonellae, a significant portion consisted of extensively drug resistant Salmonella Typhi (61.9%). Only one XDR S. Typhi was found to be resistant to azithromycin both by disk diffusion method and MIC gradient strip method, with a MIC value of 64µg/ml. The study found no discrepancy between the disk diffusion and gradient strip methods.

Conclusions: The current study found no discordance between disk diffusion and gradient strip test methods for evaluating azithromycin susceptibility among typhoidal salmonellae.

Keywords: Azithromycin, disk diffusion testing, enteric fever, Minimum inhibitory concentration, Salmonella Typhi

BACKGROUND

Typhoid fever is the leading cause of morbidity and mortality worldwide. It is a potentially lethal bacterial infection caused by Salmonella enterica serotype Typhi, if not promptly treated and managed.¹ According to recent WHO figures, typhoid fever causes up to 21 million illnesses and 161,000 fatalities annually.² Since typhoid fever is usually acquired by consuming food or water contaminated with Salmonella Typhi, nations with inadequate infrastructure and sanitation facilities are more likely to experience a high incidence of the illness. Typhoid fever is characterized by persistently high temperature, headache, nausea, abdominal pain, and diarrhea or constipation.³

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Salmonella Paratyphi A, B, or C is the cause of comparable frequently less severe paratyphoid fever.⁴ Due to the development of antimicrobial resistance among typhoidal salmonellae, typhoid fever has become difficult to treat over the years. Resistance to first and second line antibiotics i.e. chloramphenicol, ampicillin, cotrimoxazole and fluoroquinolones had led to the rise in multidrug resistant (MDR) strains leaving behind ceftriaxone as the treatment option.⁵ However, with the advent of extremely drug-resistant (XDR) strains, this treatment option has also been rendered ineffective and azithromycin and meropenem are left as antibiotics of last resort. Azithromycin has gained significant prominence in recent years as the sole oral antibiotic available for the treatment XDR S. typhi.⁶ Unfortunately, we now face the emergence of azithromycin resistance from various parts of the world including Pakistan, India, Nepal and Bangladesh.⁷ Additionally, the blatant misuse and overuse of azithromycin during the COVID-19 pandemic have only made matters worse and led to heightened concerns about the increasing resistance to the said antibiotic.⁸
Azithromycin susceptibility testing guidelines for S. Typhi were published in 2015 by the Clinical and Laboratory Standards Institute (CLSI). Though, there are still no guidelines available for the same for S. Paratyphi.\(^9\) CLSI M100 33rd edition (2023) provides both zone diameters as well as MIC breakpoints for azithromycin susceptibility testing against Salmonella Typhi. Either of the methodologies can be opted for by laboratories, depending on their feasibility. However, several studies in the past found discordance between the two methodologies when tested against typhoidal salmonellae. These studies concluded that the disk diffusion susceptibility testing method can misinterpret azithromycin sensitive strains as resistant and therefore, is not an accurate method for checking the susceptibility of azithromycin against Salmonella isolates.\(^10\)

Hence, our objective was to assess the reliability of disc diffusion test for the detection of azithromycin resistance by comparing it with the MIC gradient strips, in randomly selected typhoidal salmonellae recovered from blood cultures received in our laboratory.

**MATERIAL AND METHODS**

From March 2022 to March 2023, a prospective study was carried out in the microbiology section of Shaukat Khanum Memorial Cancer Hospital and Research Centre (SKMCH&RC), Lahore after the approval of Institutional Review Board Committee. A waiver for informed consent was also sought and approved. Using the laboratory network of SKMCH&RC, all blood culture samples from patients suspected of having typhoid fever were collected and incubated in an automated blood culture system BACT/ALERT VIRTUO (BioMérieux, France). Positively flagged bottles were subcultured onto chocolate, blood, and MacConkey agar plates. Duplicate isolates were excluded.

Using API 20E (bioMérieux), colonies suggestive of Salmonellae were identified using biochemical tests, serotyping and colony morphology. Salmonella Typhi and Salmonella Paratyphi A isolates were selected from these blood cultures by random consecutive sampling. Interpretive breakpoints for Salmonella Typhi from CLSI M100 33rd edition (2023) were extrapolated and applied to Salmonella Paratyphi A.

The Kirby–Bauer disc diffusion method on Muller–Hinton agar was used to test 231 Salmonella isolates for antibiotic susceptibility in compliance with Clinical Laboratory Standards Institute (CLSI) M100, 33rd edition (2023).\(^11\) A 90 mm Mueller Hinton agar plate containing a 15 µg disk was inoculated with 0.5 McFarland suspension of Salmonellae isolates, and the plates were then left to incubate overnight at 37°C. Zones of inhibitions were measured after 18-24 hours of incubation. A zone of inhibitions of ≥ 13 mm was considered as sensitive and ≤ 12 mm was considered as resistant.\(^11\)

Using E-test (BioMérieux), the minimum inhibitory concentrations of azithromycin were found. Mueller Hinton agar plates were inoculated with 0.5 McFarland bacterial suspensions were incubated overnight at 37°C after placing azithromycin E-strips on their surfaces. MICs were measured after 18-24 hours of incubation. According to interpretive breakpoints provided by the CLSI, isolates having MICs ≤ 16 µg/ml were considered susceptible while isolates having MICs ≥32 µg/ml were considered resistant (11). All results were reassessed by a second reader to remove any bias.

The Statistical Package for Social Sciences (SPSS) version 24.0 (IBM, Armonk, NY) was used to compile and analyze all the clinical and microbiological data. The frequencies and percentages used to represent the descriptive data were displayed. Frequencies of azithromycin MICs and distribution of XDR, MDR and multi-drug susceptible strains among Salmonella isolates were represented by using percentages and graphs and p-value of less than 0.05 was deemed statistically significant.

**RESULTS**

A total of 231 typhoidal salmonellae recovered from blood cultures were tested. Out of these, 184 isolates (80%) were Salmonella Typhi and 47 isolates (20%) were Salmonella Paratyphi A. Among the 184 Salmonella Typhi isolates; 143 (78 %), 9 (5 %) and 32 (17%) were XDR, MDR and multi-drug susceptible strains, respectively (Figure-1). Whereas, all Salmonella Paratyphi A isolates were multi-drug susceptible. The significant portion of Salmonella isolates were found to be XDR Salmonella Typhi (62%).

Analysis of demographic data showed that of the isolates, 94 (40.7%) came from female patients and 137 (59.3%) from male patients with mean age of 16.53±11.8 years. The strains originated from various parts of the country, with 48.5% (112) coming from...
Khyber Pakhtunkhwa, followed by Punjab with 38% (88), Sindh 2.5% (6), FATA 5.7% (13), Balochistan 4.9% (11) and the Federal Capital 0.4% (1).

Currently, CLSI M100, 33rd edition (2023) provides the interpretive criteria for azithromycin susceptibility testing by disk diffusion method as well as MIC testing for *Salmonella* Typhi only, the same criteria were extrapolated and applied for *Salmonella* Paratyphi A isolates. Among all *Salmonella* isolates tested, only one XDR *Salmonella* Typhi strain (0.43%) was found to be resistant to azithromycin both by disk diffusion method (no zone of inhibition) as well as the gradient strip method (MIC= 64µg/ml). The strain was recovered from the blood culture of a patient belonging to Peshawar, Khyber Pakhtunkhwa. The azithromycin resistant strain was sent for further analysis to the UK Health Security Agency reference laboratory and was found to be positive for the *mphA* gene.

The lowest and the highest MICs of azithromycin were found to be 0.75 µg/ml (*S. Paratyphi A*) and 64 µg/ml (XDR *S. Typhi*), respectively (Figure-2). The mean MIC of *Salmonella* isolates by gradient strip was found to be 2.84 ± 4.46 µg/ml whereas, the mean zone of inhibition by disk diffusion testing was 22.17 ± 2.64 mm. An inverse correlation between zone of inhibitions and MICs was found among *Salmonella* isolates with a statistically significant p-value of 0.01.

MIC50 and MIC90 were also calculated and found to be 2 µg/ml and 4 µg/ml, respectively.

The study found no discrepancy between the disk diffusion and gradient strip methods. There was no discordance in azithromycin susceptibility results obtained by both methods.

**DISCUSSION**

The emergence of XDR *S. Typhi* has posed a significant burden on the healthcare settings of low-middle income countries including Pakistan and has rendered typhoid fever difficult to treat. Consequently, carbapenems and azithromycin are left as antibiotics of last resort to treat XDR *S. Typhi*. Azithromycin being the only oral option available has gained significant importance during the last few years in the management of enteric fever caused by extensively drug resistant *S. Typhi*.

CLSI recommends either disk diffusion method or gradient strip method to evaluate azithromycin susceptibility in *Salmonella* isolates, however, data on azithromycin MICs among typhoidal salmonellae is limited from Pakistan.

According to the current study, a significant portion of *Salmonella* isolates were found to be XDR *Salmonella* Typhi (62%). Previously published data from our center reported a prevalence of 50% of XDR *S. Typhi* from all positive blood cultures.\(^{12}\) Hence, reaffirming the predominance of XDR *S. Typhi* strains in our sample group.

The current study found only one azithromycin resistant *S. Typhi* isolate (0.43%) which indicate that the rate of azithromycin resistant Salmonellae is still low in the
The isolate was also found to have the highest azithromycin MIC value (64 µg/ml) among all Salmonella isolates. A recent retrospective cross-sectional multi-centric study conducted on 150 XDR Salmonella Typhi isolates at University of Health Sciences, Lahore from January, 2012 to June, 2021 found 3 (2%) azithromycin resistant isolates with MICs of 32 µg/ml by E-test method. A comparative study conducted on 60 Salmonella isolates in the Microbiology department of Pakistan Navy Ship Shifa Hospital Karachi (PNS Shifa) from June, 2020 to December 2020, found one azithromycin resistant isolate with a high MIC of 96 µg/ml checked by E-strip method and a zone of inhibition of 9 mm on disk diffusion method.7 Another cross-sectional observational study conducted in the medicine department of Combined Military Hospital (CMH) Lahore, from April 2019 to October 2019 on 52 Salmonella isolates, also found 1 (1.9%) azithromycin resistant S. Typhi isolate with a high MIC of 64 µg/ml.8 A study conducted at a tertiary care hospital in Southern India from January, 2013 to December, 2017 on 100 Salmonella enterica strains found 6 (6%) azithromycin resistant strains with MICs ≥ 32 µg/ml.9 Another cross-sectional study performed on 66 S. Typhi isolates in Northern India found 7 (10.6%) azithromycin resistant isolates by E-test method.7 Similarly, azithromycin resistant S. Typhi isolates have also been reported in other parts of the world including Nepal and Bangladesh.10

The current study found a low resistance rate (0.432%) of azithromycin against typhoidal salmonellae. However, ongoing vigilance and surveillance is required for evaluating its resistance to minimize the public health risk. Increasing azithromycin resistance among typhoidal salmonellae in the recent past is attributable to COVID-19 pandemic. During 2020-2022, Pakistan faced dual epidemic of COVID-19 and typhoid. Azithromycin was misused during COVID-19 pandemic due to its broad-spectrum respiratory coverage. Unjudicial empirical use of azithromycin and non-adherence to antimicrobial stewardship practices may also have led to the emergence of azithromycin resistance among typhoidal salmonellae.11

The azithromycin resistant Salmonella Typhi strain in the current study was found positive for the mphA gene. It is usually located on plasmids and is the main gene involved in macrolide among Salmonella spp. Macrolide-2’-phosphotransferase encoded by mphA gene confers azithromycin resistance in Salmonella isolates.12 This gene is associated with high level azithromycin resistance among Salmonella spp. A previous study found a single high-level azithromycin resistant Salmonella strain (MIC: 64µg/ml) harbouring mphA gene.13 According to another study conducted in United Kingdom 12 out of 15 azithromycin resistant non-typhoidal Salmonella strains encoded mphA gene.14 However, literature on genetic analysis of azithromycin resistant salmonellae is scarce from Pakistan. Emergence of mphA mediated azithromycin resistance among Salmonella spp. and its ability to transmit horizontally is worrisome and renders the use of azithromycin for treating typhoid fever at risk.15

The current study found no discordance in azithromycin susceptibility results obtained by disk diffusion and E-test methods. However, few studies in the past showed discrepancies between disk diffusion and E-test methods. In 2020, a study conducted in Karachi found discordance in 10 (16.6%) out of 60 Salmonella isolates.16 Another study conducted in India found discordance in 12 (12%) out of 100 isolates.14 Similarly, in 2020 a study conducted in Karachi found discordance in 5 (0.23%) out of 2104 S. Typhi isolates.19 In 2021, a study conducted in Lahore also found discrepancy in 7 out of 150 (4.6%) isolates between disk diffusion and E-test methods.20 These studies concluded that disk diffusion method provide false resistant results and does not provide accurate results for azithromycin susceptibility testing particularly in XDR S. Typhi.

According to a study conducted in London between May, 2011 and April, 2019 concluded that E-strips for azithromycin MICs are difficult to interpret due to possible reader bias errors. So, prior training and a second reader system should be ensured at the institute level to mitigate this problem.21 As the current study showed no discrepancy between disk diffusion and E-test methods, hence, we can consider disk diffusion a reliable method for evaluating azithromycin susceptibility among Salmonella isolates as it is cost-effective and easy to interpret as compared to E-test method. Nonetheless, the present investigation has various constraints. The study is a single-centered. Additionally, as part of this investigation, we did not...
tack the clinical response of patients using azithromycin.

CONCLUSION
The current study concluded no discordance between disk diffusion and E-test methods for azithromycin susceptibility among *Salmonella* isolates. Hence, disk diffusion test can be considered a reliable method and can be used to evaluate azithromycin susceptibility among *Salmonella* isolates.

CONFICT OF INTEREST
None

AUTHOR CONTRIBUTION
Aqib Sultan: Acquisition, analysis and interpretation of data and drafting the work or revising it critically for important intellectual content and bench work.
Nasrullah Malik, Summiya Nizamuddin: Final approval of the version to be published
Nida Safdar: Acquisition, analysis of data, second reader

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