

ANALYSIS OF MINIMAL INHIBITORY CONCENTRATION (MIC) OF AZITHROMYCIN IN EXTREMELY DRUG RESISTANT (XDR) SALMONELLA ENTERICA SEROVAR TYPHI IN PRE-COVID AND AFTER ONE YEAR OF COVID-19 PANDEMIC

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ABSTRACT

Objective: To compare the Minimal Inhibitory Concentration (MIC) of Azithromycin in Extremely Drug Resistant (XDR) *Salmonella enterica* serovar Typhi (S. Typhi), Pre-COVID and after one year of COVID-19 pandemic.

Methodology: This cross-sectional study was conducted in the Department of Microbiology, Combined Military Hospital, Lahore, Pakistan, from May 2021 to April 2022. All XDR S. Typhi isolated during the study period were included and tested. Kirby –Bauer Disk Diffusion method was used for antibiotic susceptibility, and Azithromycin MIC was determined by the E-strip method. The latest Clinical & Laboratory Standards Institute (CLSI) breakpoints for Azithromycin susceptibility testing were used.

Results: A total of 384 XDR S. Typhi were included in the study. MICs of these isolates were in the ranges of 2-16ug/mL, and all were sensitive to Azithromycin as per CLSI standards. These were further compared with MICs of the pre-covid isolates. The MICs of the isolates were relatively higher as compared to pre-COVID MICs.

Conclusion: The potential Azithromycin resistance in XDR *Salmonella* Typhi is knocking at the door. Sensitivity patterns should be reported with MICs values, and if possible higher values should be screened for the presence of any potential resistance genes. Antibiotic stewardship should be strictly implemented.

Keywords: Azithromycin, COVID-19, Minimal inhibitory concentration, *Salmonella enterica* serovar Typhi XDR

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INTRODUCTION

Salmonella enterica serovars Typhi and Paratyphi A, B&C are the most common etiological agents of enteric fever. It has a worldwide distribution with a substantial morbidity burden for countries with middle and low-income

economies¹. Salmonellosis is a self-limiting disease. However, antimicrobial treatment is still needed for elderly and immune-compromised patients². Enteric fever remains a leading global cause of morbidity and mortality, particularly in low and middle-income countries, including Pakistan. The incidence of enteric fever in Southeast Asia is 110 cases/per 100000 population³.

Antimicrobial resistance (AMR) is an imminent threat to *Salmonella enterica* serovar Typhi infection treatment. According to already standardized definitions, Multidrug-resistant (MDR) *Salmonella* is defined as a strain that is

resistant to first-line recommended drugs i.e., ampicillin, trimethoprim-sulfamethoxazole, and chloramphenicol. However, the resistant (XDR) strain is the one that is resistant to all the first-line drugs as well as fluoroquinolones and third generation cephalosporins. Multidrug resistance (MDR) was first detected in the late 1980s, and the organism became resistant to first line (Ampicillin, Chloramphenicol and cotrimoxazole) therapy. The United Kingdom reported the first fluoroquinolone resistant case in 1992⁴. After the organism became resistant to first- and second-line drugs, therapeutic choices were 3rd generation cephalosporins and azithromycin. However, the organism became resistant to ceftriaxone as well (XDR), leaving azithromycin as the only oral option. Infrequent resistant cases to ceftriaxone with or without azithromycin have been reported in the last few years.

Pakistan has reported haplotype H58, *S. Typhi* extensively drug-resistant (XDR) outbreak. *Salmonella Typhi* H58 XDR isolates were reported in 2016, possess 1 AMR cassette located on chromosome (with blaTEM-1, sul2, aph(6)-Id / strB, aph(3'')-Ib / strA, dhfR, folP and cat) and another AMR cassette that is carried by IncY plasmid p60006 (with blaTEM-1, blaCTX-M-15, sul2, qnrS1, aph(6)-Id / strB, aph(3'')-Ib / strA), and have a diversity of only 2 single nucleotide polymorphisms (SNPs) among all organisms isolated during the outbreak⁵.

This led to usage of fluoroquinolones (ciprofloxacin), but decades later, resistance emerged in this group as well³. Fluoroquinolone resistance shifted the treatment options to third generation cephalosporins such as cefixime, ceftriaxone, and macrolides, including azithromycin.¹ The gradual rise in MIC of ceftriaxone against *S. Typhi* led to the major catastrophic evolution of extensively drug-resistant (XDR) *Salmonella*, which was resistant to all the first-line drugs, quinolones as well as cephalosporins. The largest outbreak of ceftriaxone-resistant *Salmonella* was reported from Hyderabad, Pakistan, in 2019⁶.

In South Asia, drug resistance to *Salmonella Typhi* is a rising concern. Empiric antibiotic therapy for febrile patients and unrestricted use of antibiotics in the community are two major reasons for drug resistance. We are not certain about the specific lineage like MDR, XDR in the case of the emergence of azithromycin resistance. A cross-sectional study from Chandigarh, India, shows that azithromycin resistance molecular basis is not identical to the strains found in Pakistan and Bangladesh and have arisen as independent strains by acquiring arcB gene. The use of a Typhoid conjugated vaccine will decrease the burden of XDR *Salmonella Typhi* in South Asia⁷.

Azithromycin is now commonly used for the treatment of enteric fever as an alternative to cephalosporins because of its cost-effectiveness, high intracellular concentration, comparatively faster fever clearance time, and good clinical response⁵. Resistance to azithromycin is infrequent but has

been reported from different parts of the world, including India, Nepal, and Bangladesh⁶.

The objective of this study was to compare the values of azithromycin MICs for XDR strains of salmonella with pre-covid era as it was being extensively used empirically during the pandemic. The knowledge generated from the comparison of MICs will allow us to assess the effectiveness of the drug against XDR salmonella Typhi in future. Furthermore, this study will add to the continuous surveillance paramount to keeping antimicrobial susceptibility trends in check and acting accordingly for antimicrobial stewardship.

METHODS

This cross-sectional study was conducted in the Department of Microbiology, Combined Military Hospital, Lahore, from 24th April 2021 to 23rd April 2022. The study was approved by the research review board (Research review board No. 306/2021). Non-probability convenience sampling technique was used to collect the samples. The sample size of 384 was calculated using Raosoft® online by keeping the confidence level of 95%, a margin of error of 5% and a population size of 1 million.

Blood culture specimens from patients belonging to all age groups, received from both inpatient and outpatient departments of the hospital were included in the study. Duplicated samples from the same patient and patients on mechanical life support were excluded from the study. The data was collected on a daily basis and was analyzed. All the samples received between 1st May 2021 to 30th April 2022 (n=384) with the growth of *Salmonella enterica* serovar Typhi were processed.

The blood culture specimens were processed by BACT/ALERT 3D system (BioMérieux, France). Positive blood cultures showing gram-negative rods were sub-cultured on blood agar and MacConkey agar (Oxoid, UK). The isolates were identified using standard microbiological techniques, based on colony morphology and biochemical tests using API 20E (BioMérieux, France)⁸. Serotyping was done using group and type-specific antisera (Remel). The in-vitro susceptibility of the test isolates was determined using the Kirby-Bauer Disk Diffusion method as well as E strips (BioMérieux, France) for azithromycin (AZM). The Muller-Hinton agar plates were incubated aerobically at 35 ±2°C for 18-24 hours in ambient air. MIC was recorded as the lowest concentration at which there was no visible growth of isolate. The CLSI recommended MIC breakpoints for AZM (susceptible ≤ 16µg/mL and resistant ≥32 µg/mL) were used for result analysis⁹. This Azithromycin MICs values data was compared with the data collected before the pandemic.

The statistical analysis was done using Statistical Package for Social Sciences (SPSS) version 23.0. Qualitative variables were summarized as mean \pm SD, while qualitative variables were summarized as frequency and percentages. Results are presented as cross-tabulations (for sensitivity patterns) and t-tests (for comparison of means). The null and alternative hypotheses are stated below:

Null hypothesis: MIC of Azithromycin is the same in pre-COVID and after one year of COVID

Alternative hypothesis: MIC of Azithromycin has increased in after one year of COVID

RESULTS

In our study total of 384 confirmed XDR Salmonella Typhi specimens were collected after performing their sensitivity for confirmation that they were XDR strains. MICs of Azithromycin in these isolates were compared with the same number (384) isolates before the COVID-19 pandemic. MIC of an isolate was done by the E Strip method. Our statistics results are presented as cross-tabulations and analysis using an independent t test.

The sensitivity pattern among pre-COVID samples revealed high sensitivity of 179 (46.6%) in the 19-59 year age group and lower sensitivity of 6 (1.6%) in the >60 years senior adult. After one year of COVID, the sensitivity pattern is highly sensitive 177(46.1%) in the 13-18 years adolescence and lower sensitive 8(2.1%) in the >60 years senior adult. (Table 1)

Cross-tabulation between age and sensitivity pattern pre and after 1 year of COVID:

Age of the patients	Pre-COVID Sensitivity pattern Sensitive
<1 year Infant	7(1.8%)
1-12 years child	171(44.5%)
13-18 years adolescence	21(5.5%)
19-59 years adult	179 (46.6%)
>60 years senior/adult	6 (1.6%)
Total	384(100%)

Age of the patients	Post COVID 1 year sensitivity pattern Sensitive
1-12 years child	80(20.8%)
13-18 years adolescence	177(46.1%)
19-59 years adult	119(31.0%)
>60 years senior adult	8(2.1%)
Total	384(100%)

We can see that the Azithromycin MIC after 1 Year of COVID has higher mean as compared to the Azithromycin MIC Pre-covid. That means, on average Azithromycin MIC after 1 Year of Covid used more than the pre-COVID. (Table 2)

	Time period	Sample Size (N)	MIC (mean)
Azithromycin	Pre covid	384	2.55
	Post Covid (1 year)	384	4.91

p-value of Levene's test for the equality of variances is .005, which is less than 0.05, so we reject the null hypothesis that the variances are equal. So, we do not assume equal variances. The p-value for t-test is .000, which is less than 0.05 (5% level of significance), so we reject the null hypothesis and conclude that the Minimal Inhibitory Concentration of Azithromycin was increased after one year of COVID. (Table III)

DISCUSSION

In our study, all isolates of XDR Salmonella Typhi were sensitive to Azithromycin on the basis of MIC values as per the latest CLSI standards. However, when we compared the values before the COVID-19 pandemic, our values were much higher in range. This shows a rise in MIC values after the indiscriminate use of this antibiotic after the start of the pandemic.

Some of our isolates had values of 12, 14, and 16ug/mL that were similar to a study conducted at Aga Khan University Hospital, Karachi, that showed MIC of 12ug/mL¹⁰. Although their isolate was phenotypically sensitive to Azithromycin. Still, on whole genome sequencing (WGS), they found genes (R717Q mutation) that cause this organism to become resistant to Azithromycin along with the genes responsible for fluoroquinolone and cephalosporin resistance. This study supports the results of our study. However, contrary to our results, a study conducted in Bangladesh reports that they isolated 32 isolates that were resistant to Azithromycin (MIC \geq 32ug/mL).

Upon whole genome sequencing (WGS), it was revealed that the resistance is encoded by *acrB* gene that causes point mutation for the efflux pump¹¹. Another study conducted in Northern India confirms the emergence of resistance against Azithromycin with similar genes as were sequenced in a study conducted in Bangladesh⁷. Another study from Nepal reports Azithromycin resistance in XDR Salmonella Typhi with higher MIC and after Whole genome sequencing (WGS) *acrB* gene point mutation¹².

Table 3. Independent Samples Test (Azithromycin_MIC)

Levene's Test for Equality of Variances	t-test for Equality of Means								
	F	Sig.	T	df	Sig.(2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
								Lower	Upper
Equal variances assumed	7.822	.005	-32.230	766	.000	-2.354	.073	-2.498	-2.211
Equal variances not assumed			-32.230	724.850	.000	-2.354	.073	-2.498	-2.211

Salmonella Typhi XDR is a constant concern, and WHO recommends continuing antimicrobial resistance surveillance for timely response and tackling the issue if any change in the resistance pattern of salmonella typhi arises¹³. Throughout the world, especially Italy and the USA many cases of XDR S. Typhi have been reported that were imported from Pakistan, so the country has been on a special watch list for spread of XDR S. Typhi¹⁴. Salmonella Typhi XDR is treated with Azithromycin and carbapenem. With the rising number of COVID-19 cases, Azithromycin gained ground for treatment of the viral infection. Self-medication has added insult to the injury and even medical practitioners have prescribed the drug without any valid justification. Although no published data in this regard is available, a Nigerian study has reported that indiscriminate mass use of Azithromycin can render other antimicrobials resistant¹⁵.

In our country, there is already an overuse of Azithromycin in fever because most of the diagnosis for typhoid fever is made clinically due to a lack of laboratories. Most of the tests performed are typhi Dot or WIDAL test which are not the gold standard. Blood culture is the gold standard, but few laboratories have that facility and can cope along with COVID-19 situation¹⁶.

This study will make clinicians aware of the situation that how quickly we can lose oral antibiotic options for the treatment of enteric fever. They will have better insight into the arising problem of antibiotic resistance in salmonella and will help implement antibiotic stewardship. They will make judicious use of antibiotics especially Azithromycin in the context of our findings.

CONCLUSION

As a result, over and indiscreet use of Azithromycin during COVID-19 pandemic as it is the only available oral option for XDR Salmonella Typhi can wreak havoc especially in an endemic country like Pakistan. Strict antimicrobial stewardship is need of the hour as Azithromycin resistance is knocking at the door. MICs of Azithromycin should be performed and the isolates

with higher MICs values in the sensitivity range should undergo gene detection for possible resistance. A large study of whole genome sequencing (WGS) should be conducted to dig out the penetration of resistance genes for Azithromycin in our country.

CONFLICT OF INTEREST

Declared none

ETHICAL APPROVAL

The study was approved by the Research Review Board, Combined Military Hospital, Lahore, vide Reference No.306/2021.

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AUTHOR'S CONTRIBUTIONS

SHK: Manuscript writing, data collection,

MA: Proof reading, supervision

AA: Manuscript editing, review

AT: Statistical analysis

AB, AT: Practical work, review