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Comparative Analysis of Phenotypic Methods for Identifying Ciprofloxacin Resistance in Salmonella Species and the Role of the aac(6')-Ib-cr Gene

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Abstract:

Background: Multidrug-resistant *Salmonella* strains are now common globally, posing a serious healthcare threat. **Aim:** Identification of ciprofloxacin resistance with molecular detection of aac(6')-Ib-cr gene in clinical isolates of *Salmonella* species. **Methodology:** A prospective, cross sectional & analytical study was conducted for a period of 12 months. *Salmonella* species identified from various clinical samples sent to the microbiology lab for culture were evaluated for ciprofloxacin using epsilometer test and Kirby Bauer's disc diffusion method and and pefloxacin susceptibility using Kirby-Bauer's disc diffusion method. Identification of aac(6')-Ib-cr gene which is molecular marker for ciprofloxacin resistance was performed using real time polymerase chain reaction. **Result:** A total of 40 *Salmonella* species bacterial isolates were identified. Out of these, 39/40 (97%) isolates were resistant to ciprofloxacin using disc-diffusion and epsilometer strip methods. Pefloxacin resistance were seen in 34/40 (85%) bacterial isolates. The aac(6')-Ib-cr gene was identified in 38/40(95%) bacterial isolates. Pearson Chi-square test indicated a statistical significant result with $p<0.05$. **Conclusion**: Pefloxacin can be used as surrogate marker for ciprofloxacin resistance in *Salmonella* species but with caution.

Keywords: *Salmonella* species, ciprofloxacin, pefloxacin, acc (6')-IB-cr gene

Introduction:

India is a nation with a wide range of spatial, societal and spiritual characteristics. But typhoid fever is widespread in the nation and major source of burden for both public and private healthcare systems. S. Divyashree et. al., (2016) suggested that, geographically, the frequency

ranges from 140 incidents of enteric fever per 1 lakh person-years in Kolkata, East India, to 273 incidents of enteric fever per 1 lakh person-years in Delhi; also, the incidence varies by age. Kuang D et. al., suggested that the incidence of ciprofloxacin resistance in *Salmonella* raised from 2.3% and 5.9 % in 2006 and 2012. The majority of the *Salmonella* isolates showed mutations in PMQR (71%) and QRDR (97.2%), indicating the widespread presence of multidrug resistance. The two most prevalent PMQR determinants were oqxA/oqxB (33.5/33%) and Aac(6')-lb-cr (62%). The other PMQR genes are oqxAB, qepA, and qaqBIII, which encode efflux pumps, and the six qnr genes (qnrA, qnrD, qnrS, qnrB, qnrC, and qnrVC) that create gyrase-protection repetitive peptides; and aac(6')-Ib-cr, which encodes an acetyl-transferase that inactivates aminoglycosides and quinolones. Salmonella bacteria that are resistant to ciprofloxacin are often linked to the aac(6')-Ib-cr gene. This gene encoded enzyme that acetylates & inactivates certain fluoroquinolone antibiotics, including ciprofloxacin, rendering them ineffective against bacterial infections. The aac(6')-lb-cr gene leads to reduced sensitivity or resistance to ciprofloxacin when it is present in *Salmonella* strains, especially those that cause infections in humans. For monitoring the spread of *Salmonella* strains resistant to ciprofloxacin and for the purpose of selecting an appropriate antibiotic therapy, the aac(6')-lb-cr gene needs to identified in clinical specimens.

In 2015, pefloxacin was developed as a surrogate marker for assessing susceptibility to fluoroquinolones which is recommended by EUCAST (European Committee on Antimicrobial Susceptibility Testing) and CLSI (Clinical and Laboratory Standards Institute). Efficacy of pefloxacin as a surrogate marker for ciprofloxacin was analyzed in the present study. We also aimed to identify the prevalence of the aac(6')-Ib-cr gene in *Salmonella* species identified from clinical samples.

MATERIALS AND METHODS:

Prospective, analytical and cross sectional study was conducted at the Department of Microbiology in SRM Medical College Hospital & Research Centre, over a course of January 2023 to December 2023. *Salmonella* species identified from various clinical samples received in the Microbiology laboratory were analyzed for the study. VITEK® 2 Biomerieux was used for

the identification and antimicrobial susceptibility of *Salmonella* species. Kirby Bauer disc diffusion for azithromycin (15 µg), ceftriaxone (30 µg), chloramphenical (30 µg), ciprofloxacin (5 µg) and pefloxacin (5 µg) was also performed on Muller Hinton Agar plate using 0.5 McFarland standards over an 18-hour incubation period at 37ºC. A total of 40 *Salmonella* species isolates were identified in the study. The 2023(M100) CLSI criteria were used in interpreting disc diffusion zone sizes of the following antibiotics (ciprofloxacin-5 µg and pefloxacin-5 µg). The interpretative criteria used for ciprofloxacin (5 µg) susceptibility was as follows zone diameter breakpoint ≤20 considered as resistant, ≥31 considered as sensitive and 21-30 considered as intermediate susceptibility. The interpretative criteria for pefloxacin were as follows resistant to pefloxacin if its zone of inhibition was ≤23mm, and considered susceptible with a zone of ≥24mm. All the 40 isolates were tested for ciprofloxacin Minimum Inhibitory Concentration by Epsilometer-strip test method. According to CLSI 2023, ciprofloxacin MIC more than ≥1 was considered resistant, <0.06 considered as sensitive and 0.12-0.5 considered as intermediate. Ciprofloxacin resistant isolates according to the MIC interpretative criteria was subjected to quantitative real-time Polymerase Chain Reaction for detecting the aac(6')-Ib-cr gene. PCR were done by using forward primer-TTGCGATGCTCTATGAGTGGCTA and reverse primer-CTCGAATGCCTGGCGTGTTT as described by Skov R et al. Presence of acc-lb-cr gene will be used for detecting ciprofloxacin resistant in *Salmonella* species. Chi-square test was used to conduct the Statistical analysis.

Result:

Out of 40 *Salmonella* isolates, 27(67%) were *Salmonella Typhi*, 11(28%) were *Salmonella Paratyphi A*, 2(5%) were *Salmonella Paratyphi B.* Of 40 *Salmonella* isolates, 39(97%) isolates were resistant to ciprofloxacin and while the remaining 1(3%) isolates was susceptible to ciprofloxacin using disc diffusion susceptibility testing and 39(97%) isolates were resistant to ciprofloxacin and remaining 1(3%) isolates were sensitive to ciprofloxacin (Fig1 & 2) using MIC E-Strip testing. Of the 40 isolates of *Salmonella* species, 34(85%) were resistant and 6(15%) were sensitive to pefloxacin using disc diffusion testing.

The analysis of aac(6')-Ib-cr gene by real time polymerase chain reaction for all 40 isolates of *Salmonella* spp., showed 38(95%) isolates had the presence of aac(6')-lb-cr gene (Fig:3). Chi-square test was used to conduct the Statistical analysis. A statistically significant correlation was

observed between the result obtained from ciprofloxacin MIC testing by E-test and ciprofloxacin susceptibility testing by disc-diffusion method, pefloxacin susceptibility testing and molecular analysis for aac(6')-Ib-cr gene. In this study, MIC was used as the gold standard method to identify ciprofloxacin susceptibility in *Salmonella* species.

Discussion:

Ciprofloxacin resistance in *Salmonella* isolates is a significant and growing concern in India. Chande C et al (2002) suggested that *S. Typhi* did not show any resistance to *Salmonella* spp. in past investigations. Thamizhmani R et al., and Medalla F et al., (2012 and 2011) suggested that there is an increasing prevalence of ciprofloxacin resistant *Salmonella* strains in India these days. Ciprofloxacin resistance in *Salmonella* species, particularly *Salmonella* enterica *serovar Typhi*, has become a significant public health issue in India. In 2015 CLSI introduced, pefloxacin as an alternative marker to assess susceptibility to ciprofloxacin and other fluoroquinolones. Pefloxacin was known to locate chromosomal regions (gyrB & gyrA, parE & parC); plasmids (qnrS, qnrB & qnrA, and aac(6')-Ib-cr) are known identify fluoroquinolone resistance more effectively than ciprofloxacin and nalidixic acid.

In this study, *Salmonella* isolates (n=40) were obtained from a variety of clinical samples. All the ciprofloxacin-resistant *Salmonella* isolates were specifically found in blood samples. This findings of the study was similar to the study by Niranjan Patil and Prashant Mule et al., were all the isolates were collected from blood sample. In this study, out of the 40 *Salmonella* isolates, 27(67%) were identified as a *Salmonella Typhi* and 11(28%) were identified as *S. Paratyphi A* and 2(5%) were *S. Paratyphi B.* This study found higher proportions of *Salmonella Typhi* isolates compared to *Salmonella Paratyphi* isolates in blood samples. In another study, the distribution of Salmonella strains was 77.9 percentage of *S. Typhi*, 21.1 percentage of *S. Paratyphi A,* and 1 percentage of *S. Paratyphi B* from blood sample. These findings are consistent with Maskey et al. (2008), who reported 71 percentage of *Salmonella Typhi* and 29 percetage of *Salmonella Paratyphi A.* Similarly, Niroula et al. (2020) found that 75% of their isolates were *Salmonella Typhi.*

In the present study, among the 40 *Salmonella* isolates, 39(97%) isolates were resistant and 1(3%) isolates were sensitive to ciprofloxacin susceptibility using MIC testing. A study

conducted by Girish et al. in 2013 the susceptibility to ciprofloxacin among the Salmonella isolates was followed up, over the years (2009, 2010 and 2011) and 84% of isolates were susceptible to ciprofloxacin in 2009, 67% were susceptible to ciprofloxacin in 2010 and 85% susceptible to ciprofloxacin in 2011. MIC rate is correlates with Syed Asim Ali Shan et al., show high resistance towards 95% to ciprofloxacin. In a study conducted by Sharma P et. al., (2017), out of 412 isolates, just 34 (8.25%) were found to be susceptible to ciprofloxacin. When comparing the ciprofloxacin susceptibility percentages between the CLSI 2015 and CLSI 2011 guidelines, it was observed that, according to the older criteria, 329 (80.03%) isolates were considered susceptible. However, with the updated criteria, only 34 (8.25%) remained susceptible, 309 (75%) were classified as having decreased susceptibility, and 69 (16.75%) were deemed resistant. Similar study conducted by Veeraraghavan B, by using the disc diffusion method, out of a total 282, 4.3%, 80.5%, and 15.2% of the isolates was found to be sensitive, intermediate, and resistant to ciprofloxacin, respectively. But in current study, there is no intermediate susceptibility to ciprofloxacin.

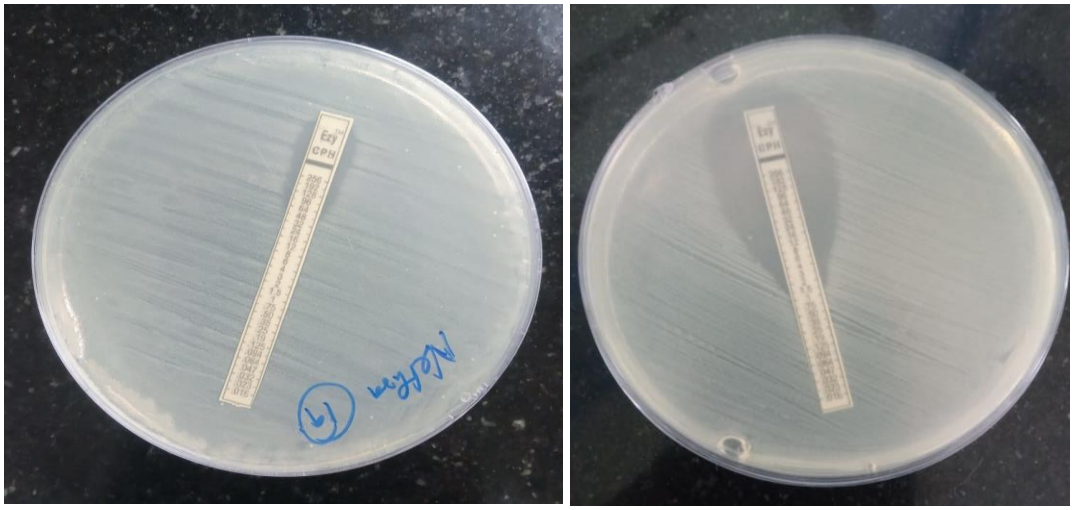
In 2015, CLSI and EUCAST have suggested pefloxacin as a surrogate marker for testing susceptibility to ciprofloxacin. According to 2023(M100) CLSI criteria for pefloxacin, ≥24 consider as sensitive and ≤23 consider as resistant. In current study, out of 40 Salmonella isolates, 6(15%) were sensitive and 36(85%) were shown resistant to pefloxacin. In another study conducted by Arunava Kali et al., among 14(100%) isolates of Salmonella 100% were resistant to pefloxacin disc diffusion method. Similarly veeraraghavan et ., suggested that out of the 282 isolates, 4.6% (n = 13) were susceptible to pefloxacin, while 95.4% (n = 271) were resistant, as determined by disc diffusion. Skov R et al (2015) and Deak E et al., (2015) suggested that pefloxacin disk diffusion separates ciprofloxacin susceptibility more effectively compared to disk diffusion, surpassing the effectiveness of ciprofloxacin disk diffusion.

In the current investigation, aac(6′)-Ib-cr gene was assessed in 40 Salmonella-isolates. Of them, 38 (95%) strains containing aac(6′)-Ib-cr gene. Different studies showed different prevalence patterns of aac(6’)-Ib-cr gene. Veldman A et. al. (2011) suggested that out of 1,215 Salmonella isolates from 13 European countries tested for PMQR, only 3(23%) of strains were identified with aac(6′)-Ib-cr gene. Sjölund-Karlsson M et al. (2010) suggested that only 6 of the 51 isolates (11.8%) that were collected in 2007 from 2,165 human Salmonella isolates collected in the

United States were discovered to possess aac(6′)-Ib-cr. In another study, out of 136 isolates only 1 ciprofloxacin resistance isolated was identified with aac(6′)-Ib-cr gene.

Chi-square test was used to conduct the Statistical analysis. In this study, Ciprofloxacin MIC was considered as the a gold standard. A statistically significant correlation was observed between ciprofloxacin Minimum Inhibitory Concentration by E-test with ciprofloxacin AST by disc diffusion method, pefloxacin AST and molecular analysis of aac(6′)-Ib-cr gene.

FIGURE: 1 FIGURE: 2



Ciprofloxacin MIC by E-strip method:

Figure 1: Ciprofloxacin MIC breakpoint (resistant) & Figure 2: Ciprofloxacin MIC breakpoint (sensitive)

Figure 3:

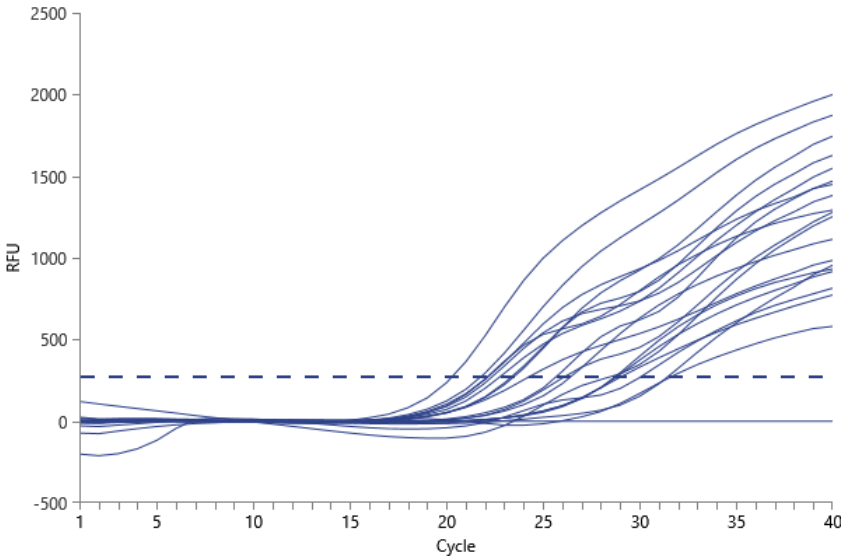


Figure 3: Molecular detection of aac(6')-Ib-cr gene by real time-PCR (this graph shows the detection of aac(6')-Ib-cr gene in 19 Salmonella isolates

Conclusion:

A significant correlation was found out between ciprofloxacin MIC value with ciprofloxacin susceptibility testing by (AST) by disk diffusion, pefloxacin AST and molecular analysis. Hence, pefloxacin can be used as surrogate marker for ciprofloxacin resistant Salmonella species but with caution. The high prevalence of aac(6′)-Ib-cr gene in ciprofloxacin resistant Salmonella species highlights the growing challenge of antimicrobial resistance.

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