

## Relationship between the Susceptibility of *Salmonella.Typhi* to Ciprofloxacin, Ofloxacin and their respective Susceptibility to Nalidixic Acid

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### Abstract

**Background:** Typhoid fever is an important cause of morbidity and mortality in many parts of the world including Pakistan. Resistance to the first line anti typhoid drugs viz chloramphenicol, cotrimoxazole and ampicillin has aggravated this situation. Quinolones are currently used as the first line antityphoid drugs, instead. Fluoroquinolones are currently recommended for patients infected with Typhi. The fluoroquinolones have shown good *in vitro* as well as clinical activity against Typhi infections.

**Materials and Methods:** It was a comparative cross-sectional conducted at Department of Microbiology UHS, Lahore, Pakistan within one year (January 2011-December 2011). A total of 100 clinical isolates of Typhi were evaluated. ATCC 9150 Paratyphi A was used as a standard strain. The bacterial isolates were preserved in microbanks (Pro-Lab Diagnostics, UK) and stored at -70°C during a period of (2007- 2011). Data was analysed through SPSS version 22.

**Results:** Of the 100 isolates, 45 strains were showing MIC  $\leq 1\mu\text{g/ml}$  which means that they were susceptible while 55 strains were intermediate having MIC  $2\mu\text{g/ml}$ . No strain was however, found resistant to ciprofloxacin according as per the CLSI 2011. As per the CLSI 2012 revised ciprofloxacin break points for disc diffusion and MIC for salmonella species. According to CLSI 2012 interpretive criteria, on disk diffusion testing 13 isolates were sensitive, 13 were resistant and 74 were intermediate to ciprofloxacin. On MIC, 55 strains were resistant showing MIC  $\geq 1\mu\text{g/ml}$  and 45 isolates were intermediate showing MIC  $0.125\text{-}0.5\mu\text{g/ml}$ . No isolate was found sensitive to ciprofloxacin according to CLSI 2012 interpretive criteria.

**Conclusion:** In conclusion, the present study showed the value of nalidixic acid susceptibility as an indirect but a certain marker of ciprofloxacin susceptibility. Nalidixic acid resistant showed increased minimum inhibitory concentration (MIC) by agar dilution method.

**Keywords:** Antibiotic resistance, Typhoid fever, Nalidixic acid resistant, ciprofloxacin susceptibility

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### Introduction:

Typhoid fever is a systemic infection caused by *Salmonella enterica* serovar Typhi (*S.Typhi*) and *Salmonella enterica* serovar

Paratyphi (*S. Paratyphi*) A, B, and C (1). Typhoidal salmonellae are gram-negative rods which belong to the family *Enterobacteriaceae*. It is a motile,

non-sporulating facultative anaerobe. The genus *Salmonella* was named after an American microbiologist Daniel Elmer Salmon and the genus was classified in to more than 2600 different serovars (2).

According to the Centres for Disease Control and Prevention, the genus *Salmonella* contains two species *S. enterica* and *S. bongori* each of which contains multiple serotypes (3). *S. Typhi* infections are widely recognized as a major cause of morbidity globally, with an estimated 21 million cases and between 200 000 and 600 000 deaths annually (4). The incidence of typhoid fever is highest in Asia and is estimated to be over hundred per hundred thousand cases/year (5).

As per estimates for the year 2000 there is a suggestion that approximately 21.5 million infections and 200,000 deaths from typhoid fever globally each year (6). South Asia is the most commonly reported region for typhoid fever during 1996 to 2005 with more than 80% of global cases and an incidence rate of 110 Cases per 100, 000 populations (7). Pakistan is a hyper-endemic area for typhoid fever and according to WHO report (2008) the incidence of typhoid fever in 5-15 years children was 412 per million in 2002 (8).

Widespread use of fluoroquinolone therapy for enteric fever has been followed by the emergence of Typhi and Paratyphi A isolates with elevated minimum inhibitory concentrations (MIC) to ciprofloxacin and ofloxacin across Asia and in parts of Africa (9). Towards the end of the last decade, it was observed that fever took longer time than before to clear and at times surprisingly failed to respond to ciprofloxacin therapy. These isolates had comparatively higher MIC of fluoroquinolones, although they were

susceptible to fluoroquinolones by the conventional disc diffusion testing and recommended MIC breakpoints. (10). Nevertheless, such strains of *S. typhi* are resistant to nalidixic acid and it was noted that clinical response to fluoroquinolones in patients infected with nalidixic acid-resistant *S. Typhi* (NARST) was inferior to the response in those infected with nalidixic acid-sensitive *S. Typhi* (NASST) strains (10). However, it is not clear whether fluoroquinolones can still be used as first-line drug for the treatment of typhoid fever, and if used whether this has any adverse impact on clinical outcomes other than treatment failure such as development of complications and morbidity assessed in terms of total duration of illness. The current MIC breakpoints for fluoroquinolones, including ciprofloxacin, for (typhoidal salmonellae) are  $\geq 1\mu\text{g/ml}$  for resistance and  $\leq 0.06$  for susceptibility (11). The present study was designed to determine the MIC of fluoroquinolones in  $\text{NA}^{\text{R}}$  and  $\text{NA}^{\text{S}}$  *S. Typhi*. It was observed that  $\text{NA}^{\text{R}}$  as well as  $\text{NA}^{\text{S}}$  *S. Typhi* have increased MIC for ciprofloxacin (MIC  $\geq 1\mu\text{g/ml}$ ) according to CLSI 2012.

#### Materials and Methods:

It was a comparative cross-sectional conducted at Department of Microbiology UHS, Lahore, Pakistan within one year. A total of 100 clinical isolates of Typhi were evaluated. ATCC 9150 Paratyphi A was used as a standarad strain. These isolates were collected from Sheikh Zayed Medical Complex Lahore, Services Hospital Lahore, Fatima Memorial Hospital Lahore, Ittefaq Hospital Lahore and Shaukat Khanum Cancer Memorial Hospital Lahore. The bacterial isolates were preserved in

microbanks (Pro-Lab Diagnostics, UK) and stored at  $-70^{\circ}\text{C}$ . All the clinical isolates were reidentified by standard morphological, cultural and biochemical profile (API -20E, bioMerieux, France). Serological confirmation was performed by using antisera (BD Difco, USA). Gram staining, Catalase test, Oxidase test, API-20E (For identification of enterobacteriaceae), Motility and Serology were performed for identification and confirmation.

### Results:

**Antimicrobial susceptibility pattern:** A total of 100 clinical isolates of *S.Typhi* were tested. Among Typhi, all isolates ( $n=100$ ) were resistant to Chloramphenicol (66%), followed by Ampicillin (66%), Co-trimoxazole (65%) and Nalidixic acid (55%) on Kirby Bauer disk diffusion according to CLSI 2011. In this study all 100 clinical isolates of *S.Typhi* were divided into two groups \* $\text{NA}^{\text{R}}$  ( $n=55$ ) and \* $\text{NA}^{\text{S}}$  ( $n=45$ ). All Isolates of *S.Typhi* ( $n=55$ ) in  $\text{NA}^{\text{R}}$  group were resistant to Nalidixic acid followed by, Chloramphenicol (78%), Ampicillin (76%) and Co- trimoxazole (75%) according to CLSI 2011 shown in (Figure:1)

In  $\text{NA}^{\text{S}}$  group ( $n=45$ ), isolates of *S.Typhi* were resistant to Ampicillin (53%), Co-trimoxazole (53%) and Chloramphenicol (51%) according to CLSI 2011 shown in (Figure: 3). In  $\text{NA}^{\text{R}}$  group, 42 (76%) isolates of *S.Typhi* were sensitive and 13 (24%) were intermediate to Ciprofloxacin, however, in  $\text{NA}^{\text{S}}$  group, all the isolates were susceptible on Kirby Bauer disk diffusion according to CLSI 2011 guidelines. According to CLSI 2012, in  $\text{NA}^{\text{R}}$  group, ( $n=55$ ) 13 (24%) strains

were resistant and 42 (76%) strains were intermediate shown in (Figure: 2)

In  $\text{NA}^{\text{S}}$  group, ( $n=45$ ) 13 (29%) strains were sensitive and 32 (71%) were intermediate shown in (Figure: 4).

Out of one hundred Typhi isolates ( $n=100$ ) it was observed that 87 (87%) isolates were sensitive ( $\geq 21$  mm) while 13 (13%) were intermediate (16-20 mm). No isolates was found resistant ( $\leq 15$  mm) to ciprofloxacin according to interpretive criteria CLSI 2011. As per the MIC, out of one hundred *S.Typhi* 45 (45%) isolates showed  $\text{MIC} \leq 1\mu\text{g/ml}$  which means that they were susceptible while 55 (55%) isolates were intermediate susceptible having  $\text{MIC} 2\mu\text{g/ml}$ . No isolates was, however, found resistant to ciprofloxacin  $\text{MIC} \geq 4\mu\text{g/ml}$  according to the CLSI 2011.

### Comparative analysis of 2011 and 2012 CLSI interpretive breakpoints

- According to CLSI 2012 interpretive criteria, out of one hundred *S.Typhi*, 13 isolates were susceptible, ( $\geq 31\text{mm}$ ) 13 were resistant ( $\leq 20\text{mm}$ ) and 74 were intermediate (16-20mm) on disk diffusion testing. Those isolates, which were resistant to ciprofloxacin on disk diffusion, were also resistant to nalidixic acid on disk diffusion as well as on MIC.
- On MIC, out of one hundred *S.Typhi*, 55 strains were resistant showing  $\text{MIC} \geq 1\mu\text{g/ml}$  and 45 isolates were intermediate showing  $\text{MIC} 0.125\text{-}0.5\mu\text{g/ml}$ . No isolate was found sensitive ( $\leq 0.064\mu\text{g/ml}$ ) to ciprofloxacin according to CLSI 2012 interpretive criteria.

**MIC determination of ciprofloxacin by agar dilution (NA<sup>R</sup> and NA<sup>S</sup> *S. Typhi* (n=100):** Agar dilution was performed to determine the MIC of ciprofloxacin for *S. Typhi*. In NA<sup>R</sup> group, out of 55 isolates, 8 (15%) were sensitive (MIC  $\leq$ 1 $\mu$ g/ml) and remaining 47 (85%) were intermediate (MIC=2 $\mu$ g/ml) whereas in NA<sup>S</sup> group out of 45 isolates, 37 (82 %) were sensitive while remaining were intermediate according to current interpretive criteria CLSI 2011. MIC ranges are given in (Table 1).

**MIC determination of ofloxacin by agar dilution NA<sup>R</sup> and NA<sup>S</sup> *S. Typhi* (n=100):** In NA<sup>R</sup> group, out of 55 isolates, only one (1%) isolate was intermediate (MIC =4  $\mu$ g/ml) while remaining all isolates were susceptible to ofloxacin whereas in NA<sup>S</sup> group, out of 45 isolates, 8 (17 %) isolates were intermediate and remaining were sensitive (MIC=2  $\mu$ g/ml) to ofloxacin according to current interpretive criteria CLSI 2011. MIC ranges are given in Table no. 2.

**MIC determination of levofloxacin by agar dilution NA<sup>R</sup> and NA<sup>S</sup> *S. Typhi* (n=100):** In NA<sup>R</sup> and NA<sup>S</sup> group, all isolates were susceptible according to current interpretive criteria CLSI 2011. MIC ranges are given in (Table 3).

### **Discussion:**

Typhoid fever remains a major health problem faced by the developing countries including Pakistan. The high rate of resistance to nalidixic acid and emergence of strains with full resistance to ciprofloxacin constitute a major problem in Pakistan. In 2000, it was estimated that over 2.16 million episodes of typhoid occurred worldwide,

resulting in 216 000 deaths, and that more than 90% of this morbidity and mortality occurred in Asia (12).

A study was conducted by WHO (13) to compare the disease burden of typhoid fever across Asia using standardized clinical and microbiological methods and surveillance procedures about the disease. The sites for higher incidences are India (493.5), Indonesia (180.3) and Pakistan (412.9), and lower incidences in the Viet Nam (24.2) and China (29.3). This data also indicated that, in the higher-incidence study sites, the incidence of typhoid fever in pre-school children aged 2–5 years was of the same order of magnitude as that for school-aged children aged 5–15 years. These findings are consistent with earlier work showing that, in high-incidence areas, the incidence of typhoid in pre-school children can approximate that for school-aged children (14).

Susceptibility testing generally adopted in the resource-poor laboratories of developing countries is limited to disc diffusion technique which may not be adequate to determine reduced susceptibility to fluoroquinolones. (15). This often requires advanced quantitative techniques such as MIC which is not available in the routine laboratories. *S. Typhi* with reduced susceptibility to fluoroquinolones and resistance to nalidixic acid require higher MICs of fluoroquinolones (1).

Testing for fluoroquinolones susceptibility according to CLSI (2011) breakpoints fails to detect reduced sensitivity to these drugs as they are considered susceptible according to CLSI (2011) interpretive criteria. Since isolates with reduced susceptibility to

fluoroquinolones may become highly resistant upon sequential accumulation of mutations in topoisomerase genes, their prediction by the use of simpler screening tools implying antibiotic discs is of great value. (16).

Fluoroquinolones treatment failure cases have been increasing. Inability to identify reduced susceptibility to fluoroquinolones by the standard disk diffusion techniques further complicates the problem. Those isolates, which have higher MICs against ciprofloxacin, result in treatment failures. Fluoroquinolones have become the treatment of choice for multi drug resistant typhoid fever. Indeed, these are the only effective oral drugs in this clinical situation. But there are reports of treatment failure to quinolones due to reduced fluoroquinolones susceptibility (17). Treatment failures with quinolones were significantly more common in patients infected with nalidixic acid resistant *S.typhi* (NA<sup>R</sup>), than in those infected with nalidixic acid sensitive *S.typhi* (NA<sup>S</sup>). Ciprofloxacin 5µg disc failed to detect these less susceptible Typhi. Some patients with typhoid fever caused by NARST that is susceptible to fluoroquinolones *in vitro*, according to current Clinical and Laboratory Standards Institute (CLSI) interpretive criteria can show a delayed response to ciprofloxacin or treatment failure (18). The failure of treatment with fluoroquinolones in the cases of *S.Typhi* and *S.Paratyphi A* in the Indian subcontinent and Southeast and Central Asia due to decreased susceptibility to ciprofloxacin is now a serious concern (10). The first case of ciprofloxacin-resistant typhoid fever was reported in 1992 in the United Kingdom (Dimitrov et al., 2007). The

first case of fluoroquinolone treatment failure in typhoid fever in Pakistan was reported in 1993 (18). Since then the incidence of such cases has been increasing. A case of ciprofloxacin treatment failure was also reported from Rawalpindi (19).

The Clinical Laboratory Standards Institute (CLSI) recently addressed new guidelines for *Salmonella* species by revising the ciprofloxacin break points in 2012. Revised CLSI guidelines for *S.Typhi* have highlighted the emerging resistance in this pathogen to the fluoroquinolones (CLSI 2012). In our study we determined the susceptibility pattern of 100 clinical isolates; \*NA<sup>R</sup> (n=55) and \*NA<sup>S</sup> (n=45) by the disk diffusion technique. Minimum inhibitory concentration was determined by agar dilution method. By the disk diffusion testing of *S.Typhi* (n=100) it was observed that 87 (87%) strains were sensitive ( $\geq 21$  mm) while 13 were intermediate (16-20 mm). No strain was found resistant ( $\leq 15$  mm) to ciprofloxacin according to interpretive criteria CLSI 2011. As per the MIC, out of 100 *S.Typhi* 45 (45%) strains were showing MIC  $\leq 1\mu\text{g/ml}$  which means that they were susceptible while 55 strains were intermediate having MIC  $2\mu\text{g/ml}$ . No strain was however; found resistant to ciprofloxacin MIC  $\geq 4\mu\text{g/ml}$  according to the CLSI 2011. This infers that Ciprofloxacin disk diffusion test cannot pick up the isolates that show an intermediate MIC values against ciprofloxacin.

According to CLSI 2011, on MIC determination it was observed that in NA<sup>R</sup> group (n=55), only 4 isolates were inhibited at MIC  $0.125\mu\text{g/ml}$ , while in NA<sup>S</sup> group (n=45), 14 isolates were inhibited at MIC

0.125µg/ml clearly indicating that although the MIC values of ciprofloxacin against all the isolates are less than the breakpoint criteria for resistance but the isolates resistant to nalidixic acid are less susceptible at MIC value of 0.125 µg/ml as compared to those which are nalidixic acid sensitive.

As per the CLSI 2012 revised ciprofloxacin break points for disc diffusion ( $\geq 31$  mm) and MIC ( $\leq 0.064$  µg/ml) for susceptible strain of *Salmonella*. CLSI has revised breakpoints for ciprofloxacin from  $\leq 1$ µg/ml in 2011 to  $\leq 0.064$ µg/ml in 2012 for susceptible strain, for intermediate from 2 µg/ml in 2011 to 0.125-0.5 µg/ml in 2012 and from  $\geq 4$  µg/ml in 2011 to  $\geq 1$ µg/ml in 2012 for resistant strain, (11). By considering this issue we revised the susceptibility pattern of 100 *S.Typhi* according to new interpretive guidelines by CLSI 2012.

According to CLSI 2012 interpretive criteria, out of 100 *S.Typhi*, 13 isolates were sensitive, ( $\geq 31$ mm) 13 were resistant ( $\leq 20$ mm) and 74 were intermediate (16-20mm) on disk diffusion testing. Those isolates, which were resistant to ciprofloxacin on disk diffusion testing, were also resistant to nalidixic acid on disc diffusion as well as on MIC test. In Another study from Pakistan shows that all the nalidixic acid-resistant isolates had a reduced susceptibility to ciprofloxacin and screening for nalidixic acid resistance is a significant method to detect reduced susceptibility to ciprofloxacin (20). This study reconfirms the occurrence of ciprofloxacin-susceptible but nalidixic acid-resistant *S. a*On MIC out of 100 *S.Typhi*, 55 strains were resistant showing MIC  $\geq 1$ µg/ml and 45 isolates were intermediate showing MIC 0.125-0.5µg/ml.

No isolate was found sensitive ( $\leq 0.064$  µg/ml) to ciprofloxacin according to CLSI 2012 interpretive criteria. In our study intermediate strain to ofloxacin were also found in agar dilution method. In NA<sup>R</sup> group, one isolate of *Typhi* was found intermediate (MIC =4 µg/ml) to ofloxacin, while in NA<sup>S</sup> group, 8 isolates of *Typhi* were intermediate (MIC =4 µg/ml) to ofloxacin. As per the MIC no strain was found to be resistant to ofloxacin and only (8%) were intermediate resistant while remaining was fully susceptible.

This infers that ofloxacin commanded more susceptibility than ciprofloxacin. However, levofloxacin showed better susceptibility than both the other quinolones here all strains were fully susceptible (MIC  $\leq 2$ µg/ml). Quantitatively determining the antibiotic MICs of the clinical isolates is not practical in routine clinical laboratories because it is expensive (E-test), time consuming process (agar dilution and broth dilution) and requires experienced personnel. Most laboratories use the disc diffusion assay, which is a qualitative assay and which is not sufficiently sensitive to screen *S.Typhi* with decreased CIP susceptibility. Findings from this study and others, on the correlation between the resistance to NA and the decreased susceptibility to ciprofloxacin will have important applications in clinical laboratories. (21). The nalidixic acid disc diffusion assay should be used as an indicator for detecting *Typhi* isolates with decreased ciprofloxacin susceptibility. It is observed that *Typhi* isolates with decreased susceptibility to ciprofloxacin will have important applications in clinical laboratories. (21). The nalidixic acid disc diffusion assay should be used as an indicator for detecting *Typhi* isolates with decreased ciprofloxacin susceptibility. It is observed that *Typhi* isolates with decreased susceptibility to ciprofloxacin will have important applications in clinical laboratories. (21). The nalidixic acid disc diffusion assay should be used as an indicator for detecting *Typhi* isolates with decreased ciprofloxacin susceptibility; however, this is now known not to be the case, and many have suggested

that decreased ciprofloxacin susceptibility is most reliably determined by measurement of the ciprofloxacin minimum inhibitory concentration (1). In our study it was observed that most of the strains in NA<sup>S</sup> group that seem to be sensitive on disk diffusion but on MIC they were intermediate susceptible. Patients with typhoid fever due to isolates with decreased ciprofloxacin susceptibility are more likely to have prolonged fever clearance times and higher rates of treatment failure (1). In the United States, Typhi with MDR and decreased ciprofloxacin susceptibility are associated with travel to the Indian subcontinent (23).

There is a separate problem with laboratory testing for reduced susceptibility to ciprofloxacin: current recommendations are that isolates should be tested simultaneously against ciprofloxacin and nalidixic acid and the isolates that are sensitive to ciprofloxacin and nalidixic acid should be reported as "sensitive to ciprofloxacin", but those isolates appear sensitive to ciprofloxacin but not to nalidixic acid should be reported as "reduced sensitivity to ciprofloxacin. Clinical laboratories should adopt the revised CLSI 2012 ciprofloxacin break points for all *Salmonella* isolates in which susceptibility testing is indicated and discuss the technical issues for laboratories using commercial antimicrobial susceptibility system (24).

### Conclusion:

In conclusion, the present study showed the value of nalidixic acid susceptibility as an indirect but a certain marker of ciprofloxacin susceptibility. Nalidixic acid resistant showed increased minimum inhibitory

concentration (MIC) by agar dilution method.

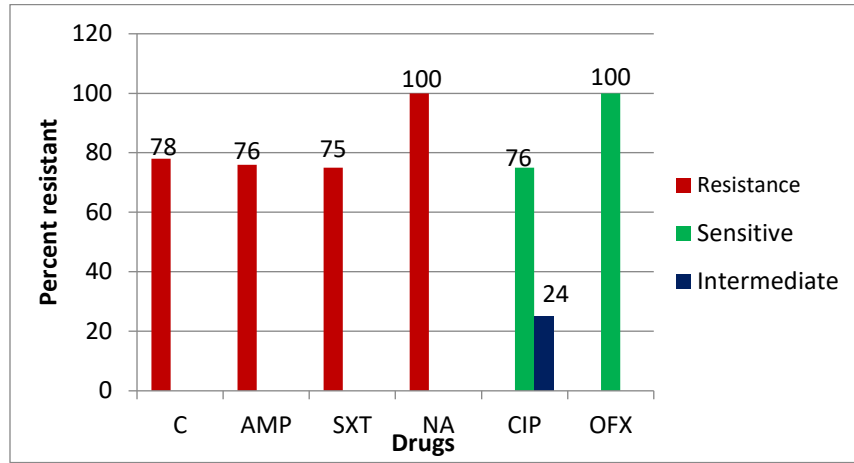
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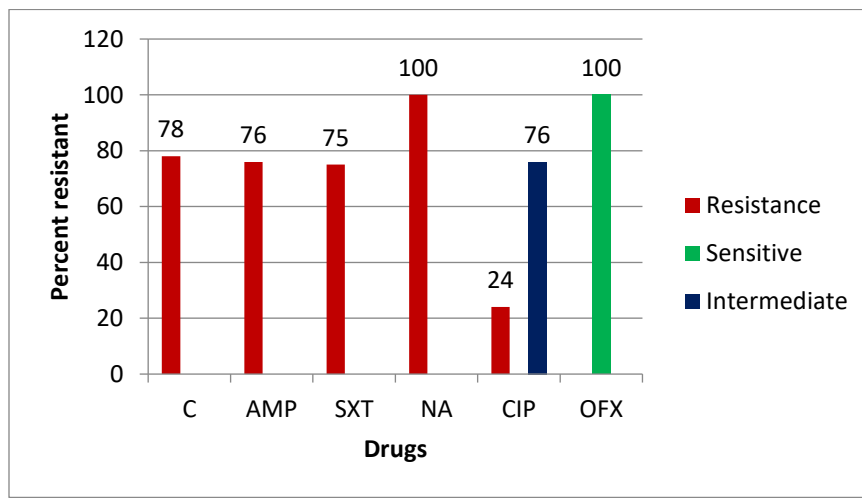


**Nalidixic acid resistant group (*S.Typhi* n=55) CLSI 2011**



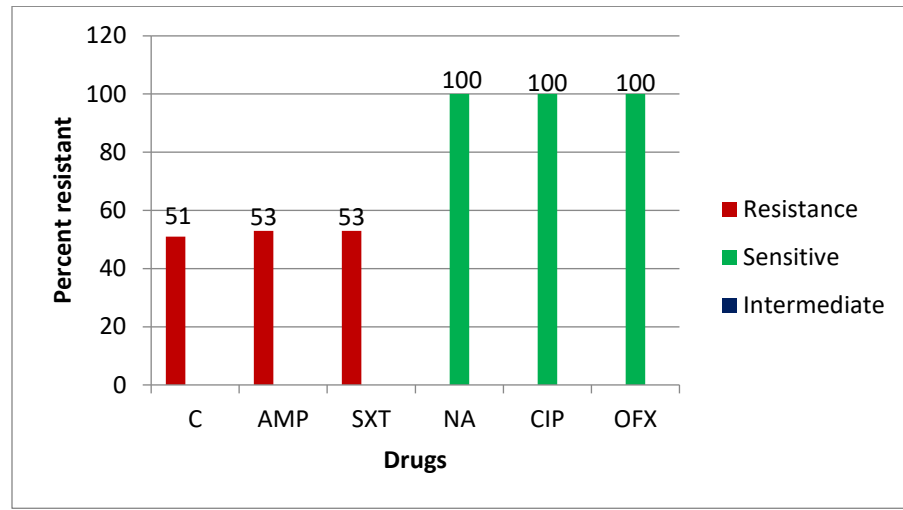
**Figure no. 1: C- Chloramphenicol, AMP-Ampicillin, SXT-Cotrimoxazole, NA- Nalidixic acid, CIP- Ciprofloxacin and OFX- Ofloxacin.**

**Nalidixic acid resistant group (*S.Typhi* n=55) CLSI 2012**



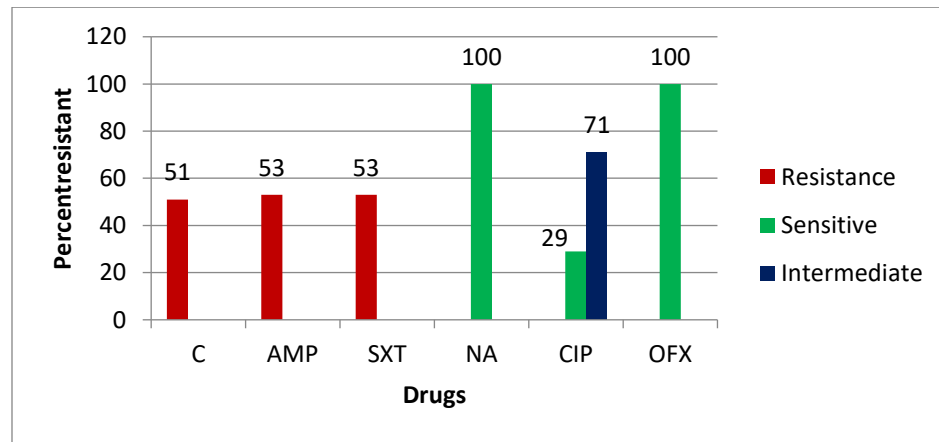
**Figure no. 2: C- Chloramphenicol, AMP-Ampicillin, SXT-Cotrimoxazole, NA- Nalidixic acid, CIP- Ciprofloxacin and OFX- Ofloxacin**

**Nalidixic acid sensitive group (*S.Typhi* n=45) CLSI 2011**



**Figure no. 3: C- Chloramphenicol, AMP-Ampicillin, SXT-Cotrimoxazole, NA- Nalidixic acid, CIP- Ciprofloxacin and OFX- Ofloxacin.**

**Nalidixic acid sensitive group (*S.Typhi* n=45) CLSI 2012**



**Figure no. 4: C- Chloramphenicol, AMP-Ampicillin, SXT-Cotrimoxazole, NA- Nalidixic acid, CIP-Ciprofloxacin and OFX- Ofloxacin**

**Table 1: Cumulative MIC of ciprofloxacin in NA<sup>R</sup> and NA<sup>S</sup> *S. Typhi* (n=100)**

Drugs	0.125µg/ml	0.25µg/ml	0.5µg/ml	1µg/ml	2.0µg/ml	4µg/ml	8µg/ml	MIC 50	MIC 90	MIC Range (µg/ml)
1.Ciprofloxacin NA <sup>R</sup> (n=55) Typhi	4	1	1	2	32	15	-	2	4	0.125-4
2.Ciprofloxacin NA <sup>S</sup> (n=45) Typhi	14	16	6	1	5	3	-	0.125	4	0.125-4

**Table 2: Cumulative MIC of ofloxacin in NA<sup>R</sup> and NA<sup>S</sup> *S. Typhi* (n=100)**

Drugs	0.25µg/ml	0.5µg/ml	1µg/ml	2.0µg/ml	4µg/ml	8µg/ml	MIC 50	MIC 90	MIC Range (µg/ml)
1.Ofloxacin NA <sup>R</sup> (n=55) Typhi	1	0	1	1	51	1	4	4	0.25-8
2.Ofloxacin NA <sup>S</sup> (n=45) Typhi	0	18	2	2	15	8	4	8	0.5-8

**Table 3: Comparative MIC of levofloxacin in NA<sup>R</sup> and NA<sup>S</sup> *S. Typhi* (n=100)**

Drugs	0.125µg/ml	0.25µg/ml	0.5µg/ml	1µg/ml	2.0µg/ml	4µg/ml	8µg/ml	MIC 50	MIC 90	MIC Range (µg/ml)
1. Levofloxacin NA <sup>R</sup> (n=55) Typhi	1	0	1	0	50	3	-	2	2	0.125-4
2. Levofloxacin NA <sup>S</sup> (n=45) Typhi	0	6	23	5	2	9	-	2	8	0.25-4