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SUSCEPTIBILITY PATTERN OF *SALMONELLA TYPHI* CAUSING ENTERIC FEVER TOWARDS DIFFERENT ANTIMICROBIALS

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ABSTRACT

Resistant to therapeutic regimen of enteric fever is a global problem. Such a resistance has led to treatment failure of Enteric fever. Therefore, it is necessary to study the sensitivity and resistance pattern of the drugs used in Enteric fever. This study was conducted to determine the susceptibility pattern of *Salmonella* Typhi causing enteric fever towards different antimicrobials. In this study, out of 145 isolates, 108 were confirmed to be *S.* Typhi by different Gram's staining and along with different biochemical tests. In this study, *S. Typhi* was tested for 5 antibiotics i.e. Ciprofloxacin, Ofloxacin, Levofloxacin, Ceftriaxone and Chloramphenicol. This study showed the highest sensitivity for Chloramphenicol (97.22%), Ceftriaxone (79.62%), levofloxacin (68.51%), Ofloxacin (50.92%) and Ciprofloxacin (2.77%). Ciprofloxacin was the most resistant drug against *S.* Typhi. A total of 19 isolates were found to be multi drug resistant isolates (MDR). The MIC value for ciprofloxacin ranged from 55µg/ml to 100µg/ml. Among the 10 isolates, all the isolates were resistant to Ciprofloxacin (MIC ≤1µg/ml). The MIC value ranged from 12.5µg/ml to 100µg/ml for ofloxacin. All the 10 isolates were resistant to Ofloxacin as CLSI standard (≤2µg/ml). MIC for Ceftriaxone was found to be 25µg/ml. Three out of ten isolates were found to be sensitive, 5 were intermediate and 2 isolates showed resistant MIC value(≤8µg/ml sensitive). Resistance to most antibiotics is a major problem worldwide and is becoming a challenge to treat the infections. Hence, antibiotic susceptibility should be done periodically to provide the rational treatment of enteric fever.

KEYWORDS: Sensitivity, Fluroquinolones, Ceftriaxone, Chloramphenicol, MIC.

INTRODUCTION

Enteric fever is an important public health problem in developing countries including Nepal. A changing antibiotic susceptibility pattern of *Salmonella* Typhi and emergence of multi drug resistance has increased to a great concern. Enteric fever is a systemic infection caused by the human-adapted pathogens *Salmonella* Typhi and *Salmonella* Paratyphi A, B, and C. These organisms are important causes of febrile illness in crowded and impoverished populations with inadequate sanitation that are exposed to unsafe water and food (Whitaker *et al*., 2009). *Salmonella Typhi* is the gram-negative enteric bacillus belonging to the family Enterobacteriaceae. It is a motile, obligate, facultative anaerobe (Denyer et al., 2008).

Multidrug-resistant *S. Typhi* (MDRST) is epidemiologically defined as strains resistant to any two antimicrobials in vitro even if the antimicrobials tested are known to be clinically ineffective. A more useful definition of MDRST is reserved for strains of *S. Typhi* resistant to all three first-line antityphoidal antimicrobial agents, namely ampicillin, chloramphenicol, and trimethoprim-sulphamethoxazole (Khanal *et al*., 2007).

Traditional drugs such as chloramphenicol, ampicillin and co-trimoxazole were the most effectively used first line drugs for the treatment of enteric fever. However, during the late 1980s and early 1990s the occurrence of multidrug-resistant (MDR) *S. Typhi* strains resistant to chloramphenicol, ampicillin and co-trimoxazole, led to the use of fluroquinolones, particularly ciprofloxacin and third generation cephalosporin for the treatment of enteric fever (Adhikari *et al*., 2012).

The quinolones used in therapy of enteric fever are ciprofloxacin, levofloxacin and ofloxacin. Extendedspectrum cephalosporin, such as cefipime, cefpodoximeproxetil, ceftriaxone and cefixime, have shown promise as therapies for the treatment of enteric fever. Resistance is also emerging to extended-spectrum cephalosporin: ceftriaxone, cefixime, cefipime (Capoor and Nair, 2010).

Enteric fever continues to be a major health problem despite the use of antibiotics and the development of newer antibacterial drugs. The causative organism *Salmonella Typhi* has rapidly gained resistance to antibiotics like ampicillin, chloramphenicol and cotrimoxazole, and also to previously efficacious drugs fluroquinolones. The variation in the susceptibility

patterns reported for *S.Typhi*, it is important to constantly monitor it so as to provide suitable guidelines for treatment(Madhulika *et al*., 2004).

Enteric fever, a systemic infection caused by the bacteria *Salmonella Typhi* and *Salmonella Paratyphi* A, is endemic in Kathmandu, Nepal. Kathmandu, the capital city of Nepal, has been previously coined an enteric fever capital of the world. Several studies have poignantly emphasized the significant burden of enteric fever within the local population and in travellers visiting the area. The population of Kathmandu is increasing and available figures suggest that enteric fever caused by *S.Typhi* show no significant signs of decreasing (Karkey *et al*., 2008).

Improved understanding of the pathogenesis, immune control and microbiology of *S. Typhi* infection can help accelerate the development of improved vaccines and diagnostic tests necessary for disease control (Hohmann *et al*., 2010).

Antibiotics are the most prescribing medicines to wide variety of diseases. Hence irrational use of antibiotic leads to resistance day by day. Multiple-drug resistance occurs when bacteria are resistant to more than one antibiotic. This is generally the rule rather than the exception among resistant bacteria. This situation has largely occurred through the sequential use of multiple different antibiotics. The first antibiotic began by selecting a single resistance gene. Eventually, however, bacteria resistant to the first antibiotic picked up resistance to others as they were introduced into the environment. Antibiotics are used tremendously without any microbiological evidences of suspected organisms; hence antibiotic resistance is increasing day by day. Hence, susceptibility pattern of is necessary for better therapy of the infectious diseases.

Enteric fever caused by *S.Typhi* remains endemic in many areas of the developing world, causing millions of infections. The recommendation to use fluroquinolones as first line therapy for enteric fever, irrespective of sensitivity patterns has had profound public health implications for developing countries. Extended-spectrum cephalosporins are also used for treating such infections. Multidrug-resistant strains of *S.Typhi* (MDRST) are epidemiologically defined as strains resistant to any two antimicrobials. A more useful definition of MDRST is reserved for strains resistant to all three first-line antityphoidal antimicrobial agents namely ampicillin, chloramphenicol and Trimethoprim-sulphamethoxazole. With the emergence of MDRST, fluoroquinolones have gained

importance for the treatment of enteric fever in recent years. Now, different studies showed the reduced susceptibility to fluoroquinolones. Now this is seen in third generation cephalosporin also. Antibiotic-resistant strains of *S.Typhi* are becoming prevalent throughout the world; this has resulted in modifications of treatment approaches. Therefore, considering all these problems, this study is carried out to find the susceptibility pattern of clinical isolates of *S. Typhi* against antimicrobials.

METHODS AND METHODOLOGY

METHODS

This study was conducted at National Model College for Advanced Learning (NMCAL) Microbiology Labotarory, Kathmandu from 2070/2/12 to 2070/7/11. A total of 145 samples were collected from Kathmandu Model Hospital, Bagbazaar and 108 clinical isolates were identified for *Salmonella Typhi* in our laboratory.

ANTIBIOTIC SUSCEPTIBILITY TESTING

The antimicrobial susceptibility testing of the isolates towards various antimicrobial discs was done by modified Kirby- Bauer disc diffusion method as recommended by Clinical and Laboratory Standards Institute (CLSI) using Mueller Hinton Agar (MHA).

Using a sterile loop a single isolated colony of which the sensitivity to be determined was touched and inoculated into nutrient broth tube and incubated at 37°C for 4 hours. After incubation, the turbidity of the suspension was matched with the turbidity standards of Mc-Farland No. 0.5.

Using sterile swab, a plate of Mueller Hinton Agar was inoculated with the bacterial suspension using carpet culture technique. The plate was left for about 5 minutes to let inoculums to dry. Using sterile forceps, appropriate antimicrobial discs 6mm diameter was placed on the inoculated plates. After overnight incubation, the diameter of each zone of inhibition in mm was measured and results were compared with the standard zone interpretative chart provided by company. The organisms showing resistant to two and more two groups of antibiotics were taken as Multi drug resistant isolates.

2.4 DATA ANALYSIS

All the primary data of sensitive, resistance, MIC were presented in tables and graphs using MS Excel 2007, wherever applicable. The MIC value was shown in scatter plot.

FLOW CHART OF METHODOLOGY

Clinical isolates of *Salmonella Typhi*

↓

Subculture in nutrient agar and incubated at 37^{o}C

↓

Gram staining and Biochemical tests for verification

↓

Antibiotic susceptibility test on MHA by Kirby Bauer technique

↓

Determination of zone of inhibition

↓

MIC determination of isolates by Tube dilution method

RESULT

IDENTIFICATION OF ISOLATES

Out of 145 clinical isolates, 108 of them were confirmed to be *S.Typhi* using Gram's staining along with different biochemical tests such as catalase, oxidase, Triple Sugar Iron Agar (TSIA), urease, citrate, Sulphide, Indole, Motility (SIM), Methyl Red (MR), Voges-Proskauer (VP). For *S.Typhi the* confirmatory tests were obtained as below.

- Gram stain: Gram –ve
- Catalase test: +ve
- Oxidase test: -ve
- Triple Sugar Agar (TSIA : Alk/Acid
- Gas: -ve
- Urease: -ve
- Citrate test: -ve
- Sulphide: +ve
- Indole test: -ve
- Motility: motile
- Methyl Red (MR): +ve
- Voges-Proskauer (VP) test: -ve
- O/F: Fermentative

ANTIBIOTIC SUSCEPTIBILITY PATTERN

Hundred eight isolates were tested for their antimicrobial susceptibility towards 5 antibiotics (Chloramphenicol, ceftriaxone, Ciprofloxacin, ofloxacin and levofloxacin by disc diffusion method while MIC value of only 10 isolates for 3 antibiotics (Ceftriaxone, Ciprofloxacin and ofloxacin) were determined by broth dilution method.

DISC DIFFUSION ZONE DIAMETER

The sensitivity pattern of the organism obtained by disc diffusion method for each of the antibiotics is interpreted sensitive or resistant as per Zone interpretative chart. Out of the 5 antimicrobials tested for the *S.Typhi* isolates using disc diffusion method, Chloramphenicol Showed the highest sensitivity profile. Chloramphenicol showed the 97.22% sensitive, 0.925% intermediate and 1.85% resistance pattern against *S.Typhi* whereas Ceftriaxone showed the 79.62% sensitive, 12.03% intermediate and 8.33% resistance pattern. Similarly, levofloxacin showed the 68.51% sensitive, 28.70% intermediate and 2.77% resistance pattern followed by Ofloxacin showed the 50.92% sensitive, 38.88% intermediate and 10.18% resistance pattern. However, Ciprofloxacin was found to be most resistant drug. Only 2.77% of organism was sensitive towards ciprofloxacin, 28.70% were intermediate and 68.51% was resistant

MDR ISOLATES OF *Salmonella Typhi*

Out of 108 isolates of *S.Typhi*, 18 isolates were resistant to 2 groups of drugs and only 1 isolate was resistant to 3 groups of drugs .Therefore those isolates that are resistant to 2 or more groups of drugs were MDR isolates of *Salmonella Typhi*. Therefore, a total of 19 isolates of *Salmonella Typhi* were found be Multi drug reistant isolates (MDR).

Minimum Inhibitory Concentration

Minimum Inhibitory Concentration values were determined by broth dilution method for the 10 randomly selected isolates for three antibiotics, (Ceftriaxone, Ciprofloxacin and Ofloxacin). The isolates were interpreted as resistant or sensitive as per CLSI guidelines. Among the 10 isolates, 3 isolates are sensitive to ceftriaxone according to CLSI standard MIC value of Ceftriaxone ≤8 µg/ml and were classified as sensitive, 5 were intermediate compared to CLSI MIC value 16-32 µg/ml which was classified as resistant and 2 were resistant compared to CLSI standard ≥32 µg/ml (Figure: 2).

For Ciprofloxacin, among 10 isolates all had their MIC value ≥4µg/ml; hence none of them were sensitive according to CLSI guidelines. MIC value of Ciprofloxacin was ≥4 µg/ml was classified as resistant (Figure: 2).

For Ofloxacin, among 10 isolates all had their MIC value ≥8µg /ml; hence none of them were sensitive according to CLSI guidelines. MIC value of was≥8 µg/ml for all 10 isolates and was classified as resistant (Figure: 2).

MIC distribution by scatter plot

Scatterplot diagram for ciprofloxacin

Among the 10 isolates, 3 had MIC value of 100µg/ml for ciprofloxacin, 3 had 55µg/ml, 1 had 25µg/ml, 1 had 12.5µg/ml and 1 had 3.125µg/ml. Here, most of the isolates scatter in the range 55µg/ml to 100µg/ml.

Scatterplot diagram for Ofloxacin

Among the 10 isolates, 8 had MIC value of 25µg/ml for Ofloxacin and 2 had 12.5µg/ml. In this plot, most isolates were scatter in the range 25µg/ml.

Scatter diagram for Ceftraixone

Among the 10 isolates, 1 had MIC value of 100µg/ml for ceftriaxone ,1 had 50µg/ml,4 had 25µg/ml, 2 had 12.5µg/ml, 1 had 6.125µg/ml. In this plot, most isolates distribute in the range 25µg/ml.

APPENDIX-I

Gram Staining reaction

Sample no.	Grams' reaction

- **Biochemical test**

Sample no.	Catalase	Oxidase	O/F	SIM			MR	VP	Citrate	Urease	TSIA
				S	I	M					

[O/F= Oxidative/Fermentative, SIM= Sulphide(S), Indole(I), Motility(M), MR= Methyl Red, VP= Voges-Proskauer, TSIA= Triple Sugar Iron Agar

Antimicrobial susceptibility testing Worksheet

Organism Code	Reference Zone of diameter (mm)			Observed Zone of diameter (mm)	Remarks
	Sensitive	Intermediate	Resistant	Isolates	

Note: S=Susceptible, I= Intermediate, R= Resistance

- **Details of the Antibiotic Susceptibility Testing:**

S.N.	Organism code	CTR	C	CIP	OF	LE
1	A1	31	30	15	16	18
2	A2	31	30	21	20	20
3	A3	25	30	23	24	25
4	A4	28	30	15	16	29
5	A5	20	28	14	15	-
6	A6	34	33	15	15	17
7	A7	30	32	15	15	19
8	A9	32	30	22	23	22
9	A11	26	30	15	15	11
10	A12	30	34	15	15	20
11	A13	34	27	12	14	15
12	A14	26	27	22	21	23
13	A15	20	26	19	21	26
14	A16	29	28	15	16	17
15	A17	26	27	24	22	26
16	A18	25	31	16	14	17
17	A19	26	27	26	22	25
18	A20	34	32	16	15	19
19	A21	26	30	18	18	22
20	A22	25	30	14	14	18
21	A23	25	33	32	31	29
22	A24	31	27	15	15	16
23	A25	26	27	13	13	17
24	A26	23	30	13	14	19
25	A27	24	26	13	20	21
26	A28	25	29	20	13	16
27	A29	15	29	14	11	16
28	A30	20	26	19	21	21
29	A31	19	29	11	14	15
30	A33	20	26	12	12	15
31	A36	25	24	14	13	16
32	A37	26	-	23	19	21
33	A38	23	26	13	13	16
34	A39	22	20	13	12	16
35	A40	26	29	14	19	17
36	A41	20	21	20	18	19
37	A42	21	26	13	12	16
38	A43	20	32	14	12	15
39	A45	24	24	13	12	14
40	A46	28	25	22	19	22
41	A48	27	27	14	13	16
42	A49	24	28	15	14	18
43	A50	19	22	18	16	19

44	A51	19	25	13	12	16
45	A52	-	26	23	24	25
46	A54	-	-	10	-	9
47	A56	25	27	13	14	16
48	A58	25	30	18	14	17
49	A59	25	23	24	22	22
50	A60	26	29	18	14	19
51	A61	26	26	26	20	23
52	A63	29	25	16	12	15
53	A64	31	32	17	15	17
54	A65	20	22	15	12	15
55	A66	24	31	25	21	22
56	A67	25	25	24	19	21
57	A68	23	23	22	21	20
58	A69	25	21	13	18	16
59	A72	25	30	25	20	23
60	A78	26	27	23	22	23
61	A79	25	30	16	14	18
62	A82	26	25	24	19	21
63	A84	23	30	25	21	22
64	A86	25	30	25	21	18
65	A91	26	22	23	19	19
66	A94	26	29	24	22	25
67	A96	24	32	25	18	24
68	A97	27	30	22	20	21
69	A98	28	25	20	22	22
70	A100	22	27	25	19	23
71	A101	23	26	25	17	18
72	A102	25	25	26	24	22
73	A103	26	22	19	14	15
74	A104	21	24	31	19	20
75	A105	25	29	25	20	18
76	A106	30	30	32	15	16
77	A107	26	33	27	22	19
78	A108	40	35	20	19	20
79	A109	28	30	20	17	19
80	A111	29	30	19	16	19
81	A112	24	30	18	18	19
82	A113	25	20	20	19	18
83	A114	28	30	20	18	20
84	A115	30	31	19	18	23
85	A116	20	27	16	13	16
86	A117	29	29	16	14	16
87	A118	38	39	26	22	24
88	A119	31	30	20	15	18
89	A121	32	30	20	16	22
90	A122	25	26	15	12	14
91	A123	30	28	19	16	18

92	A124	23	25	15	13	16
93	A125	30	28	16	13	16
94	A126	25	23	15	13	21
95	A127	24	30	16	14	16
96	A128	13	21	16	14	17
97	A131	32	31	17	15	18
98	A133	20	30	19	16	18
99	A134	30	30	16	15	16
100	A135	28	29	15	13	16
101	A136	29	28	17	14	16
102	A139	30	15	30	13	15
103	A140	30	30	25	27	24
104	A141	10	19	20	22	24
105	A142	30	30	19	15	19
106	A143	26	25	16	14	15
107	A144	28	28	18	15	19
108	A145	19	29	19	15	19

CTR- Ceftriaxone, C- Chloramphenicol, CIP- Ciprofloxacin, OF- Ofloxacin, LE- Levofloxacin

- **Guidelines of Antimicrobial Susceptibility Testing**

Inhibition zone diameter size interpretive standards with equivalent MIC breakpoint for *Salmonella* Typhi by CLSI (2012) interpretation chart.

Antimicrobial agent (Disc potency)	**Diameter of zone of inhibition(mm) and Antimicrobial disc equivalent MIC break point (µg/ml)**		
	Susceptible	**Intermediate**	**Resistant**
Chloramphenicol(30µg)	≥18mm (≤8 µg/ml)	13-17mm (16 µg/ml)	≤12mm (≥32 µg/ml)
Ciprofloxacin (30µg)	≥31mm (≤1 µg/ml)	21-30mm 2 µg/ml	≤20mm (≥4 µg/ml)
Levofloxacin (5µg)	≥17mm (≤2 µg/ml)	14-16mm (4µg/ml)	≤13mm (≥8 µg/ml)
Ofloxacin (5µg)	≥16mm (≤2 µg/ml)	13-15mm (4 µg/ml)	≤12mm (≥8 µg/ml)
Ceftriaxone(30µg)	≥23mm (≤ 8µg/ml)	20-22mm (16-32µg/ml)	≤19mm (≥64 µg/ml)

APPENDIX-II

Working Procedure

- **Gram-Staining Procedure**

First derived by Hans Christian Gram ring the late 19th century, the Gram-stain can be used effectively to divide all bacterial species into large groups: those that take the basic dye,

crystal violet (Gram-positive) and those that allows the crystal dye to was out easily with the de-colorizer alcohol or acetone (Gram-negative). The following steps are involved in Gram-stain.

- A thin film of the materials to be examined was prepared and dried.
- The material on the slide was heat fixed and allowed to cool before staining.
- The slide was flooded with crystal violet stain and allowed to remain without drying for 1 minute.
- The slide was rinsed with tap water, shaking off excess.
- The slide was flooded with iodine solution and allowed to remain on the surface without drying for twice as long as the crystal violet was in contact with the slide surface.
- The slide was rinsed with tap water, shaking off excess.
- The slide was flooded with alcohol acetone de-colorizer for 10 seconds and rinsed immediately with tap water until no further color flows from the slide with the de-colorizer. Thicker smear requires more aggressive decolorizing.
- The slide was flooded with counter-stain (safranine) for 1 minute and washed off with tap water.
- The slide was blotted between two clean sheets of bibulous paper and examined microscopically under oil immersion at 100X.

➢ **Antibiotic susceptibility testing by disc diffusion method**

Principle

Standardized inoculum of bacteria is swabbed onto the surface of a Mueller Hinton agar (MHA) plate. Filter paper disc impregnated with antimicrobial agents are placed on the agar. After overnight incubation, the diameter of the zone of inhibition is measured around each disc. By referring to the tables in the CLSI disc diffusion standard, a qualitative report of susceptible, intermediate or resistant is obtained.

Procedure

- Bring agar plates and discs to room temperature before use. Agar plates may be removed from refrigerator and placed in 35˚C ambient air incubator with lids slightly ajar to evaporate excess moisture. Do not leave in incubator for longer than 30 min.
- Inoculum preparation.

Using a loop or swab, transfer colonies as follows

- ✓ Direct colony suspension method: pick several colonies from a fresh (18-24 hr.) nonselective agar plate to broth or 0.9% NaCl.
- ✓ Log phase method.

- ❖ Pick four or five isolated colonies to 3.0 to 5.0 ml of nutrient broth.
- ❖ Incubate at 35˚C for 2 to 8 hr. until growth reaches the turbidity at or above that of a 0.5 McFarland standards.

- ✓ For either the log phase or direct colony suspension method, vortex well and adjust turbidity visually with sterile broth or 0.9% NaCl to match a 0.5% McFarland standard.

- Inoculation of agar plates

- ✓ Within 15 minutes of adjusting turbidity, dip a sterile cotton swab into the inoculums and rotate against the wall of the tube above the liquid to remove excess inoculums.
- ✓ Swab entire surface of agar plate three times, rotating plates approximately 60˚ between streaking to ensure even distribution. Avoid hitting the slides of the plate to avoid aerosols. Finally, run swab around the edge of the agar to remove any excess moisture.
- ✓ Allow inoculated plate to stand for 3-15 minutes before applying discs.

- Application of discs

- ✓ Apply disc to agar surface with dispenser or manually with sterile forceps.
- ✓ Apply gentle pressure with sterile forceps or needle to ensure complete contact of disc with agar.
- ✓ Do not place discs closer the 24mm from center to center (no more than 12 discs on 150 mm plates and 5 discs on 100 mm plates.
- ✓ Do not relocate disc once it has made contact with agar surface. Instead place a new disc in another location on the agar.

- Incubation

- ✓ Invert plates and incubate within 15 minutes of disc application.
- ✓ Incubate for 16 to 18 hours at 35˚C in an ambient air incubator.

- Reading plates

- ✓ Read plates only if lawn of growth is confluent or nearly confluent.

- ✓ Hold inverted plate a few inches above a black nonreflecting surface.
- ✓ Illuminate plate with reflected light.
- ✓ Use a sliding caliper or ruler held on the back of the plate to measure the diameter of inhibition zone to nearest whole millimeter.
- ✓ Discrete colonies growing within the inhibition zone may represent a mixed culture or resistant variants; subculture single colonies from the primary culture plate, and re-test for susceptibility. If the discrete colonies are still apparent, measure the colony- free inner zone.

- Interpretation and Reporting

Use criteria specified by the Zone Interpretation Chart to interpret the zone of inhibition for each antimicrobial agents and report categorical result as either susceptible (S), intermediate (I), or resistant (R).

Precautions

The following sources of error should be investigated to verify that

- Zone diameters were measured and transcribed correctly;
- The turbidity standard has not expired, is stored properly, meets performance requirements, and was adequately mixed prior to use;
- All materials used were within their expiration dates and stored at the proper temperature;
- The incubator is at proper temperature and atmosphere;
- Other equipment's used (e.g., pipettes) are functioning properly;
- Discs were stored desiccated and at proper temperature;
- Inoculums suspensions were prepared and adjusted correctly; and inoculum for the test was prepared from a plate incubated for the correct length of time and in no case more than 24 hours old.

➢ **Determination of MIC by broth dilution method**

Principle

MICs are widely used to establish the susceptibility of organisms that give borderline results in disc tests, for tests on organisms where disc tests may be unreliable, and when a more accurate result is required for clinical management. MIC scores are important in diagnostic laboratories to confirm resistance of microorganisms to an antimicrobial agent, to monitor the activity of new antimicrobial agents and also used to determine the MIC breakpoints.

Procedure

- Preparation of antibiotic stock solutions and dilution range**.**

✓ Suitable range of antibiotic concentrations was chosen for the organisms to be tested.

- Preparation of Inoculum

  The inoculum should be adjusted so that it contains 10^7 and 10^8cfu/ml. The following procedure describes a method for preparing the desired inoculums by comparison with a 0.5 McFarland standard.

✓ Preparation of the McFarland standard

0.5 ml of 0.048 M $BaCl_2$ (1.17% w/v $BaCl_2.2H_2O$) was added to 99.5ml of 0.18 M H_2SO_4 (1%v/v) with constant stirring. The standard was then distributed into cap tubes of the same size and with the same volume. The tubes were sealed tightly to prevent loss by evaporation and were stored protected from light at room temperature. The standard was vigorously agitated before use.

✓ Preparation of inoculum

At least four morphologically similar colonies are touched with a sterile loop. It was then transferred to the 2ml nutrient broth with constant shaking and incubated at 35-37˚C for about 2 hours. The turbidity was then compared to the O.5 McFarland standard.

✓ Adjustment of the organism suspension to the density of the McFarland standards

The density of the organism suspension prepared was adjusted to equal that of the 0.5 McFarland standards by sterile distilled water. To aid comparison, the test and standard was compared against a white background with a contrasting black line. Suspensions should contain between 10^7 and 10^8cfu/ml.

- Preparation of broth dilution tubes

✓ Eleven capped tubes of the same size each containing 2ml nutrient broth were arranged for each antibiotic.

✓ Two tubes served as a control- negative control and positive control. Remaining was coded with their numbers.

✓ 2ml volumes of each antibiotic dilution were transferred to the tubes except positive control.

- Inoculation

✓ 50µL of the prepared inoculums equivalent to a 0.5 McFarland standard were added to the tubes except negative control. The contents of the tubes were mixed thoroughly.

- Incubation conditions

  The tubes were incubated at 35-37˚C for 18-20 hour.

- Reading and interpretation

  The MIC endpoint was read as the lowest concentration of antibiotic at which there is no visible growth.

Precautions

- The incubator is at proper temperature and atmosphere.
- Inoculum suspensions were prepared and adjusted properly.
- All the materials used were within their expiration dates and stored at the proper temperature.

APPENDIX-III

Methodology of biochemical tests used for identifications of bacteria

- **Catalase Test**

This test is performed to demonstrate the presence of catalase, an enzyme that catalyses the release of oxygen from hydrogen peroxide. During aerobic respiration, in the presence of oxygen, microorganisms produce hydrogen peroxide, which is lethal to the cell itself. The enzyme catalase is present in most cytochrome containing aerobic and facultative anaerobic bacteria, the main exception being *Streptococcus* spp.

Procedure: A small amount of a culture from Nutrient Agar plate was taken in a clean glass slide and about 2-3 drops of 3% H_2O_2 was put on the surface of the slide. The positive test in indicated by the formation of active bubbling of the oxygen gas. A false positive reaction may be obtained if the culture medium contains catalase (e.g. Blood Agar) or if an iron wire loop is used.

- **Oxidase Test**

This test is performed for the detection of cytochrome oxidase in bacteria which catalyzes the transport of electrons between electron donors. In the presence of redox dye Tetramethyl-*p*-

phenylenediamine dihydrochloride, the cytochrome oxidase oxidizes it into a deep purple colored end product Indophenol which is detected in the test. The test is used for screening species of Neisseria, Alcalignenes, Aeromonas, Vibra, Campylobacter and Pseudomonas which give positive reactions and for excluding the Enterobacteriaceae, all species of which give negative reactions.

Procedure: A piece of filter paper was soaked with few drops of oxidase reagent (Whatman's No. 1 filter paper impregnated with 1% tetramethyl-*p*-phenylenediaminedihydrochloride). Then the colony of the test organism was smeared on the filter paper. The positive test is indicated by the appearance of blue-purple color within 10 seconds.

- **Indole Production test**

This test detects the ability of the organism to produce an enzyme: 'tryptophanase' which oxidizes tryptophan to form indolic metabolites: indole, skatole (methyl indole) and indole acetic acid. The enzyme tryptophanase catalyses the deamination reaction attacking the tryptophan molecule in its side chain and leaving the aromatic ring intact in the form of indole.

Procedure: A smooth bacterial colony was stabbed on SIM (SulphideIndole Motility) medium by a sterile stab wire and the inoculated media was incubated at 37^{o}C for 24 hours. After 24 hours incubation, 2-3 drops of Kovac's reagent was added. Appearance of red color on the top of media indicated indole positive. Indole if present combines with the aldehyde present in the reagent to give a red color in the alcohol layer. The color reaction is based on the presence of the pyrrole structure present in indole.

- **Methyl Red test**

This test is performed to test the ability of an organism to produce and maintains stable acid end product from the fermentation of glucose to give a red color with the indicator methyl red and to overcome the buffering capacity of the system. Medium used in the study was Clark and Lubs medium (MR/VP broth, pH 6.9). Methyl red is an indicator which is already acid and will denote changes in degree of acidity by color reactions over a pH range of 4.4 – 6.0

Procedure: A pure colony of the test organism was inoculated into 2 ml of MRVP medium and was incubated at 37^{o}C for 24 hours. After incubation, about 5 drops of methyl red reagent

was added and mixed well. The positive test was indicated by the development of bright red color, indicating acidity and negative yellow color.

- **Voges-Proskauer (VP) test**

The principle of this test is to determine the ability of some organisms to produce acetyl methyl carbinol, a neutral end product (acetoin) or its reduction product 2, 3 – butanidiol during fermentation of carbohydrates. An organism of the Enterobacteriacease group is usually either methyl red positive and Voges- proskauser- negative or methyl red negative and Voges- Proskauer positive. The Vogesproskauer test for acetoin is need primarily E. coli from Klebsiella and Enterrobacter species.

Procedure: A pure colony of the test organism was inoculated into 2 ml of MRVP medium and was incubated at 37^{o}C for 24 hours. After incubation, about 5 drops of barritt's reagent was added and shaken well for maximum aeration and kept for 15 minutes, positive test is indicated by the development of pink red color.

- **Citrate Utilization test**

This test is performed to detect an organism utilizes citrate as a sole source of carbon for metabolism with resulting alkalinity. The medium used for citrate fermentation (Simmon's Citrate medium) also contains inorganic ammonium salts. organisms capable of utilizing citrate as its sole carbon source also utilizes the ammonium salts present in the medium as its sole nitrogen source, the ammonium salts are broken down to ammonia with resulting alkalinity.

Procedure: A loopful of test organism was streaked on the slant area of Simmon's Citrate Agar medium and incubated at 37^{o}C for 24 hours. A positive test was indicated by the growth of organism and change of media by green to blue, due to alkaline reaction. The pH indicator Bromothymol blue has a pH range of 6.0 – 7.6, i.e. above pH 7.6; a blue color develops due to alkalinity of the medium.

- **Motility test**

This test is done to determine if an organism was motile or non-motile. Bacteria are motile by means of flagella. Flagella occur primarily among the bacilli, however a few cocci forms are motile. Motile bacteria may contains a single flagella. The motility media used for motility test are semisolid, making motility interpretations macroscopic.

Procedure: Motility of organism was tested by hanging drop and cultured method. In cultural method, the test organism was stabbed in the SIM medium and incubated at 37^{o}C for 48 hours. Motile organisms migrate from the stab line and diffuse into the medium causing turbidity. Whereas non-motile bacteria show the growth along the stab line, and the surrounding media remains colorless and clear.

- **Triple Sugar Iron(TSI) Agar Test**

The TSI agar is used to determine the ability of an organism to utilize specific carbohydrate incorporated in the medium (glucose, sucrose and lactose in concentration of 0.1%, 1.0% and 1.0% respectively), with or without the production of gas (indicated by cracks in the media as well as an sir gap at the bottom of the tube) along with determination of possible hydrogen sulfide production (detected by production of black color in the medium). A pH indicator (phenol red) included in the medium can detect acid production from fermentation of these carbohydrates and it gives yellow reaction at acidic pH, and red reaction to indicated an alkaline surrounding.

Procedure: The test organism was streaked and stabbed on the surface of TSI and incubated at 37^{o}C for 24 hours. Acid production limited only to the butt region of the tube is indicative of glucose utilization, while acid production in slant and butt indicates sucrose or lactose fermentation. The results are interpreted as follows:

- ✓ Yellow (Acid)/ Yellow (Acid), Gas, H_2S → Lactose/Sucrose fermenter, H_2S producer.
- ✓ Red (Alkaline) / Yellow (Acid), No Gas, No H_2S → Only Glucose, not lactose/Sucrose fermenter, not aerogenic, No H_2S production
- ✓ Red (Alkaline) / No Change → Glucose, Lactose and Sucrose non- fermenter.
- ✓ Yellow (Acid)/ No Change → Glucose-Oxidizer.
- ✓ No Change/ No Change → Non-fermenter

- **Urea Hydrolysis test**

This test demonstrated the urease activity present in bacteria which decomposes urea, releasing ammonia and carbon dioxide. Ammonia thus produced changes the color of indicator (phenol red) incorporated in the medium.

Procedure: The test organism was inoculated in a medium containing urea and the indicator phenol red. The inoculated medium was incubated at 37^{o}C overnight. Positive organism

shows pink red color due to the breakdown of urea to ammonia. With the release of ammonia the medium becomes alkaline as shown by a change in color of the indicator to pink.

- **Oxidative/Fermentative Test**

In Oxidative/Fermentative test end product that is produced in aerobic and anaerobic process can be detected. Carbohydrates are complex organic molecules containing C, H and O in the ratio $(CH_2O)_n$. The carbohydrates are classified as monosaccharide, disaccharides and polysaccharides. Monosaccharide are simple sugars containing 3-7° C atoms e.g. Glucose, Fructose etc. Disaccharides are composed of a monosaccharide unit e.g. Sucrose etc. The polysaccharides contain 8 or more monosaccharide units e.g. Starch, cellulose, glycogen etc. During the process of catabolism i.e. breakdown or degradation of complex organic molecules, certain amount of energy is released. Most of the microorganisms have the capability of catabolizing carbohydrate.

Both the extra cellular and intracellular enzymes are necessary for the degradation of carbohydrate. Extra cellular enzymes degrade the large complex carbohydrate into simpler compounds which can pass to the cell. The intracellular enzymes catabolize these simpler compounds to generate energy. The end product of this degradation is used in glycolysis for the generation of energy.

Glycolysis is one of the metabolic pathway in which simple sugar is converted in a form of energy as ATP. If glucose, after entering a cell is catabolized aerobically the process is called oxidative metabolism where molecular O_2 serves as the final electron acceptor.

If Glucose is catabolized anaerobically, the fermentative metabolism takes place where organic molecules serve as the final electron acceptor. The metabolic end products of carbohydrate fermentation can either be the organic acids like lactic, formic or acetic or organic acid and gas like H_2 or CO_2.

Whether an organism is oxidative or fermentative can be determined by using Hugh and Leifson's medium (O/F medium). In this method, two tubes are used, one open to the air and the other sealed with paraffin to create anaerobic condition. By utilizing glucose results in production of acid which changes the color of medium to yellow.

Change in color of medium to yellow in both tubes suggests fermentation while in only open tube suggests oxidation. Certain microorganisms do not use glucose but instead utilizes

nitrogen source, here tryptone. In such case, the medium changes to blue color due to the production of alkaline end products.

Procedure: Two O/F tubes were taken and labelled with the name of given organism. With the help of sterile inoculating wire the organism was stabbed the organism in both O/F medium.one tube was sealed with 1ml of sterile paraffin oil to create anaerobic condition. Then incubation was done in37˚C.The color developed in both tubes after proper incubation was observed.

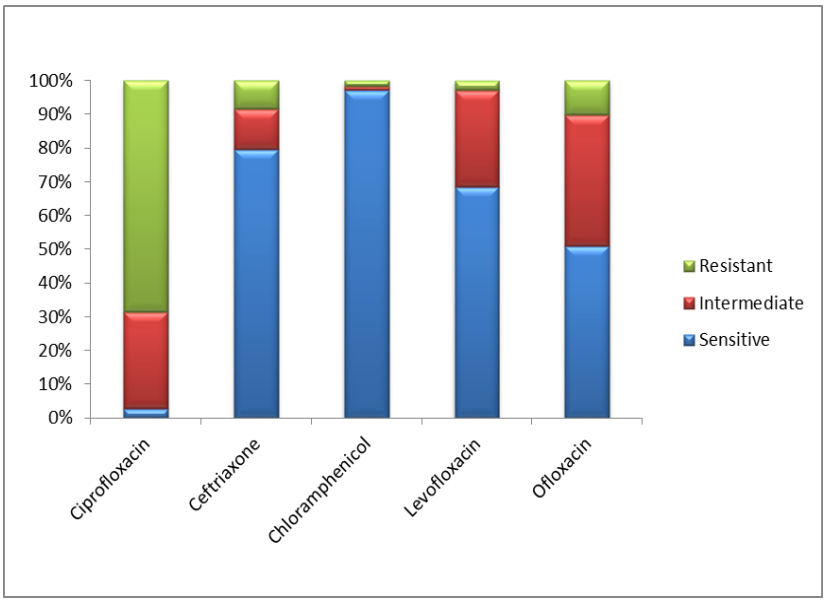

Fig: 1 Susceptibility pattern of Antimicrobials

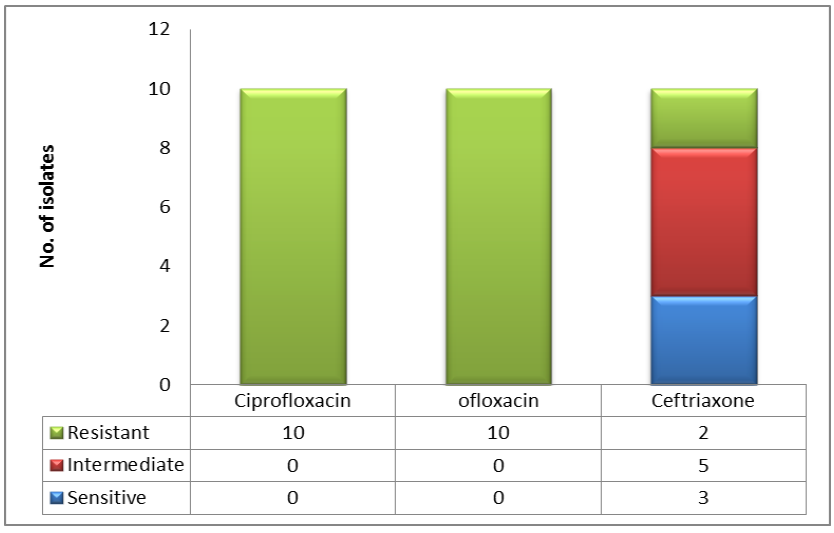

Fig: 2 MIC distributions among the *S. Typhi*

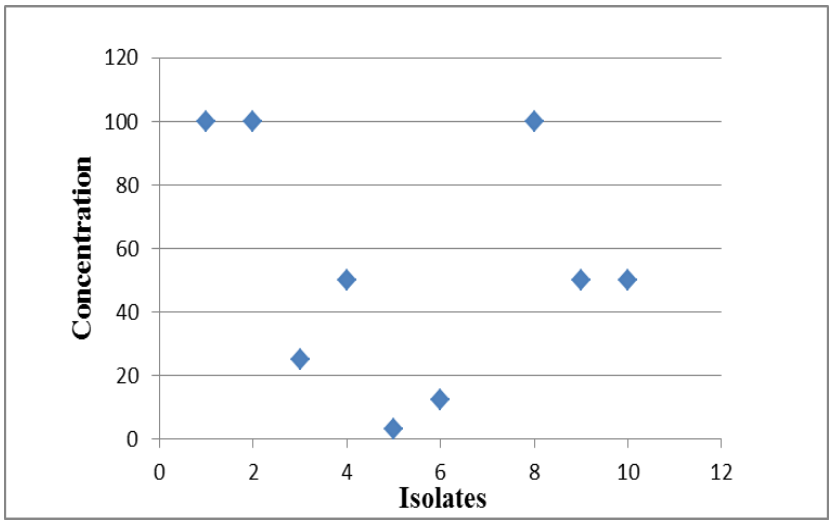

Fig: 3 Scatter plot of Ciprofloxacin

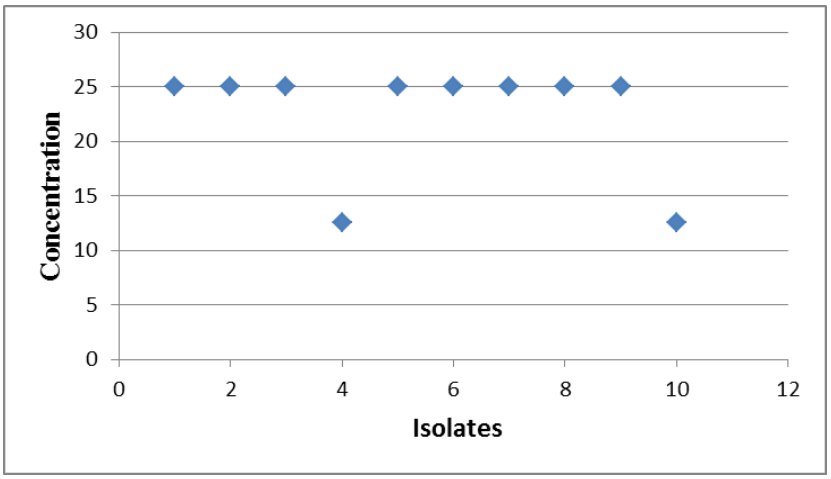

Fig: 4 Scatter plot of Ofloxacin

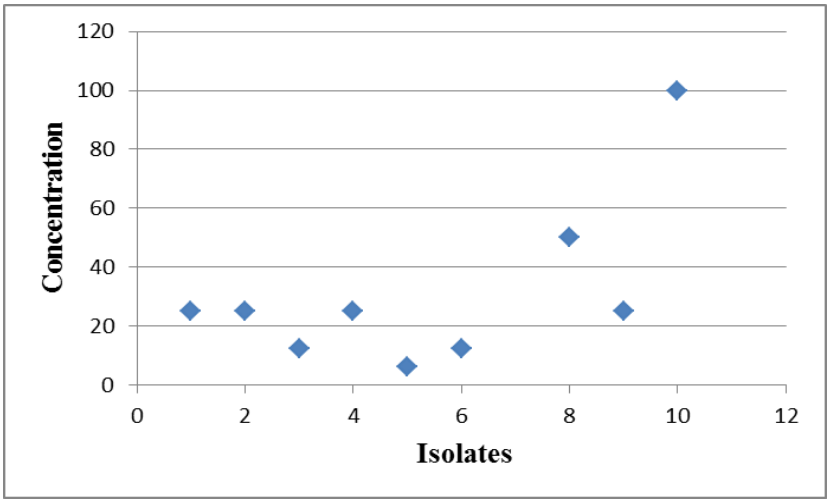

Fig: 5 Scatter plot of ceftriaxone

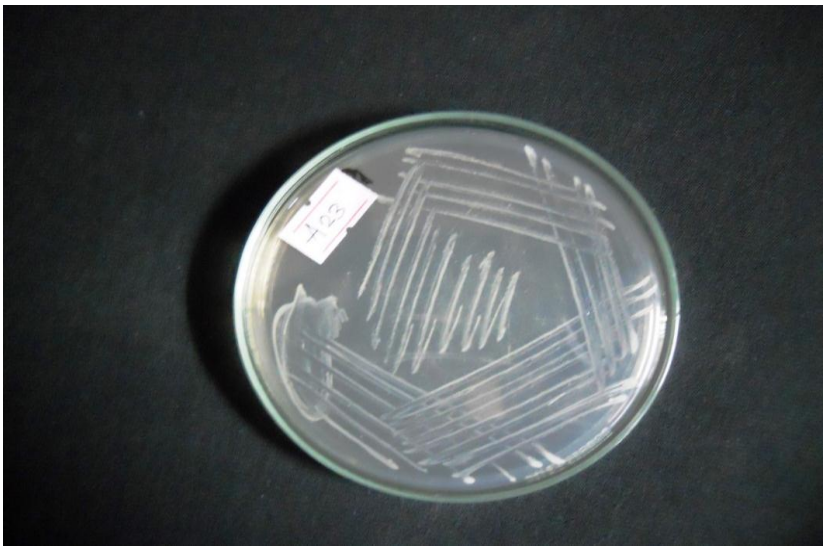

Figure 7: Subculture of S.Typhi

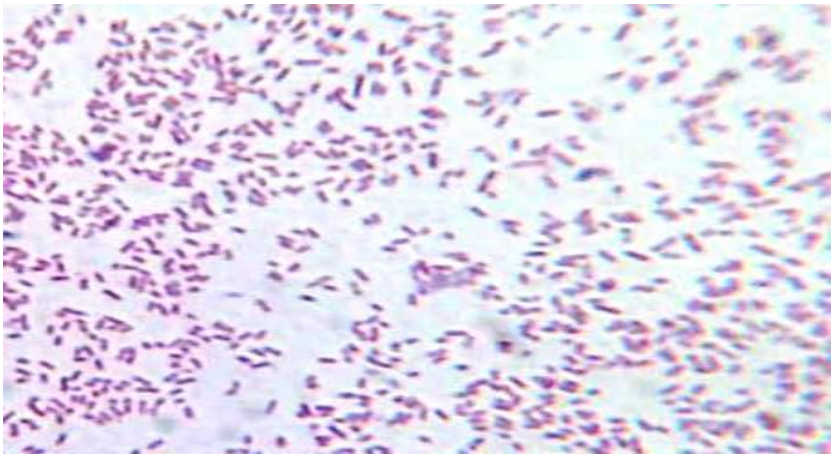

Figure 8: Gram's staining test *S.Typhi*

Figure 9: Biochemical tests of S.Typhi

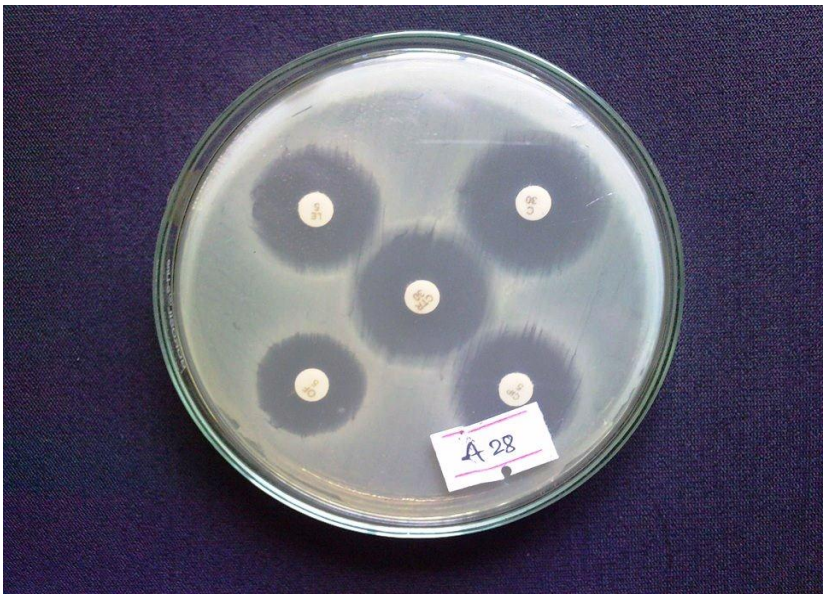

Figure 10: Antimicrobial susceptibility of *S. Typhi*

DISCUSSION

In this study, a total of 108isolates of *S.Typhi* were identified from different Gram's staining and biochemical tests collected from blood culture of patients and their antimicrobial susceptibility patterns were determined. The resistance and sensitivity profiles of *S.Typhi* showed variable results among five anti-microbial agents tested. In this study, out of 108 isolates, only 3 isolates were sensitive to ciprofloxacin, 31 isolates are intermediate and 74 isolates are resistant to ciprofloxacin. In the study only 2.77% isolates are sensitive to ciprofloxacin. A study conducted by Prajapati, *et al*. 2008 in Kanti Hospital, Nepal found that out of 195 total positive samples, ciprofloxacin was least sensitive among other drugs. (86.6%), this was similar to this study. The sensitivity rates of isolates have shown an increasing trend of resistance to ciprofloxacin indicating wide use of the drug in treating different kinds of infections (Prajapati *et al*., 2008).

A study by among 388 isolates of *S. Typhi* , 50 isolates(11.7%), were sensitive , 368 isolates(88.7%) were intermediate and 10 isolates (2.3%) were resistant Ciprofloxacin . They reported a high level resistance of ciprofloxacin against *S.Typhi*, which can be correlate with this study (Ahmed *et al*., 2006).

In contrast to this study, Ackers *et al* showed that among 74 *S.Typhi* isolates in US, all the isolates were found to be sensitive ciprofloxacin (Ackers *et al*., 2000**).** This might be due the geographical variations between the two countries and high level of sanitation in the developed countries like US. Previous study of Madhulika *et al* showed that, out of total 157

isolates of *S.Typhi* ,129 ciprofloxacin sensitive which vary from the present study (Madhulika *et al*., 2004). This variation may be due to the geographical variation.

In contrast to this study, another study from Nepal showed the antimicrobial susceptibility patterns of *S. Typhi* of 132 isolates in which all the isolates were susceptible to Ciprofloxacin (Khanal *et al*., 2007).

In this study, Chloramphenicol was the most sensitive drug against *S. Typhi*. It showed the 97.22% sensitivity against *S. Typhi*, and 1.85% resistant. Similar result was obtained from previous study in Chennai who reported an 86% sensitivity of Chloramphenicol against *S. Typhi.* (Krishnan *et al*., 2009). In the study, in Nigeria by Adabara, *et al*., 2012 showed that among 45 isolates all are sensitive to Chloramphenicol which was nearly similar result in the present study i.e. 97.22%.(Adabara *et al*., 2012). This study was also supported by Amatya, *et al*., 2007 conducted in Kathmandu Model Hospital of Nepal who showed 97.4% were sensitive and 2.6% resistant among 78 isolates *S. Typhi* (Amatya *et al.,* 2007). In contrast to this study, a study conducted in Mexico showed that, out of 493 strians of *S. Typhi*, 452 (91.7%) were resistant to chloramphenicol. It might be due to the due to the excessive use of Chloramphenicol at that time (Olarte and Galindo, 1973)

In this study, ceftriaxone is also another potent drug. Among 108 isolates, 86(79.62%) isolates were sensitive to ceftriaxone and 9(8.33%) isolates were resistant to ceftriaxone. As similar result was shown by Muthu, *et al*. 2011 98.5% sensitivity to ceftriaxone among 133 isolates (Muthu *et al*., 2011). Also from Northern India reported, 87% to 90% sensitivity of ceftriaxone which is similar to the this study (79.62%) (Gautam *et al*., 2002).

A study conducted in Kathmandu, Nepal showed that 100% sensitive to ciprofloxacin and ceftriaxone agaisnst *S.Typhi*. This contradicts this study in case of ciprofloxacin which showed only (2.77%). But similar to ceftriaxone (Kansakar et al., 2005).

In this study, out of 108 isolates 55 isolates were found to be sensitive to ofloxacin (50.92%). Prajapati, *et al*. 2008 showed that *Salmonella Typhi* was found to be most sensitive to ofloxacin (93.5%), which was different from this study (Prajapati *et al*., 2008). Similarly, study from Nigeria reported 100% Sensitivity for ofloxacin among 45 isolates. This variation may be due the less use of this drug in that area that they reported in the study, while in our country ofloxacin is also used frequently. Levofloxacin was another drug of fluroquinolones

group with greater spectrum of activity among previous fluroquinolones. In this study, levofloxacin showed 68.51% sensitivity, 31% intermediate and 2.77% resistant.

In this study MIC values were determined in randomly selected 10 isolates for 3 drugs Ciprofloxacin, Ofloxacin, and ceftriaxone. According to CLSI breakpoints ciprofloxacin and ofloxacin were completely resistant. According to scatter plot, MIC value for ofloxacin was found to be 25mcg/ml, for ciprofloxacin was 55mcg/ml-100mcg/ml and for ceftriaxone was 25mcg/ml. Similar result was obtained in over a 5-year period; study from India reported that instances of ciprofloxacin-resistant enteric fever further increased from 0.6 to 15.2 %. The MIC value for ciprofloxacin increased from 0.125mg/ml. Decreased therapeutic efficacy of quinolones is attributed to their widespread and indiscriminate use in humans (Nagshetty *et al*., 2009).

In this study, ciprofloxacin MIC Value was found to be 55mcg/ml and 100mcg/ml whereas Saha *et al*., 2006, showed highly ciprofloxacin-resistant (MIC, 512mcg/ml) strains of *Salmonela Typhi* in Dhaka, Bangladesh, which is very higher value than this study. The emergence of this highly resistant strain in Bangladesh may be due to the widespread use of ciprofloxacin in a population (Saha *et al*., 2006).

On contradictory to this study, previous study on Chennai showed the MIC Value for *Salmonella Typhi* for Ciprofloxacin, Ofloxacin and Ceftriaxone. The MIC of ciprofloxacin, ofloxacin and ceftriaxone were in the recommended range of susceptibility as given by NCCLS, 14 (28%) strains had MIC of ciprofloxacin greater than 0.5 ug/ml with 4 strains having an MIC of 1.56 ug/ml; 25 (50%) strains had MIC of ofloxacin greater than 0.5 ug/ml and 20 (40%) strains had MIC of ceftriaxone greater than 0.5 ug/ml. They account that high levels of MIC of ciprofloxacin may lead to the treatment failure cases. The rising levels of MIC of ofloxacin and ceftriaxone in *S. Typhi* is also of concern. They recommend that MIC levels of ofloxacin and ceftriaxone should be monitored along with ciprofloxacin in treatment failure cases of enteric fever (Sekar *et al*., 2003).

Brown, *et al.,* 1996 showed among twelve isolates of *S. Typhi* isolated in Vellore, India had reduced susceptibility to 4-quinolones (MIC of ciprofloxacin 0.256 mg/L) which was contrast to the range of MIC value of present study but is similar in terms of resistance. Such a variable data may be due to the different strains of isolates (Brown *et al*., 1996).

In this study, out of 108 isolates, a total of 19 isolates were found to be multidrug resistant isolates i.e. resistant to fluroquinolnes, Chloramphenicol and ceftriaxone. A Surveillance studies by John A crump *et.al* demonstrated considerable geographic variation in the proportion of *S. Typhi* isolates that are MDR in the same region, with sites in India, Pakistan, and Vietnam having higher rates of MDR isolates than sites in China and Indonesia. In the United States, *S. Typhi* with MDR and decreased ciprofloxacin susceptibility are associated with travel to the Indian subcontinent (Crump and Mintz, 2010).

CONCLUSION

The *in vitro* susceptibility pattern of 108 isolates of *S.Typhi* showed highest sensitivity to Chloramphenicol (97.22%), then Ceftriaxone (79.62%), followed by Levofloxacin (68.51%), Ofloxacin (50.92%) and lastly Ciprofloxacin (2.77%). All the 10 isolates showed higher MIC values which were found to be 25µg/ml for Ofloxacin and Ceftriaxone and 55-100 µg/ml for Ciprofloxacin. This showed that Ciprofloxacin was found to be resitant against *S. Typhi*. Therefore, antimicrobial surveillance should be done periodically to monitor the current susceptibility patterns in hospitals.

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