Prevalence and Patterns of Antimicrobial Resistance in *Salmonella enterica* serovar Typhi and *Salmonella enterica* serovar Paratyphi, Bangladesh

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DECLARATION

This is to declare that the research work embodying the results reported in this thesis entitled "**Prevalence and Patterns of Antimicrobial Resistance in *Salmonella enterica* serovar Typhi and *Salmonella enerica* serovar Paratyphi, Bangladesh**" submitted by Shafina Nowshin Nuzhat, has been carried out under the joint supervision and able guidance of Dr. Mahbubur Rahman, Physician, Clinical Microbiologist & Molecular Biologist, Consultant, Scientist & Head, Respiratory & Antibiotic Resistance Labs, Ex-Head, Department of Laboratory Services & Clinical Microbiology, icddr,b and Professor M. Mahboob Hossain, Microbiology Program, BRAC University in partial fulfilment of MS in Biotechnology, at BRAC University, Dhaka. It is further declared that the research work presented here is original, has not been submitted anywhere else for any degree or diploma.

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Dedicated to

My Beloved Parents

And

My Respected Supervisors

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ABBREVIATIONS

mg = milligram

gm = gram

L = Liter

µL = microliter

pH = Negative logarithm of hydrogen in concentration

% = percentage

Conc = concentration

et al = Associates

etc = Etcetera

Fig = Figure

S= Susceptible

I = Intermediate

R= Resistant

NA= Not available

NA= Nalidixic acid

spp = species

MHA = Mueller Hinton Agar

Amp = Ampicillin

C = Chloramphenicol

SXT = Trimethoprim- sulfamethoxazole

CIP = Ciprofloxacin

AZM = Azithromycin

CRO = Ceftiaxone

CFM = Cefixime

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ABSTRACT

Salmonella enterica serovar Typhi (*S.* Typhi) and *Salmonella enterica serovar* Paratyphi *(S.* Paratyphi) are two gram negative bacilli, responsible for enteric fever (typhoid and paratyphoid, respectively). Both of the fever, mainly typhoid caused by *S.* Typhi sometimes takes place in epidemic form in developing countries like Bangladesh. The morbidity and mortality rates of this disease can be reduced by effective antimicrobial therapy. But resistance to antibiotic is a never ending process that generate severe problem to treat this life threatening disease. The aim of our study was to assess the patterns of antimicrobial resistance and their changing trends in *S.* Typhi and *S.* Paratyphi that are endemic in Bangladesh. Blood and stool samples were cultured from the patients with high and continuous fever in icddr,b Dhaka hospital. One hundred *S.* Typhi, 21 *S.* Paratyphi A and 1 *S.* Paratyphi B were identified by biochemical and serological tests. Antimicrobial susceptibility test was carried out to observe the resistant patterns of these isolates against eight selected antibiotics. Minimal Inhibitory Concentration (MIC) of azithromycin, ciprofloxacin and nalidixic acid was determined using E-test and agar dilution technique. Seventy eight percent and 77% *S.* Typhi isolates were susceptible to ampicillin and chloramphenicol respectively where 100% *S.* Paratyphi was susceptible to those two antimicrobials. 77% isolates of *S.* Typhi were susceptible to trimethoprim-sulfamthoxazole where all *S.* Paratyphi isolates were susceptible to this antibiotic. All the *S.* Typhi and *S.* Paratyphi isolates were susceptible to cefixime and ceftriaxone. The majority of *S.* Typhi (87%) and *S.* Paratyphi (91%) were resistant to nalidixic acid and moderately susceptible to ciprofloxacin (83% of *S.* Typhi and 91% of *S.* Paratyphi). *S.* Typhi (83.84%) isolates were susceptible to azithromycin where as 91% *S.* Paratyphi were resistant to azithromycin determined by both disc diffusion and E-test. The findings suggested that multidrug resistant (resistant to ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole) *S.* Typhi and *S.* Paratyphi were still a problem in Bangladesh. In this study, 18%, 5% and 4% of *S.* Typhi were resistant to four, three and two drugs respectively but multidrug resistant *S.* Paratyphi was not detected. Nalidixic acid resistant strains were susceptible to ciprofloxacin for the past few years but the susceptibility to ciprofloxacin was low (ciprofloxacin susceptible *S.* Typhi and *S.* Paratyphi were only 13 and 5%). In addition this study showed that azithromycin can be used for the treatment of typhoid fever but not paratyphoid fever. However, cefixime and ceftraixone showed the highest susceptibility to both bacteria for treating enteric fever at present circumstances in Bangladesh.

Chapter-1
Introduction

1.1. General Introduction

Salmonella is one of the most important pathogens in the family *Enterobacteriaceae*. *Salmonella* organism is responsible for causing an acute infection of the intestine which is known as Salmonellosis, remain a worldwide health problem (Bowmer, 1988). In human, salmonellosis is considered as one of the most common and economically important zoonotic diseases.There are 3 main kinds of Salmonellosis in human, enteric fever (typhoid and paratyphoid) and gastroenteritis (non-typhoidal) (Pelczar*et al*., 2006). Enteric fever is a potentially fatal multi-systemic illness caused primarily by *Salmonellaenterica* serotype Typhi (*S*. Typhi) and to a lesser extent, *Salmonella enterica* serotype Paratyphi *(S*. Paratyphi) A, B and C (Mandal, 1979). *S.* Typhi and *S*. Paratyphi are a facultative anaerobe and a gram negative rod, and rod shaped bacillus. The only host of *S*. Typhi and *S.* Paratyphi are humans and very rarely domestic animal. Most often this pathogenic organisms are transmitted orally via food or beverages handled by an often asymptomatic individual—a carrier, who chronically sheds the bacteria through stool or, less commonly, urine (Earampamoorthyand Koff, 1975; Ali *et al*.,2006; Ram, 2007). *S*. Paratyphi is more commonly transmitted in food from street vendors. People are affected by enteric fever (typhoid and paratyphoid) via such transmission. Both typhoid and paratyphoid fever are considered as major causes of morbidity in the developing world. Centre for disease control and prevention (CDC), USA-2013 has estimated 22 million cases of typhoid fever and 200,000 related deaths occur worldwide each year; an additional 6 million cases of paratyphoid fever are estimated to occur annually (Anna and Eric,2013). However, the highest proportion has been reported in Asia, followed by Africa and Latin America (Kumar*et al.*,2013). Bangladesh is a densely populated developing country where typhoid fever is around the year problem which sometimes takes epidemic proportions in this country. The incidence of typhoid is 3.9 episodes/1,000 person-years in an urban slum of Bangladesh (Brook*et al*., 2005). The morbidity and mortality rate of this disease indicates that this infection is life threatening for human beings, especially for people of developing the world.

Effective antimicrobial therapy reduces the rate of morbidity and mortality. Early antibiotics therapy has transformed a previously life threatening illness of several weeks

duration. Without therapy, the illness may last for 3–4 weeks and case-fatality rates may exceed 10%. With appropriate treatment, clinical symptoms subside within a few days, fever recedes within 5 days, and mortality is reduced to approximately 1% (Perilla *et al.*, 2002).

Antibiotics such as ampicillin,chloramphenicol, trimethoprim–sulfamethaxazole, nalidixic acid, azithromycin, ciprofloxacin, cefixime, ceftriaxone, ofloxacin, gatifloxacin,imipenem, meropenem etc. are choice of drugs for treating both typhoid and paratyphoid fever. But resistance to drugs is such a never ending process through which organisms can evade themselves from the effect of such antibiotic. Besides because of the ready availability of over-the-counter antibiotics and subsequent resistance to these drugs in areas of endemicity, enteric fever is becoming harder to treat.

Depending on the evaluation of resistant organisms, antibiotics are suggested for successful treatment of typhoid and paratyphoid. Chloramphenicol, ampicillin, and trimethoprim-sulphamethoxazole became prevalent in some Asian countries during the late 1980s and early 1990s and resistant *S.* Typhi and *S.* Paratyphi emerged as a significant therapeutic problem. In most strain the resistant organisms developed by plasmids (Jacoby *et al*., 1980).Ciprofloxacin and ofloxacin have for some years been the drugs of choice for enteric fever, but resistance to these drugs has become very common in South Asia and has sporadically been reported in Sub-Saharan Africa. Nalidixic acid sensitivity has to be carried out in all enteric fever organisms isolated from South Asia, and if resistance is noted ciprofloxacin should not be used. Injectable third-generation cephalosporins are often the empiric drug of choice when the possibility of fluoroquinolone nonsusceptibility is high. Azithromycin is increasingly used to treat typhoid fever (Anna *et al*., 2013).On the other hand, such typhoid serovers often become susceptible to particular antibiotics after a certain period of time. For typhoid and paratyphoid although same antibiotics are used but susceptibility to particular antibiotic of these bacteria may vary. Thus both *S.* Typhi and *S.* Paratyphi have a varying degree of susceptibility to different antibiotics. Such susceptibility may change due to selective pressure, plasmid transfer or resistant gene transfer after sometimes or at the time of treatment.

It is, therefore, important to determine antimicrobial resistance patterns which are essential in recommending treatment (Perilla *et al*., 2002). Laboratory techniques are used to determine the resistant and susceptible organisms to specific antibiotics which may reduce the rate of mortality of enteric fever.

Disc diffusion method has been carefully standardized by NCCLS (an international, interdisciplinary, nonprofit, educational organization that develops updated consensus standards and guidelines for the healthcare community on an annual basis), which is effective to detect the antimicrobial resistant and susceptible strain of typhoid and paratyphoid, to avoid treatment failure. But through disc diffusion, it is not possible to determine at which concentration a drug is bactericidal or bacteriostatic. Thus MIC is (minimal inhibitory concentration) of a particular drug for *S*. Typhi and *S*. Paratyphi is an effective method to determine antibiotic concentration for treatment of enteric fever. MIC can be done using two dilution method– Broth dilution and Agar dilution method. In addition Epsilometer test (E-test) is considered as much advanced diffusion method to estimate the minimal inhibitory concentration (MIC) (Perilla *et al.,* 2002). Our aim of this study was to analyze changes in the resistance patterns of *S*.Typhi and *S*.Paratyphi for some commonly used antibiotics using disc diffusion method and also to determine of MICs of selected antibiotics. Such analysis will be helpful in near future for the treatment of typhoid and paratyphoid fever by effective antimicrobial therapy, which will reduce the rate of morbidity and mortality typhoid and paratyphoid fever.

Historical Background

S. Typhi has been a major human pathogen for thousands of years, survives in conditions of poor sanitation, crowding, and social chaos. It may have responsible for the Great Plague of Athens at the end of the Peloponnesian War (Papagrigorakis *et al.,* 2007). The name *S*. Typhi is derived from the ancient Greek typhus, an ethereal smoke or cloud that was believed to cause disease and madness. In the advanced stages of typhoid fever, the patient's level of consciousness is truly clouded (Christie, 1987). Marry Mallon was the most egregious carrier of typhoid fever, also known as Typhoid Mary. She was identified as first American carrier in 1907. With the development of vaccinations and advances in public sanitation and hygienic conditions most developed countries typhoid fever

gradually reduced throughout the first half of the 20th century. Antibiotics were introduced in clinical practice in 1942, greatly reduced mortality in developed countries.

1.2. Microbiology, diagnostic and pathogenesis

Salmonella is one of the most important pathogens in the family *Enterobacteriaceae*. The genus *Salmonella* has two species: *Salmonella enterica* and *Salmonella bongori*(Su& Chiu, 2007; Murray*et al.*, 2007.) *Salmonellaenterica* further divided into six subspecies- *Salmonella enterica* subspecies enterica (I), *Salmonella enterica* subspecies salamae (II*), Salmonella enterica* subspecies arizone (IIIa), *Salmonella enterica* subspecies diarizone (IIIb), *Salmonella enterica* subspecies houtenae (IV), *Salmonella enterica* subspecies indica (VI).

The usual habitat for subspecies enterica (I) is warm-blooded animals (Su& Chiu, 2007; Porwollik,2004). The usual habitat for subspecies II, IIIa, IIIb, IV, and VI is cold-blooded animals and the environment (Murray*et al*., 2007). All species of *Salmonella* can infect humans. *Salmonella enterica* subspecies *enterica* has about 2610 different serotypes; the most well-known being serotypes Typhi, Paratyphi, Enteritidis, Typhimurium and Choleraesuis (Su& Chiu, 2007). *Salmonella enterica* subspecies *enterica* serotype Typhi (*S.* Typhi) and *Salmonella enterica* subspecies *enterica* serotype Paratyphi (*S*. Paratyphi) both are responsible for enteric fever. Mainly the serotypes are classified by three surface antigens: the flagellar "H" antigen, the Oligosaccharide "O" antigen and the polysaccharide "Vi" antigen (found in*S.*Typhi and *S*. Paratyphi C serotype).

There are two major categories of H antigens, called phase 1 and phase 2. *Salmonella* that can express only one "H" antigen consequently have motile and non-motile phenotypes and are termed monophasic, whilst isolates that lack any "H" antigen expression are termed non-motile. Though*S*. Typhi and *S*. Paratyphi cause enteric fever with similar symptoms, they are variable on the basis of such antigenic property. Even depending on such antigenic property, *S*. Paratyphi is characterized into *S.*Paratyphi A, *S.* Paratyphi B, and *S*. Paratyphi C.

The antigenic differences among different serotype of *Salmonella* can be better understood by Kauffman –White Classification. Thus the basic difference between *S.*

Typhi and different strain of *S*. Paratyphi can be focused. In Kauffman –White Scheme those serover with particular O antigens in common are collected into "O" groups and arranged alphabetically by "H" antigens within the group. The following table represents the names of few *Salmonella* serovars, together with their antigenic formula.

Table 1:Kauffman-White Classification

Group	Serovar	Somatic"O" antigen	Flagellar (H) antigens	
			Phase 1	Phase 2
A	*S*. Paratyphi A	1, 2, 12	a	-
B	*S*. Paratyphi B	1, 4, 5, 12	b	1, 2
	S. Typhimurium	1, 4, 5, 12	i	1, 2
	S. Gloucester	1, 4, 12, 27	i	i, w
C1	*S*. Paratyphi C	6, 7, Vi	c	1, 5
	S. Cholerasuis	6, 7	c	1, 5
C2	*S*. Newport	6, 8	e, h	1, 2
D	*S*. Typhi	9, 12, Vi	d	-
	S. Enteritidis	1, 9, 12	g, m	-
E	*S*. Anatum	3, 10	e, h	1, 6
F	*S*. Aberdeen	11	i	1, 2
G	*S*. Poona	13, 22	z	1, 6

This antigenic formula is written as for ex: for *S*.Typhi9, 12,Vi:d: 0.

1.2.1. Morphology and physical properties

The morphology of the *S*.Typhi and *S*.Paratyphi corresponds with the general characteristics of the *Salmonella*. *S.* Typhi and *S.* Paratyphi are a facultative anaerobe and a gram negative and rod-shaped bacillus that are 1-3um, in length and 0.5-0.7um in thickness (Huckstep *et al.*, 2002). They are no spore forming usually motile with petrichous flagella and fimbriae. Temperature range is 28^{o}C - 50^{o}C, optimum temperature is 42^{o}C - 45^{o}C .Only *S.* Typhi and *S.* Paratyphi C possess polysaccharide capsule or Vi antigen. The Vi antigen can be destroyed at 100^{o}C. The growing temperature is 37 °C it can be killed by a temperature of 60°C for 15 minutes rapidly by boiling (Virginia Bioinformatics Institute, 2009). The range of pH is 4 – 9 and Optimum pH is 6.5 - 7.5. *S.* Typhi will generally be killed at a pH level of less than 2.5 (Gorden and Small, 1993).

1.2.2. Diagnosis

Blood cultures are the standard diagnostic method; provided a large volume of blood is cultured (15 ml in adults), they are positive in 60% to 80.0% of patients with typhoid (Parry, 2002). The culture of bone marrow is more sensitive. Bone marrow culture increases the diagnostic yield to about 80% of cases. Thus the result is positive in 80.0% to 95.0% of patients with typhoid, even patients who have been taking antibiotics for several days, regardless of the duration of illness (Wain *et al*., 2001). Blood cultures are less sensitive than bone marrow cultures because of the lower numbers of microorganisms in blood as compared with bone marrow (Wain *et al.*, 2001). The sensitivity of blood culture is higher in the first week of the illness, is reduced by prior use of antibiotics, and increases with the volume of blood cultured and the ratio of blood to broth (Hoffman *et al.,* 1986).

A stool culture is not usually positive during the early phase of the disease. The sensitivity of stool culture depends on a number of feces culture, and the positivity rate increases with the duration of the illness. Stool cultures are positive in 30 percent of patients with acute typhoid fever. For the detection of carriers, several samples should be examined because of the irregular nature of shedding (Wain *et al*., 2001).

The role of Widal's test is controversial, because the sensitivity, specificity, and predictive values of this widely used test vary considerably among geographic areas. The Widal test is an old serologic assay for detecting IgM and IgG to the O and H antigens of *Salmonella*. The test is unreliable but is widely used in most developing countries because of its low cost (Lanata *et al*., 1983). Newer serologic assays for *S.* Typhi infection are occasionally used in outbreak situations and are somewhat more sensitive and specific than the Widal test, but are not an adequate substitute for blood, stool, or bone marrow culture (Anna and Eric, 2013).

However, most laboratories identify *Salmonellae* by a combination of antigenic and biochemical reactions. Only *S.* Typhi, *S.* Paratyphi C, and some strains of *Salmonella*. Dublin possesses the Vi capsular polysaccharide antigen which can be rapidly detected by slide agglutination studies (Hashimoto *et al.,* 1995). For *S.* enterica serotype Typhicarriers, sensitivity by slide agglutination is 70 to 80 percent, with a specificity of 80.0 to 95.0 (Lanata *et al.,* 1983). DNA probes and polymerase-chain-reaction protocols have been developed to detect *S.* Typhi and *S.* Paratyphi directly in the blood (Song *et al.,* 1993).

For diagnosis in laboratory generally, the selective media like MacConkey (Mac), Salmonella-Shigella (S-S) agar, Deoxycholate citrate agar (DCA) are used which favored *Salmonella* and Shigella over coliform organism. S-S media is highly positive for selection of *Salmonella* by suppressing gram positive. Besides nowadays for the enteric organism, selective media Hekton enteric is used in a laboratory. As both *S.*Typhi and *S.* Paratyphi are non-lactose fermented, they can be detected rapidly MacConkey, Deoxycholatemedia. Though expensive than S-S media, but blood agar is also used for detection of *Salmonella*, where the size of the colony is moderately large (e.g.2.3 mm in diameter), moist and white circular disc.

Biochemical test

To aid in the more definitive identification of bacteria a series of biochemical tests that can be used to differentiate even closely related *Salmonella*.

S. Typhi and *S.* Paratyphi are slightly variable in biochemical properties. Both of the enteric fever causing bacilli can produce acid by fermenting glucose, mannose, and xylose.But *S.* Typhi that is none gas producer or produce minimal gas, where *S.* Paratyphi produces gas by fermentation. On the other hand, *S.* Paratyphi A does not produce H_2S, where *S.* Typhi variable produces H_2S; *S.* Paratyphi B generates H_2S in a higher amount.

Table 2: Characteristics biochemical reactions of common *Salmonella* organisms

Organism	Motility	Lac	Glu	Mann	Dulcite	Xyl	Inositol	Litmus milk	H_2S
S. Typhi	+	−	A	A	−	A	−	A	±
S. Paratyphi A	+	_	AG	AG	AG	−	−	A	−
S. Paratyphi B	+	_	AG	AG	AG	AG	AG	A- Alk	+
S. Paratyphi C	+	_	AG	AG	AG	AG	−	A-Alk	+
S. Typhimurium	+	_	AG	AG	AG	AG	AG	A-Alk	+
S. Enteritidis	+	_	AG	AG	AG	AG	−	A-Alk	+
S. Cholerasuis	+	_	AG	AG	AG	AG	−	A-Alk	+

N.B: Lac= Lactose; Glu=Glucose; Mann=Mannose; Xyl=Xylose; A=Acid; AG= Acid and Gas, − = nochange; ± =variable; Alk= Alkaline

1.2.3. Pathophysiology

S. Typhi and *S.* Paratyphi enter the host's system primarily through the distal ileum. They have specialized fimbriae that adhere to the epithelium over clusters of lymphoid tissue in the ileum (Peyer's patches), the main relay point for macrophages traveling from the gut into the lymphatic system. The bacteria then induce their host macrophages to attract more macrophages (Raffatellu*et al.,* 2006).

S. Typhi has a Vi capsular antigen that masks PAMPs, avoiding neutrophil-based inflammation, while the most commons *S.*Paratyphi A, does not. This may explain the greater infectivity of *S.* Typhi compared with most of its cousins (De Jong *et al.*, 2012). Typhoidal *Salmonella* co-opts the macrophages' cellular machinery for their own reproduction (Ramsden *et al*., 2007) as they are carried through the mesenteric lymph nodes to the thoracic duct and the lymphatic's and then through to the reticuloendothelial tissues of the liver, spleen, bone marrow, and lymph nodes. Once there, they pause and continue to multiply until some critical density is reached. Afterward, the bacteria induce macrophage apoptosis, breaking out into the bloodstream to invade the rest of the body (Parry *et al.,* 2002).The bacteria then infect the gallbladder via either bacteremia or direct extension of infected bile. The result is that the organism re-enters the gastrointestinal tract in the bile and reinfects player patches. Bacteria that do not re-infect the host are typically shed in the stool and are then available to infect other hosts (Christie, 1987; Parry *et al*., 2002).Chronic carriers are responsible for much of the transmission of the organism. While asymptomatic, they may continue to shed bacteria in their stool for decades. The organisms sequester themselves either as a biofilm on gallstones or gallbladder epithelium or, perhaps, intracellular, within the epithelium itself. An inoculum as small as 100,000 organisms of *S.* Typhi causes infection in more than 50% of healthy volunteers (Levine *et al.,* 2001). The infectious dose is variable, but thought to be in the range 10^5 - 10^9 cells, depending on the strain involved and the susceptibility of the host. *S.* Paratyphi requires much higher inoculums to infect, and it is less endemic in rural areas.

1.2.4. Complications:

The classic signs of enteric fever include fever, toxemia, delirium, abdominal pain, constipation, and hepatosplenomegaly (Punjabi *et al.,* 1988). **First week**: Fever occurs in 75-85% of patients in the first week and is often initially remittent but becomes steady. The individual's temperature often rises to as high as 103-104°F (39-40°C). Constipation often develops early and is likely due to obstruction at the ileocecal valve by swollen Payer patches. It may last for the entire duration of illness (Punjabi *et al.,* 1988). End of the First week: At approximately the end of the first week of illness, about a third of patients develop bacterial emboli to the skin known as rose spots. These are considered a classic symptom of typhoid fever. Rose spots constitute a subtle, extremely sparse (often <5 spots), salmon-colored, blanching, truncal, maculopapular rash with 1 to 4 mm lesions that generally resolve within 2-5 days; however, relative bradycardia and a dicrotic pulse are also common during this stage of illness (Parry *et al*., 2002).

The second week: During the second week of illness, the patient is toxic-appearing and apathetic with sustained fever. The abdomen is slightly distended, and soft splenomegaly is common (Parry *et al*., 2002).

The third week: In the third week, the patient grows more toxic and anorexic with significant weight loss. The patient may have a thread pulse, tachypnea, conjunctivitis, and crackles over the lung bases (Parry *et al.,* 2002). The patient may enter into a typhoid state of apathy, confusion, and even psychosis. Patients may develop polyneuropathy. Abnormal cerebrospinal fluid should prompt a search for a different cause. Meanwhile, the patient commonly has pronounced abdominal distension. Some individuals may produce liquid, foul, green-yellow diarrhea (pea soup diarrhea). At this stage, the patient may die from overwhelming toxemia, myocarditis, intestinal hemorrhage, or perforation due to necrotic Peyer's patches (Parry *et al.,* 2002). Rare complications of enteric fever include pancreatitis, meningitis, orchitis, and osteomyelitis.

The fourth week: During the fourth week, the fever, mental state, and abdominal distension slowly improve over a few days, but intestinal complications may still occur in surviving untreated individuals. Weight loss and debilitating weakness persist last

months. Relapses occur in 10% of patients, mostly during the first 2-3 weeks of convalescence (Parry *et al*., 2002).*S.* Paratyphi A causes the same syndrome as *S*.Typhi but appears to be a relative newcomer. It may be taking over the *S.* Typhi niche, in part, because of immunological naivete among the population and incomplete coverage by vaccines that target *S.* Typhi. *S.* Paratyphi B causes sporadic gastroenteritis and, less frequently, paratyphoid fever (Desenclos, 1996).

1.3. Epidemiology

1.3.1. Worldwide incidence, prevalence, and mortality of typhoid fever

Typhoid fever is considered as the global health problem (Bhan*et al*., 2005).Globally 27 million cases of enteric fever occur each year. According to the World Health Organization (WHO) about 21 million people are affected by typhoid fever in each year with a mortality rate of 1-4% (Bhan*et al*., 2005; Crump *et al.*, 2004).A report of WHO – 2004 showed the incidence of typhoid is high (>100 cases per 100,000 population per year) in south-central Asia, Southeast Asia, and southern Africa; medium (10 to 100 cases per 100,000 per year) in the rest of Asia, Africa, Latin America, and Oceania, except for Australia and New Zealand; and low in the other parts of the world (<10 cases per 100,000).90% of typhoid fever occurs in South East Asia. The annual attack rate ranges from 358 to 1100 per 100,000 populations (Crump *et al*., 2004; Ochiai *et al.*, 2008; Pandey *et al.*, 1990). According to best global estimates, the worldwide death rate is 600000 (Sattar *et al*., 2012).90% of typhoid fever occurs in South East Asia. The annual attack rate ranges from 358 to 1100 per 100,000 populations (Crump *et al.*, 2004; Ochiai *et al.*, 2008, Pandey *et al*., 1990). According to best global estimates, the worldwide death rate is 600000 (Sattar *et al.*, 2012)

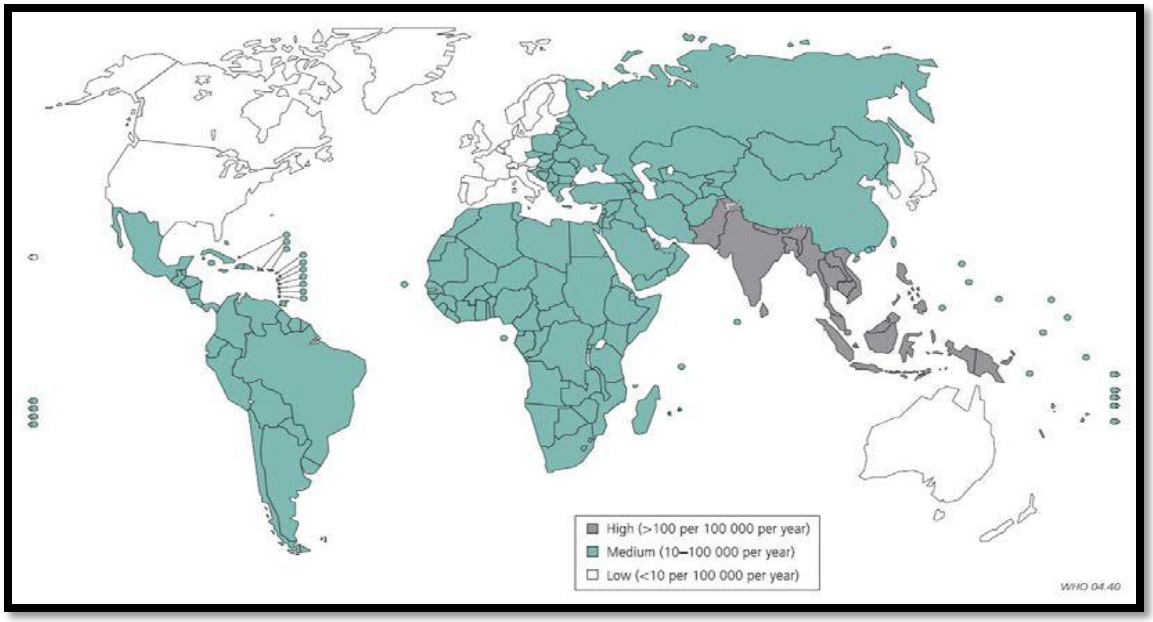

Fig 1:Global incidence of typhoid (Crump*et al*., 2004)

1.3.2. Typhoid in developed countries

In developed countries, such as in USA and U.K upgraded sanitation and successful antibiotic treatment as well as vaccination have gradually reduced the incidence of typhoid fever. In 1920, about 35,994 cases of typhoid fever were reported. In 2006, there were only 314 (Ryan *et al.,* 1989). In the United States, typhoid fever has become less frequent. It mostly affects travelers and immigrants into the USA. In a review of laboratory-confirmed cases reported to the Centers for Disease Control and Prevention (CDC) between 1999 and 2006, there were about 1900 cases of *S*. Typhi infection (Ryan *et al*., 1989). In South East England, an outbreak of typhoid from September 1897 to January 1898, affected nearly 2000 people, 143 of whom died (Stanwell and Smith, 1997). The prevalence of this disease is reported to be just one case per 1, 00,000 populations in U.K. (Bir, 2001). Between 2006 and 2010, approximately 500 cases of typhoid were reported in the UK. In an article in China, it has been reported that from 1988 to 2007, 19,000 cases of typhoid and paratyphoid were reported, with a total of 7 deaths in China. The numbers for the range of annual occurrence rate and death rate were 17.68 per one hundred thousand and 0.36 per thousand, respectively (Xu *et al*., 2009).

Although rare, outbreaks have also been reported in other European countries including Italy, Tajikistan, Turkey, Russia and the Ukraine international (Aypak, 2010; Rizzo, 2008; WHO, 2006). The provision of clean water and good sewage systems as well as antibiotic treatment and vaccination led to a dramatic decrease in the incidence of typhoid in the developed countries (Sattar *et al*., 2012).In developed countries; approximately 80 percent typhoid cases are associated with travel. The risk of travel to the Indian subcontinent (estimated rate >100 cases per million travelers), or Southeast Asia and Africa (estimated 5 to 14 cases per million travelers) is significantly higher (*Mermin et al.*, 1998; CDC, 2013). In contrast to that seen in the rich countries a recent epidemiologic study showed that south-east and south-central Asia are the regions of highest endemicity with rates greater than 100/100,000 cases per year; the rest of Asia, Africa, Latin America, the Caribbean and Oceania (except Australia and New Zealand) are the next highest with incidence rates of 10-100/100,000 and Europe, North America and the rest of the developed world have low rates of disease (Public health operational guidelines for typhoid and paratyphoid, 2012).

1.3.3. Typhoid in developing countries

Today most of the burden of this disease occurs in the developing world, where sanitary conditions remain poor (Parry *et al.*,2002). According to Crump *et al,* 2004 the crude incidence of typhoid fever cases in Africa as 50/100,000 persons and in Asia as 274/100,000 persons, because of poor socio-economic condition such as lack of provision of safe drinking water and sanitation lower in most parts of Africa than in South-East and South-Central Asia (Crump *et al.*, 2004).

On the Indian subcontinent, Maximum incidence of typhoid has been reported in Pakistan (451.7 per 100,000 persons/year) followed by India (214.2 per 100,000 persons/year), reported in 2008 (Ochai *et al*., 2008).Typhoid fever remains the 4th main cause of death in Pakistan (WHO, 2006). A study on the large-scale community in an Indian urban slum reported the prevalence of typhoid as high as 2 per 1,000 population per year for children under five, and 5.1 per 1,000 population per year for children under ten (Walia *et al.*,2006). Another study in Northern India reported that the majority of incidence occurred in children aged 5 to 12 years and 24.8% of cases were in children up to 5 years

of age (Walia*et al.,* 2006). In three urban slums in Karachi, Kolkata and North Jakarta, the prevalence of blood-confirmed typhoid fever cases among children 5-15 years of age ranged from 180 cases to 494 cases per 100 000 persons (Gilman*et al*., 1975). In Vietnam, the incidence rate is 198 per 100,000 in the delta region of Vietnam .In South Vietnam, typhoid fever remains a considerable intestinal infection. Between 1990 and 1993, among 15 districts in the South of the country, a total per year of 3,853 to 9,179 cases was registered: from 8 to 31 led to death(Nguyen *et al*., 1993). Annual incidences in Papua New Guinea and Indonesia reach about 1200/100,000 population(Typhoid fever, Ptolemy Project, 2006)

A series of typhoid fever outbreaks have been surging through central and southern Africa since early November 2011.On a February 2012, typhoid outbreak occurred in Bindura City, Zimbabwe, in the Mashonaland province (Alfred, 2012).

1.3.4. Typhoid fever in Bangladesh

Bangladesh, located in South Asia, has a population that is mostly insolvent; thus, it is probable that typhoid incidence is usually high, yet little is known about the distribution of this disease in Bangladesh. Typhoid fever is around the year problem which sometimes takes epidemic proportions in this country.Monsoon months have the highest disease occurrences followed by the pre-monsoon and post-monsoon season (Dewan *et al*., 2013).The reasons behind such occurrences are unsafe water supply, defective sewage system and unhygienic food handling practice (Rahman *et al.,* 2011).

In Bangladesh half of typhoid cases are resistant to commonly used antibiotics leading to typhoid case fatality rates as high as 30%.(WHO,2011). A bacteremia typhoid fever incidence of 3.9 episodes/1,000 person-years reported during fever surveillance in a Dhaka urban slum (Brook *et al.,* 2005).

1.3.5. Epidemiological distribution on typhoid fever related to age and sex

Typhoid fever, in general, is age specific. Attack rates are highest in persons younger than 20 years or older than 70 years; however, the highest rate is found in infants (130 isolates per 100,000) (Parry *et al.,* 2002). In more recent years, prospective studies have

shown that typhoid fever is highest in adolescents and young adults. Highest attack rate occurs in children aged 8-13 years (Parry *et al.,* 2002).

In China, the age range was 20-50 years old with a large regional distribution and it was highly prevalent in winter and spring months (Xu*et al.,* 2009). In Bangladesh, a population-based study suggested that children and young adults had the highest age-specific rates of all enteric infection (Brook*et al.,* 2005).Peak incidence has been reported to occur in children 5–15 years of age; however, in regions where the disease is highly endemic, children <5 years of age may have among the highest infection rates (Brook *et al*., 2005). Another study in Dhaka reported that Prevalence of enteric fever is high among the patients of school going age group (66.67%), habituated with unsafe drinking water (58.33%) and junk foods (72.92%) (Rahman *et al*., 2011).A review of icddr, b Dhaka hospital's records of typhoid patients admitted from January 2010 to December 2012 showed that typhoid fever is prevalent throughout the year in Dhaka. Young children and young adults are the common sufferers of this illness, while older patients with typhoid fever are rare among the admitted patients in icddr, b, Dhaka hospital (Typhoid fever in Bangladesh, 2011). Another study during 2005-2009 in Dhaka metropolitan revealed that the age-specific incidence rate was highest for the0–4 years age group (277 cases), followed by the 60+ years age group (51 cases), then there were 45 cases of 15–17 years, 37 cases of 18–34 years, 34 cases of 35–39 years and 11 cases for 10–14 years per 100,000 people (Dewan *et al.,* 2013). Typhoid fever incidence is more commonly seen in male than female. The male-female ratio of typhoid cases has found to be 1.36, and the median age of the cases was 14 years within Dhaka metropolitan (Dewan *et al*., 2013).

1.3.6. Worldwide prevalence of Paratyphoid fever

Paratyphoid fever is also considered as an enteric fever but it is milder than typhoid. The incidence of paratyphoid fever is increasing gradually worldwide (Ochiai, 2005). It is estimated 6 million cases of paratyphoid fever occur, worldwide annually (Centre for Disease Control, 2012). Paratyphoid fever occurs sporadically or in limited outbreaks but is most likely under-reported (Heyman,2008) .The ratio of disease caused by *S.* Typhi compared to that of *S.* Paratyphi A and B is estimated to be about 4:1 (Alberta Health

Public Health Notifiable Disease Management Guidelines Paratyphoid Fever, 2013). *S.* Paratyphi A is prevalent in India and other Asian countries, Eastern Europe and South America and Africa. *S.* Paratyphi is common in Eastern Europe, Britain, North America and *S.* Paratyphi C in Eastern Europe and Guyana. *S.* Paratyphi B is rare and C is very rare. In 2000, 5.4 million cases of paratyphoid fever caused by *S.* Paratyphi A have been reported (Heyman, 2008).

1.3.7. Incidence of Paratyphoid in different countries

Approximately 150 cases of paratyphoid fever are reported each year in the United States, most of the cases are observed in recent travelers. The risk of paratyphoid fever is increasing among travelers to southern and Southeast Asia. Paratyphoid fever occurs sporadically or in limited outbreaks but is most likely under-reported. The incidence of paratyphoid remains very low in Canada. In 1999, *S.* Paratyphi infection was removed from the nationally sporadic disease list as a separate entity and was included under *Salmonella* infections (Alberta Health Public Health Notifiable Disease Management Guidelines Paratyphoid Fever, 2013). Between 2006 and 2010 there was an average of 106 *S.* Paratyphiisolates reported from Canada. The majority of isolates were identified as *S.* Paratyphi A (Public health agency of Canada, 2012). Between 200 and 2012 in Canada, 93/104 cases reported travel outside Canada (e.g., traveled to South Asia), an additional 6/104 cases reported recent immigration and for 5/104 cases there was no information available. *S.* Paratyphi A remains the main cause of paratyphoid fever in England, Wales and Northern Ireland and an average of 192 paratyphoid cases reported each year from 1995 to 2005. In 2005, 61incidence of *S.* Paratyphi A infection were associated with travels to India, and 31 travels to Pakistan (Health protection agency, 2011). Later, from 1 May 2006 to 30 April 2007, 224 *S.* Paratyphi A infections have been reported and 100% of the patients had travel histories (Health protection agency, 2011). In the first eight months of 2013, numbers of *Salmonella* Paratyphi A infections among travelers returning toFrance from Cambodia increased greatly while several other countries also reported imported cases although to a lesser extent.

*S.*Paratyphi A is increasingly important as the causative agent (50% of *Salmonella* bloodstream isolates) of enteric fever in Asia (Health protection agency, 2013). It is the

second major cause of enteric fever in Asia, Middle East, Africa and South America after *S.*Typhi. *S.* Paratyphi A is more prevalent in Pakistan, Nepal, India and China.

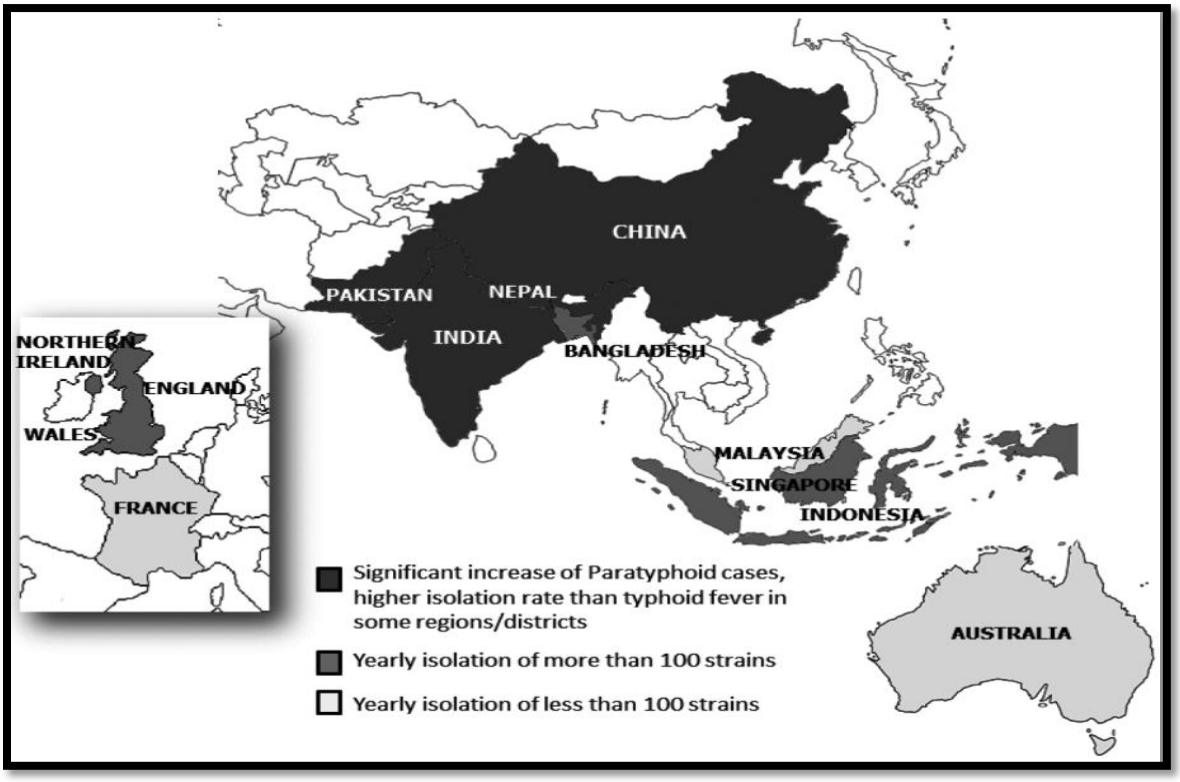

Figure2:Distribution of *S.* Paratyphi A in different countries in Asia and Europe. (Alberta Health Public Health Notifiable Disease Management Guidelines Paratyphoid Fever, 2013)

In China, the incidence of paratyphoid fever has also increased rapidly as reported in San Jiang county and Quanzhou county in 1995 (Yang, 2008). During the period of 2002-2007 in Shenzhen, of China, 70.3% of 91 patients in Shenzhen People's Hospital were diagnosed with *S.* Paratyphi A infection (Wu *et al.,* 2010). In India, no major outbreak of paratyphoid fever has been recorded, although it was implicated to cause 3% - 17% of enteric fever cases before October 1995.Besides in 1996, 36 cases of paratyphoid fever were reported in residence areas of new Delhi, India within 1 month period (September-October) (Mendiratta, 2004). Similarly, 23.5% enteric fever incidence in Calcutta from September 2004- August 2004 (Gupta *et al.*, 2009) and 23.3% in Chennai between 2007 - 2009 was due to *S.*Paratyphi A. In Pakistan there was a huge increase of *S.*Paratyphi A infection in Rawalpindi (Woods, 2006; Shirakawa, 2006) and Karachi since 199 while in

Nepal a great isolation rate of *S*.Paratyphi A observed during summer. Although a higher prevalence of *S*.Paratyphi A compared to *S*.Typhi (with the ratio of 39:30 from total 69 isolates) has been reported in autumn.In Patan Hospital, Kathmandu2677 *S*. Paratyphi A strains were isolated from blood of patients (accounted for 29.3% of enteric fever) between 1993 and 2003. Later, during January- August 2004, the isolation rate of *S*. Paratyphi A increased and accounted for 32.8% of enteric fever in the same hospital (Maskey *et al.,* 2008).A case finding the study of *S*. Paratyphpi B infection between 15 August and 30 November 1993; a pair matched case-control study; an environmental investigation at a processing plant that produced a raw goats' milk cheese incriminated in the outbreak; phage typing and genotyping of food and human *S*. Paratyphi B isolates (Desenclos *et al*., 1996). The outbreak began during the second week of August 1993 and continued until the second week of November. The isolation rate was highest among infants and children aged 1 to 5 years .In France, *S*. Paratyphi B from very important. Unlike *S*. Typhi, *S*. Paratyphi C is pathogenic for animals as well as for humans. This pathogen is only a minor cause of enteric fevers in South America, Asia, and the United States. However, in certain parts of Africa, occasional outbreaks of enteric fever caused by *S*. Paratyphi C have been reported (Daniels *et al.,* 1989). Like typhoid fever paratyphoid fever also biased to age and sex. Paratyphoid is more common in adults. A study in Kolkata reported that the case of paratyphoid fever was lower (0.8/1000/year) and the mean age of paratyphoid fever was older compared to typhoid fever (incidence 1.4/ 1000year/mean age 14.7 years) (Sur*et al*., 2007). In Bangladesh prevalence of paratyphoid fever is still not severe as typhoid. In Dhaka, Bangladesh 8 Paratyphoid fever incidence were reported among 48 cases of enteric fever in the year 2003 (Sheikh *et al*., 2010). Another study showed only 5 out of 89 patients with enteric fever were paratyphoid fever incidence (Naheed *et al*., 2010).

1.4. Reason of Resistance

Antimicrobial agents have saved the human race from a lot of suffering due to infectious disease burden. Millions of people would have infected with infectious diseases, without antimicrobial agents. Resistance by microorganisms to antimicrobial agents is an old phenomenon. They have developed mechanisms that enable them to respond to selective

pressure exerted by various environments and competitive challenges, through which they survive. A human being has been depriving the diseases causing organisms of their habitat by using antimicrobial agents.These microorganisms have developed resistance mechanisms to act against this offensive for their survival. The problem of antimicrobial resistance was never taken to be such a threat to the management of infectious diseases, prior to the 1990s. But gradually treatment failures have increasingly being seen in health care settings against first-line drugs and second-line drugs or more Globally antimicrobial resistance among disease-causing organisms is a serious threat to infectious disease management at present (Anı´bal *et al.,* 2010). Increasing prevalence of resistance has been reported over the years in different regions of the world especially in developing countries (Byarugaba, 2005). So, use of antibiotics is the single most important factor responsible for increased antimicrobial resistance (Aarestrup *et al.,* 2001; Byarugaba, 2004).

Some common reasons for increasing resistance levels include the following:

- Substandard or irregular use of antimicrobials for prophylaxis and treatment of infection,
- Disobedience to infection-control practices,
- Prolonged hospitalization increased number and duration of intensive-care-unit stays,
- Multiple comorbidities in hospitalized patients,
- Greater use of invasive devices and catheters,
- Lack of effectiveness in infection-control practices, transfer of colonized patients from hospital to hospital,
- Categorization of colonized patients in long-term-care facilities,
- Antibiotic use in agriculture and household chores, and
- Increasing national and international travel.

The extent of antibiotic resistance is dependent on the following:

- The mechanisms as a result of selective pressure either from antibiotic use or otherwise, the population of organisms that spontaneously acquire resistance
- The rate of entry from the community of those resistant organisms into health care settings, and
- The proportion that is transfer from person to person.

The antimicrobial resistant patterns are divided into two classes

- Phenotypic resistance
- Genetic resistance (Chetana *et al*., 2013)

1.4.1. Phenotypic resistance

The emergence of a phenotype resistant to antimicrobial agents depends on various factors of a host: degree of resistance expression, the capability of a microorganism to tolerate resistance mechanism, initial colonization site, and other factors (Chetana *et al.,* 2013). Genetic changes or the acquisition of resistance genes, or to mutations in elements relevant for the activity of the antibiotic, are the reason for the development of antibiotic resistance. In some situations resistance can be obtained without any genetic alteration; this is called phenotypic resistance. Non-inherited resistance is linked up with specific processes such as growth in biofilms, a stationary growth phase or persistence. These situations might occur at the time of infection but they are not usually considered in classical susceptibility tests at the clinical microbiology laboratories. It is a transient non-inherited form of resistance (Corona *et al.,* 2013).

1.4.2. Genetic Resistance

Genetically bacteria can achieve antimicrobial resistance in two ways

1. Intrinsic or Inherent Resistance
2. Acquired Resistance

1.4.2.1. Inherent (Natural) Resistance

Intrinsic resistance is considered to be a natural and inherited property with high predictability. Once the identity of the organisms is known, the aspects of its anti-microbial resistance are also recognized. It is the innate ability of bacterial species to resist activity of a particular agent, which allows tolerance to the particular drug. In this case, a resistant gene may present in particular bacterial species that is naturally transmitted. Inherent resistance can also be called "insensitivity" since it occurs in organisms that have never been susceptible to that particular drug. Such natural insensitivity can be due to:

a) Lack of affinity of the drug for the bacterial target

b) Inaccessibility of the drug into the bacterial cell

c) Extrusion of the drug by chromosomally encoded active exporters

d) Innate production of enzymes that inactivate the drug

For example, Streptomyces has some genes responsible for resistance to its own antibiotics; Gram-negative bacteria are vancomycin-resistant due to lack of uptake of resulting from the inability of vancomycin to penetrate the outer membrane.

1.4.2.2 .Acquired Resistance

Acquired resistance means gaining the ability to resist the activity of a particular antimicrobial agent of which it was susceptible. It can be achieved from the acquisition of foreign genes or from mutation of genes involved in normal physiological processes and cell structure. Genes can be transferred by three ways – Transduction, Conjugation, and transformation (Džidic *et al.*, 2008; Alekshun and Levy, 2007; Hawkey, 2008).

Basically, resistance genes are acquired in two ways

a. Vertical gene transfer

b. Horizontal gene transfer

a. Vertical gene transfer

Vertical gene transfer mainly involves spontaneous mutation and then the transfer of mutated resistant genes (Toddar, 2008-2012). Chromosomal mutations are quite rare (mutation frequency is about 10^{-8}- 10^{-9}microorganisms). Such mutation commonly determines resistance to structurally related compounds.

These mutations occur as errors of replication or an incorrect repair of damaged DNA. They are called spontaneous mutations or growth-dependent mutations (Giedraitienė *et al.,*2011).Once the resistance genes have developed, they are transferred through replication to all the bacterial progeny directly. This is called vertical gene transfer. Thus in this pattern of gene transfer, a mutation in bacterial chromosome impart resistance to a member of the bacterial population. For example, only a single point mutation in the rpoB gene is associated with a complete resistance to rifampicin (Giedraitienė *et al.,* 2011).In a case of *S.*Typhi and *S.* Paratyphi reduced susceptibility to fluoroquinolones involve chromosomal genes and associated point mutations in target gene encoding DNA gyrase (gyr A or gyr B) or topoisomerase (parC and parE) (Anıbal *et al.,* 2010).

b. Horizontal gene transfer

Another mechanism beyond spontaneous mutation is responsible for the acquisition of antibiotic resistance. Lateral or horizontal gene transfer (HGT) is a process whereby genetic material contained in small packets of DNA can be transferred between individual bacteria of the same species or even between different species (Bennett, 2008).There are at least three possible mechanisms of Horizontal gene transfer, equivalent to the three processes of genetic exchange in bacteria:

i. Transduction (via bacteriophages and integrons)

ii. Transformation (via incorporation of chromosomal DNA, plasmids into a chromosome) (Giedraitienė *et al.,* 2011).

iii. Conjugation (via plasmids and conjugative transposons),

i. Transduction

Transduction is the transfer of genes by bacteriophages. By bacteriophage, any bacterial gene is exchanged in transduction process. In restricted or specialized transduction only limited number of genes can be exchanged. Antibiotic resistance gene can be transferred by general transduction as the passive transfer. In most cases, gene transfer is between members of the same bacterial species.

ii. Transformation

Transformation is a process where parts of DNA are taken up by the bacteria from the external environment. This DNA is normally present in the external environment due to the death and lysis of another bacterium. This phenomenon is observed naturally in some species and can be induced artificially in others by using different techniques.

iii. Conjugation

Conjugation is often regarded as the major reason for the spread of antibiotic resistance in closely associated bacteria (Baharoglu*et al.,* 2010; Brisson-Noel, 1988; Grohmann and Muth, 2003).

Conjugation occurs when there is direct cell-cell contact between two bacteria (which need not be closely related) and transfer of small pieces of DNA called plasmids takes place. This is thought to be the main mechanism of horizontal gene transfer (Todar, 2008-2012). The transfer of resistance genes through plasmid is more effective than chromosomal mutation (Alekshun and Levy, 2007).Most plasmids are double-stranded circular DNA but their size may differ from 2–3 kb to plasmids, which encode up to 10% of the host cell chromosome (Alekshun and Levy, 2007) Plasmids(R plasmid) encode genes that produce resistance to main classes of antimicrobial agents (cephalosporin, fluoroquinolones, and aminoglycosides), and virulence determinants that a cell to survive in the environment of lethal antibiotic dose (Hawkey, 2008; Mayer*et al.,* 1995). The conjugation occurs via sex-pilli .Through sex pilli the non-chromosomal genetic material is transferred from donor (male) to recipient (female) cell. The conjugation is mediated by *tra* genes. This conjugative plasmid (R plasmid) contains about 40 genes for production of sex – pilli and the machinery to transfer the plasmid. It is a common

mechanism of gene transfers in Gram-negative as well as Gram – positive bacterial populations. In *S*.Typhi and *S*.Paratyphi a single plasmid is responsible for coding for multidrug resistance (ampicillin, Chloramphenicol, and Trimethoprim-sulfamethoxazole resistance). This 100,000 to 120,000 kD plasmid belongs to the incompatibility group H1 and is highly transmissible. This multidrug-resistant plasmid is transferred by 66.6% strains during a conjugative recombination (Raveendran *et al*., 2010).

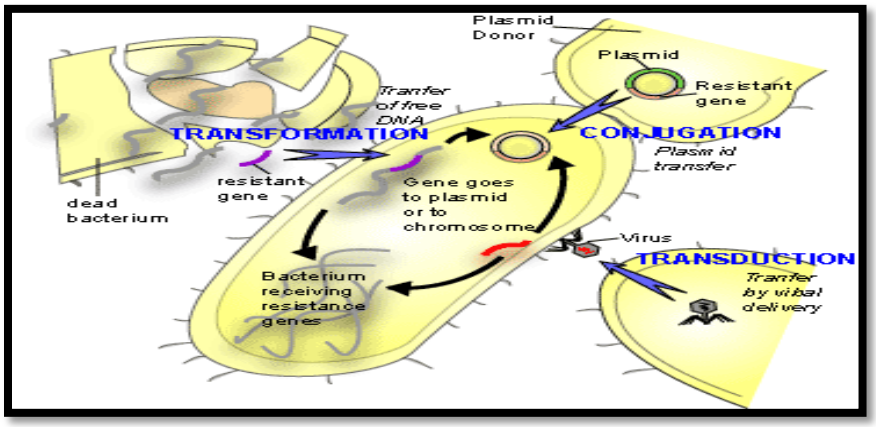

Fig 3: Main three mechanism of bacterial gene transfer (transduction, transformation and conjugation) (Toddar, 2008-2012).

Transposition

Transposition is another different gene transfer process where, transposon (also called mobile genetic element) a small mobile genetic element containing specific DNA sequence, able to transpose itself. Transposons carrying antibiotic resistance gene can move from one bacterial chromosome to another bacterial chromosome without being a part of the plasmid, chromosome or bacteriophage (Cohen, 1976).Transposons can be integrated into plasmids or the host's chromosome, encompass small elements called insertion sequences (IS elements), transposon. They have terminal repeat sequences that play a role in recombination and recognize a protein (for example, transposase or recombinase) that is necessary to insert or remove a transposon from specific genome regions (Alekshun and Levy, 2007; Bennett, 2008; Hawkey, 2008). Transposons are transferred by conjugation, transformation, or transduction (e.g., mecA gene is found in MRSA) and spread quicker than genes in chromosomes. Transposition is proposed to be

responsible for the rapid evolution of multiple drug-resistant bacterial strains. Studies on transposable genetic elements in bacteria have not only given insight into the spread of antibiotic resistance but also into the process of DNA movement. There is various type of transposon for ex: composite transposons, complex and conjugative transposons. (Lupski,1987)

Conjugative transposons have characteristic features of plasmids and can help to transfer endogenic plasmids from one microorganism to another (Raghunath, 2008; Tolmasky, 2000; Hawkey, 2008). This transposable element can move from the chromosome of one bacterium to another without being a part of plasmid or bacteriophages.

A conjugative transposon is widespread in nature and this transposition occurs in a conservative manner. Since they provide a mechanism for dispersing antibiotic resistance determinants among pathogenic bacteria, conjugative transposons are of great medical importance. Many of the clinically isolated multiple-antibiotic-resistant gram-positive bacteria (streptococci and staphylococci) in which relatives of Tn916 have been identified carry no detectable plasmids (Buu and Horaud, 1985). This strongly suggests that spread of antibiotic resistance results from the movement of conjugative transposons. An example of conjugative transposon includes Tn916 contain tetracycline and minocycline resistant genes and Tn1545 contain tetracycline, minocycline, erythromycin, and Kanamycin resistant genes,are self-transferrable (Giedraitienė *et al*, 2011).

Another kind of transposon is composite (compound) Transposon, which is flanked by insertion sequences or by inverted Repeats. For example, Tn5 contains (*Kan, Bleo, Str*) kanamycin, bleomycin streptomycin resistant genes (kanamycin bleomycin and streptomycin resistant gene) .A complex transposon is large (>5 kb), flanked by short terminal inverted repeats, and specifies transposes and the recombinase. For example, Tn1 and Tn3 carry β-lactamase resistant gene Tn7 contains streptomycin resistant gene, Tn1546 contains glycopeptides (Giedraitienė *et al.,* 2011).

Site-specific Integration

Antibiotic resistance genes can integrate at a unique site within the conserved DNA segment of an integron (biossonette and PH, 1992). Integrons are generally non-mobile and are often located on mobile genetic elements like transposons and plasmids that could serve as vehicles for the inter- and intra-species transmission of genes (Domingueset *et al*., 2012; Fluit, 2004). Bacterial integrons are gene capture systems. Integron encodes three main components in the 5´ conserved segment: an enzyme integrase (gene *int*) that serves as a specific recombination system to insert or to remove a new gene cassette, specific recombination site (*attI* site), and a promoter that starts gene transcription (Giedraitienė *et al.*, 2011). The gene cassettes in the integrons are mainly antibiotic resistance genes which are expressed by a common promoter that ensures the correct expression of these cassettes. A recent study showed that most of the integrons that have been sequenced and characterized contain at least one acquired resistance gene (Fluit, 2004). The number of gene cassettes can vary in an integron but so far the highest number of gene cassettes observed is eight. Integrons are differentiated on the basis of sequence similarity. There are at least five classes of integrons with class I integrons being the most studied and characterized. They have found to present on many gram-negative genera associated with the gut microbiota such as *Acinetobacter, Escherichia coli, Salmonella* and much more (Fluit and Schmitz, 2004). The class II integrons are often associated with Tn7 family of transposons and also found in *Salmonella* and *Shigella*. Class III integrons are very similar to the other two classes but are related to the Tn402 transposon. The class IV and V have been identified in association with the development of trimethoprim resistance in Vibrio species (Ravi*et al.,* 2014).Various evidence exists that show integrons moving through bacterial species and transferring multidrug resistance properties. The diversity of class I integrons and gene cassettes in *Enterobacteriaceae* are found in hospital wastewater. Integrons generated new linkages for antibiotic resistance mostly at areas of high exposure to antibiotics. Thus, antimicrobial selective pressure plays a major role in acquiring resistance genes. The ancestral integrons may not have contained many resistance genes but with the increase in antibiotic usage, most integrons sequenced to date harbor the *qac* and *sul1* genes that confer resistance to biocides and sulfonamides respectively (Ravi*et al*., 2014).

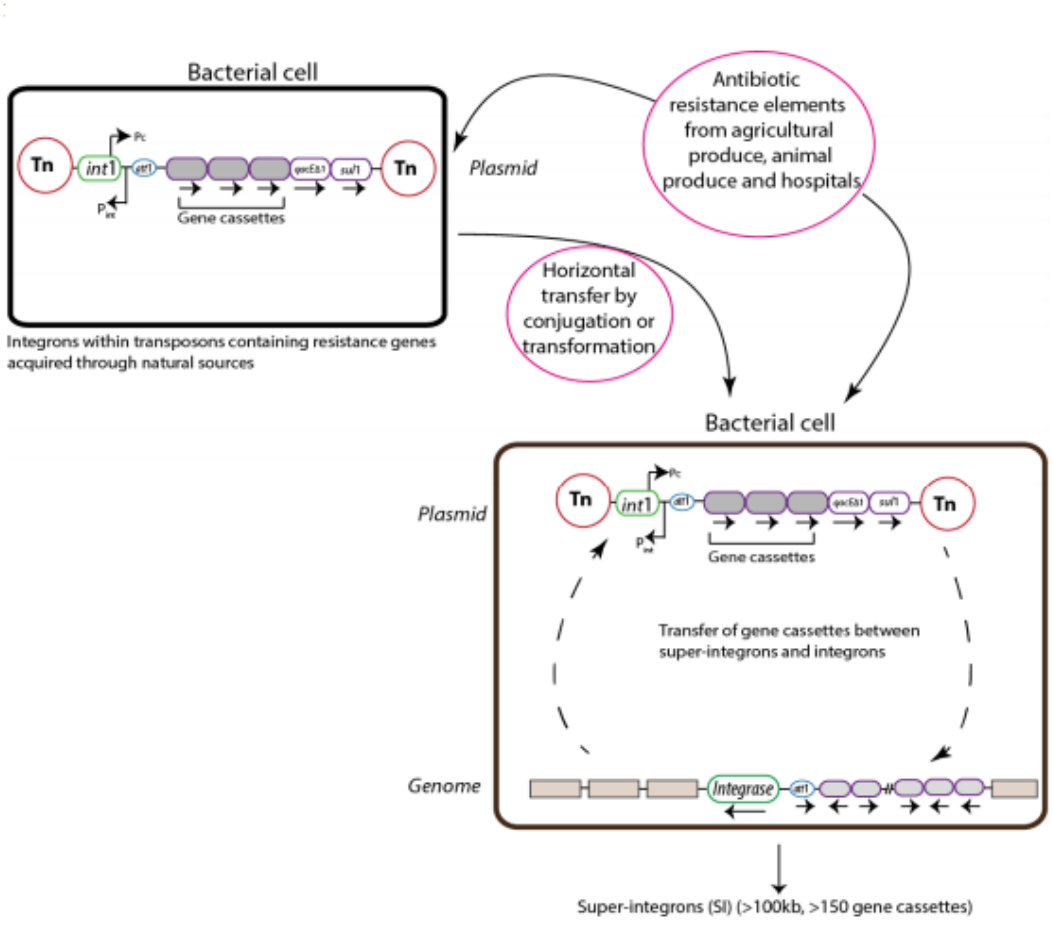

Fig 4: Transfer of antibiotic resistance genes through integron (Ravi*et al.*, 2014)

The figure gives a schematic representation of the transmission of integrons Transposons. TN =transposon, *int*1= responsible for the integration, *att*1=attachment of the gene cassettes. The *qacE*Δ1 and *sul*1 =genes encode resistance to quaternary ammonium compounds and sulfonamides, respectively. The gray areas represent the gene cassettes with varied functions. Pint = integrase (*int*1), Pc = promoter for captured gene cassette

1.4.3. Antimicrobial Resistance mechanism by biochemical means

A Bacterium uses several biochemical types of resistance mechanisms, after gaining resistance genes to protect it from various antimicrobial agents. Different antimicrobial agents act in a several ways. In order to understand these mechanisms as well as the chemical nature of the antimicrobial agents, it is essential to understand the ways how resistance against them develops

These include generally:

- Interference with the cell wall synthesis –(penicillin, ampicillin, cephalosporin)
- Inhibition of protein synthesis – (Macrolides, chloramphenicol, oxazolidinones)
- Interference with nucleic acid synthesis –Quinolones, Rifampicin,
- Inhibition of metabolic pathway – Sulfonamide (sulfamethoxazole) and trimethoprim
- Disorganizing cell membrane – Cyclic lipopeptide daptomysin (Dzidic *et al*., 2008).

Through different means microorganisms have achieved resistance to a drug, primarily based on the chemical structure of the antimicrobial agent and the mechanisms through which the agents acted. The resistance mechanisms, therefore, depend on which specific pathways are inhibited by the drugs and the alternative ways available for those pathways that the organisms can modify to get a way around in order to survive (Anı´bal *et al.*, 2010). Understanding the resistance mechanism has become the significant biochemical issue.

The main biochemical mechanisms that bacteria use for defense are as follows:

1. Antibiotic inactivation
2. Target modification
3. Efflux pump and outer membrane permeability
4. Bypass of antibiotic inhibition

1.4.3.1. Antibiotic inactivation

The defense mechanisms within the category of antibiotic inactivation include the production of enzymes that degrade or modify the drug itself. Biochemical strategies are - hydrolysis, redox mechanisms and by transfer of group (Dzidic *et al*., 2008).

a. Antibiotic inactivation by hydrolysis

Several enzymes are involved in destroying antibiotic activity by targeting and cleaving the bonds. These enzymes can often be excreted by the bacteria, inactivating antibiotics before they reach their target within the bacteria. The classical hydrolytic amidases are the b-lactamases that cleave the b-lactam ring of the penicillin and cephalosporin antibiotics (Dzidic *et al*., 2008).

Many Gram-negative and Gram-positive bacteria produce such enzymes, and more than 300 different b-lactamases have been identified. The most clinically important hydrolytic enzymes are produced by gram-negative bacteria (Wickens and Wade, 2005) and genes of those enzymes are coded on chromosomes and plasmids. Genes that encode *β*-lactamases are transferred by transposons but also they may be found in the composition of integrons (Jacoby and Munoz-Price, 2005). *β*-Lactamases hydrolyze nearly all *β*-lactams that have ester and amide bond, e.g., penicillin, cephalosporin, monobactams, and carbapenemsSerine *β*-lactamases – cephalosporinases, e.g. AmpC enzyme – are found in *Enterobacter* spp (Giedraitienė *et al*., 2011).

Extended-spectrum *β*-lactamases (ESBLs) produce resistance to all penicillins, third generation cephalosporins (e.g. ceftazidime, cefotaxime, ceftriaxone) and aztreonam, but not cephamycins (cefoxitin and cefotetan) and carbapenems. ESBL are most commonly found in *Enterobacteriaceae.* Examples of other hydrolytic enzymes include esterase that has been linked to macrolide antibiotic resistance and ring-opening epoxidase causing resistance to fosfomycin (Džidic*et al*., 2008).

B.Antibiotic inactivation by redox reaction

Oxidation and reduction reactions are used by pathogenic bacteria as the resistance mechanism against antibiotics. *Streptomycesvirginiae*, producer of the type A streptogramin antibiotic virginiamycin M1, protects itself from its own antibiotic by reducing a critical ketone group to an alcohol at position 16 (Džidic*et al*., 2008).

C.Antibiotic inactivation by transfer of group

The most diverse family of resistant enzymes is the group of transferases. These enzymes inactivate antibiotics (chloramphenicol, macrolides or rifampicin, aminoglycosides) by chemical substitution such as by addition of adenylyl, phosphoryl or acetyl groups to the periphery of the antibiotic molecule. The modified antibiotics are prevented from binding in their binding to a target (Džidic *et al.,* 2008).

1.4.3.2. Target Modification

The second major resistance mechanism is the modification of antibiotic target site for which the antibiotic cannot bind to the target properly. Because of essential cellular functions of target sites, organisms cannot avoid antimicrobial action by dispensing with them entirely.

a. Alternation of peptidoglycan structure

An excellent selective target for the antibiotics is the peptidoglycan component of bacterial cell wall. Consequently, enzymes involved in synthesis and assembly of the peptidoglycan component of the bacterial cell wall provide excellent targets for selective inhibition. Decreased affinity to b-lactam antibiotics is the result of mutations in the penicillin-binding domain of penicillin-binding proteins (PBPs) (Džidic *et al.,* 2008).

b. Interfering synthesis of protein

A wide range of antibiotics interferes with protein synthesis on different levels of protein metabolism. The resistance to antibiotics that interfere with protein synthesis (tetracyclines, macrolides, aminoglycosides, chloramphenicol, fusidic acid, mupirocin, oxazolidinones, streptogramins,) or transcription via RNA polymerase (the rifamycins) is achieved by modification of the specific target. The macrolide, lincosamide and

streptogramin B group of antibiotics block protein synthesis in bacteria by binding to the 50S ribosomal subunit .Mutations in 23S rRNA close to the sites of methylation have also been associated with resistance to the macrolide group of antibiotics in a range of organisms (Džidic *et al.,* 2008).

c. Interference of DNA synthesis

The mechanism of resistance involves modification of two enzymes: DNA *gyrase* (also known as topoisomerase II) (genes *gyr*A and *gyr*B) and topoisomerase IV (parC and parE) (Kim *et al*., 2002). Mutations in genes gyrA and parC are followed by replication failure, and then quinolones/ fluoroquinolones cannot bind. The most common mutation in E. coli gyrA causes a reduced drug affinity for modified-DNA complex, and MIC is higher (Giedraitienė *et al*., 2011). Quinolones (ciprofloxacin) bind to DNA gyrase A subunit (Garau *et al.,* 2004). Chromosomal mutations mainly responsible for the quinolones resistance in addition plasmid-mediated and point mutation-related (in genes gyrA and parC) resistance to quinolones has reported as well (Giedraitienė *et al*., 2011).

1.4.3.3. Efflux pump and outer membrane (OM) permeability

The efflux pumps are the membrane proteins that export antibiotics out of the cell and keep its intracellular concentration at the low level. Reduced outer membrane permeability results in reduced uptake. The reduced uptake and active efflux induce low-level resistance in many clinically important bacteria (Nikaido, 1994).

a. Efflux pumps

Efflux pumps affect all classes of antibiotics, mainly the macrolides, tetracyclines, and fluoroquinolones because these antibiotics inhibit different aspects of protein and DNA biosynthesis and therefore must be intracellular to exert their effect. Efflux pumps vary in both their specificity and mechanism (Nikaido and Zgurskaya, 1999). Some have drug specificity. Many efflux systems are multidrug transporters that are capable of expelling a wide spectrum of structurally unrelated drugs, thus contributing significantly to bacterial multidrug resistance (MDR) (H.W. and Veen,1997). Inducible multidrug efflux pumps are responsible for the intrinsic antibiotic resistance of many organisms, and mutation of

the regulatory elements that control the production of efflux pumps can lead to an increase in antibiotic resistance.

b. Outer membrane (OM) permeability changes

Gram-negative bacteria have an outer membrane consisting of an inner layer containing phospholipids and an outer layer containing the lipid a moiety of lipopolysaccharides (LPS). This composition of the outer membrane (OM) slows down penetration of drugs and transport across the outer membrane is achieved by porin proteins that form water-filled channels. Drug molecules can penetrate the outer membrane employing one of the following modes: by diffusion through porins, by diffusion through the bilayer or by self-promoted uptake. The mode of entry employed by a drug molecule largely depends on its chemical composition. For example, hydrophilic compounds either enter the periplasm through porins (e.g. b-lactams) or self-promoted uptake (aminoglycosides). Antibiotics such as b- -lactams, chloramphenicol, and fluoroquinolones enter the Gram-negative outer membrane via porins. As such, changes in porin copy number, size or selectivity alters the rate of diffusion of these antibiotics. Strains of *E. coli* and *S.* Typhimurium defective in LPS have been found to be at least 4-fold more susceptible to erythromycin, roxithromycin, clarithromycin and azithromycin than the wild-type strains (Džidic *et al*., 2008).

1.4.3.4. Bypass of antibiotic inactivation

By producing alternative target (usually enzyme) bacteria often develop their resistance to antibiotic. For example, MRSA produces an alternative PBP). This mode of resistance is observed in many trimethoprim-sulfonamide-resistant bacteria. Resistance to trimethoprim and sulphonamides is caused by reduced sensitivity and affinity of altered enzymes dihydropteroate synthetase (DHPS and dihydropteroate reductase (DHFR) to trimethoprim and sulphanamide. The alternative target allows bacteria to survive by adopting the role of the native protein (Džidic *et al*, 2008).

1.5. Objective of the Study

The present investigation has been designed with the following objectives:

- To determine the antimicrobial resistance pattern of commonly used antibiotics among *Salmonella enterica* serovar Typhi and *Salmonella enterica*serovarParatyphi isolated from blood and stool culture in Bangladesh.
- To find out the main susceptibility profiles of *S.* Typhi and *S.*Paratyphi serotypes of clinical isolates.
- To determine the Minimum Inhibitory Concentration (MIC) of ciprofloxacin, nalidixic acid, and azithromycin

Chapter-2
Method and Material

2.1. Clinical Specimens

In this study blood and stool samples were collected as clinical specimens randomly from patients who attended the Clinical Research and Service Centre of the icddr, b, Dhaka from 26th June 2013 to 17th May 2014. Isolation and identification were carried out in at Clinical Microbiology Laboratory icddr, b Dhaka. In this dissertation the study was carried out at Respiratory & Antibiotic Resistance Labof icddr, b.

2.2. Isolation of *Salmonella* spp

In Dupont 1.5 ml of child blood was collected and 5ml of adult blood was collected in blood culture broth. For stool culture sample of stool was collected in a clean container. Samples were inoculated onto Mackonkey, Chocolate and blood and S-S agar and kept overnight at 37°C. After incubation plate was examined for non-lactose fermenting pale colonies.

2.3.1. Identification of *Salmonella*spp

Suspected lactose fermenting pale colonies were subjected to the biochemical test in order to identify *Salmonella enterica* serovar Typhi (*S.* Typhi) and *Salmonella enterica* serovar Partyphi (*S.* Paratyphi).

2.3.2. Biochemical Test

Following biochemical tests were performed for identification

Kligler's Iron Agar (KIA) test

The KIA test tube contains a large amount of lactose and the small amount of glucose, pH indicator (red in a base and yellow in acid). It also contains iron which is precipitated as black sulfide if H_2S is produced. The test is used to identify unknown organisms on the basis of the changes in the properties of these ingredients.

•**Lactose (+ or −):** The surface of the slant of KIA tube has an aerobic condition. By fermentation of high concentration of carbohydrate (lactose) presents in this tube produces acid. As a result, for lactose fermentative organisms the slant becomes yellow

in color and for not- lactose fermentative organisms color of the slant changes to red in color.

• **Glucose (+ or −):** The butt of the tube has an anaerobic condition where the fermentation of trace amount of glucose produces acid that changes the pH. For glucose positive organisms the color of butt changes to yellow in color.

• **H_2S (+/-):** Hydrogen sulfide production is detected by the blackening of the tube.

• **Gas (+/-):** Production of gas from glucose or lactose is detected by the formation of the bubble in the butt.

Method: In this study, KIA medium was used to perform this test. Using a sterile straight wire an isolated colony was touched and then stabbed straightly into the soft agar of KIA and also onto the surface of the slant. The inoculated tube was then incubated at 37°C for 18-24 hrs.

Motility Indole Urea test

This test includes a semisolid motility indole urea medium which is used for the differentiation of *Enterobacteriaceae* that include three separate tests in one tube. The following characteristics determine in this test.

• **Motility**: Motility of organisms is detected by the growth along the time of inoculation on.

• **Indole**: Formation of indole is detected by the formation of redline between indole reagent and medium.

• **Urea:** Production of urea is detected by the formation of the pink color of the medium.

Method: One suspected colony was selected and touched using a sterile straight wire and then stabbed into agar very carefully down the tube without moving. The tube was incubated at 37°C for 18-24 hrs. At the following day, tubes were taken out from the incubator and 2-3 drops of Kovac's reagent was put into the tube to detect indole production.

Citrate Test

Citrate test is used for the identification of organisms on the basis of their ability to utilize sodium citrate to form oxalate .In this media citrate is the only carbon source. Citrate agar slant contains sodium citrate (only carbon source) and ammonium ion (the sole nitrogen source). In this media bromothymol blue is a pH indicator which turns into green at pH < 7.0 and blue at pH > 7.6 organisms that utilize citrate for energy.

Method: Using a sterile loop an isolated colony was touched and inoculated on the surface of the Simmons Citrate agar medium and incubated at 37°C for 18-24 hrs.

2.4. Serological test to confirm *S.* Typhi and *S*. Paratyphi

The serological study was performed by slide agglutination test. The dry and clean glass slide was marked into several parts with a way pencil. A drop of normal saline was placed on each block. An isolated colony of *Salmonella* species was touched with a sterile loop and suspended in normal saline on the slide. Commercially available Salmonella antisera (Murex diagnostic reagents, UK) were added to the suspension. After back and forth movement of the slide, agglutination was observed two minutes later against diffuse high, Stereotyping was done using specific antiserum.

2.5. Identification of *S.* Typhi and *S*. Paratyphi

By biochemical and serological tests *S.* Typhi and *S.* Paratyphi isolates were characterized. The isolated strains were preserved in 3ml of T1N1 agar to use them for further analysis.

2.6. Determination of antimicrobial susceptibility test by modified Kirby-Bauer (Bauer *et al*., 1966) method

Susceptibility of isolates to different antimicrobial agents was measured in vitro by employing modified Kirby-Bauer (Bauer*et al.,* 1966) method. This test is frequently used to determine the drug sensitivity of microorganisms isolated from an infectious process and to interpret their disease potential. This method allows the rapid determination of efficacy of a drug by measuring the diameter of the zone of inhibition that result from diffusion of the agent into the medium surrounding the disc.

Following media and commercially available antibiotic disc were used for this test.

Media:
Mueller- Hinton agar medium was used

Antibiotic disc:

Table 3: The antimicrobial disc used in this study with their potency gave bellow:

1. Ampicillin (Amp)	(10 µg)
2. Chloramphenicol (C)	(30 µg)
3. Trimethoprim- sulfamethoxazole (SXT)	(1.25 µg + 23.7 µg)
4. Azithromycin (Azm)	(15 µg)
5. Nalidixic acid (NA)	(30 µg)
6. Ciprofloxacin (CIP)	(5 µg)
7. Cefixime (CFM)	(5 µg)
8. Ceftriaxone (CRO)	(30 µg)

2.6.1. Preparation of inoculums for disc diffusion tests

Bacterial strain was sub-cultured in media (MacConkeyand Salmonella – Shigellamedia) to obtain the pure colony. A single colony was then inoculated into 2ml Muller-Hinton broth with a sterile loop. The broth was then incubated at 37°C for 3-4 hrs to obtain the young culture. The turbidity of actively growing broth culture was adjusted to a McFarland 0.5 (3×10^8 CFU/ml). McFarland 0.5 was used as a standard for turbidity testing after growth in broth. This standard 0.5 McFarland was prepared by adding 0.5 ml of 0.048 M $BaCl_2$ (1.75%$BaCl_2$, $2H_2O$) to 99.5 ml of 0.36 NH_2SO4 (1%) with Mueller – Hinton broth.

2.6.2. Inoculation of agar plates for susceptibility test.:

To streak on the surface of agar medium , a sterile , nontoxic cotton swab was dipped into the tube containing young culture and the excess broth was purged by pressing and rotating the swab firmly against the inside of the tube above the fluid level. The swab was then streaked evenly in three directions over the surface of the agar plate to obtain uniform inoculums. A final sweep was made around the agar rim with the cotton swab. This plate was then allowed to dry for 3-5 minutes.

Before the discs were applied. Antibiotic-impregnated discs were then applied to the surface of the inoculated plate with sterile forceps .In each plate four discs were placed at 30 mm apart from each other and 15 mm from the edge of the plate. All discs were gently pressed down onto the agar with forceps to ensure complete contact with the agar surface. Within 15 minutes after the discs were applied, the plates were inverted and placed in an incubator at 37°C.

2.6.3. Examination of agar plates

After 16-18 hrs of incubation, the plates were examined and a ruler measured the diameters of the zones of complete inhibition to the nearest whole millimeter. The zone diameters for individual antimicrobial agents were then translated into susceptible, intermediate or resistant by comparing standard CLSI (Clinical and Laboratory Standards Institute) guideline -2013 and BSAC (British Society for Antimicrobial Chemotherapy)-2013 guideline.

CLSI -2013 guideline and BSAC-2013 guideline, the interpretative standard of zone diameter for *Enterobacteriaceae*, which was used in this study, is given in the Table: 4 and Table: 5

Table 4: CLSI- guideline 2013

Antimicrobial agents	CLSI Guideline -2013			
	Resistant (R)	Intermediate (I)	Sensitive (S)	Disc Size
Ampicillin (Amp)	≤13	14-16	≥17	10µg
Chloramphenicol (C)	≤12	13-17	≥18	30µg
Azithromycin (Azm)	NA	NA	NA	NA
Nalidixic Acid (NA)	≤13	14-18	≥19	30µg
Cefixime (CFM)	≤15	16-18	≥19	5µg
Ceftriaxone (CRO)	≤19	20-22	≥23	30µg
Trimethoprim - sulfamethoxazole (SXT)	≤10	11-15	≥16	1.25/23.7µg
Ciprofloxacin (CIP)	≤20	21-30	≥31	5µg

[NA= Not Available]

Table 5: BSAC guideline -2013

Antimicrobial agents	BSAC -2013			
	Resistant (R)	Intermediate (I)	Sensitive (S)	Disc Size
Ampicillin (Amp)	≤14	-	≥15	10µg
Chloramphenicol (C)	≤20	-	≥21	30µg
Azithromycin (Azm)	≤18	-	≥19	15µg
Nalidixic Acid (NA)	≤17	-	≥18	30µg
Cefixime (CFM)	≤19	-	≥20	5µg
Ceftriaxone (CRO)	≤23	24-27	≥28	30µg
Trimethoprim- sulfamethoxazole (SXT)	≤15	-	≥16	1.25/23.7 µg
Ciprofloxacin (CIP)	NA	NA	NA	1µg

[NA= Not Available]

There are differences in the standard of zone diameter breakpoint and disc size of some antibiotics between CLSI guideline and BSAC-2013. On the basis of such differences, a comparative analysis was done for both *S.* Typhi and *S.* Paratyphi.

Table 6: A variation of zone diameter breakpoints between CLSI-2013 and BSAC-2013

Antimicrobial agents	CLSI Guideline -2013			Disc size	BSAC- 2013			Disc size
	R	I	S		R	I	S	
Ampicillin (Amp)	≤13	14-16	≥17	10µg	≤14	-	≥15	10µg
Chloramphenicol (C)	≤12	13-17	≥18	30µg	≤20	-	≥21	30µg
Azithromycin (Azm)	NA	NA	NA	NA	≤18	-	≥19	15µg
Nalidixic Acid (NA)	≤13	14-18	≥19	30µg	≤17	-	≥18	30µg
Cefixime (CFM)	≤15	16-18	≥19	5µg	≤19	-	≥20	5µg
Ceftriaxone (CRO)	≤19	20-22	≥23	30µg	≤23	24-27	≥28	30µg
Trimethoprim - sulfamethoxazole (SXT)	≤10	-	≥16	1.25/23.7 µg	≤15	-	≥16	1.25/23.7 µg
Ciprofloxacin (CIP)	≤20	21-30	≥31	5µg	NA	NA	NA	1µg

2.6.4. Determination of minimal inhibitory concentration (MIC)

Disc diffusion method provided a statistical data on the susceptibility pattern of bacterial isolates to antimicrobial agents. Then minimal inhibitory concentration (MIC) of ciprofloxacin, azithromycin, and nalidixic acid was determined. (MIC, lowest concentration of antibiotic which inhibits the growth of bacteria by 99.5%) (Barry *et al.*, 1985).For MIC of nalidixic acid, agar dilution technique was used, whether E-test (Epsilometer test), a more advanced method was used to determine MIC of ciprofloxacin and azithromycin

2.7. Determination the MIC of azithromycin and ciprofloxacin

The E-test strip is a plastic strip with a predefined antimicrobial agent concentration gradient immobilized on one side and continuous MIC scale covering 15, 2 fold dilutions on the opposite side; it allows MIC determination on agar media.

Table7: Standard concentration or dilution of Ciprofloxacin and Azithromycin are used in MIC test

Antimicrobial Agents	Standard Concentration Used in MIC test (µg/ml)
Azithromycin	0.016- 256
Ciprofloxacin	0.002-32

2.7.1. Preparation of inoculums and inoculation of the media

Bacterial culture strains were sub-cultured on MacConkey, MHA (Mueller Hinton Agar) and S-S (Salmonella - Shigella) media to obtain the isolated pure colony. Each isolated colony was incubated into 2ml Mueller-Hinton broth with a sterile loop and the broth was incubated at 37°C for 3-4 hrs. The turbidity of broth culture was adjusted to a McFarland 0.5. McFarland 0.5 was used as a standard for turbidity testing after growth in broth. The broth culture was inoculated on Muller-Hinton agar plate (4mmthick agar) with a sterile

cotton swab to obtain confluent growth. The E-test strip of standard strength was placed on the inoculated plate with the sterile needle. All strips were gently pressed down onto the agar with a needle. All strips were gently pressed down onto the agar with a needle and were placed firmly. In each plate 2 strips – ciprofloxacin and azithromycin strips were placed. Then the plates were inverted and inoculated at 37°Cfor 18-24 hrs.

Thirteen *S.* Typhi isolates and 14 *S.* Paratyphi isolates were tested for MIC determination of ciprofloxacin. To determine the MIC for azithromycin 10 *S.* Typhi and 16 *S.* Paratyphi isolates, those were resistant to azithromycin in disc diffusion, were tested.

2.7.2. Reading Result

After overnight incubation, the interaction of the antimicrobial agent gradient and the test bacteria gives rise to elliptical inhibitory zones. The intersection of the growth ellipse margin with the E-test strip gradient indicated the MIC of the drug for the organism.

2.8. Determination of Minimal Inhibitory Concentration of nalidixic acid using agar dilution method.

Dilution methods are used to determine the minimum inhibitory concentrations (MICs) of antimicrobial agents and are the reference methods for antimicrobial susceptibility testing against which other methods such as disc diffusion are calibrated. In dilution tests, microorganisms are tested for their ability to produce visible growth on series of agar plates (agar dilution) or in microplate wells of broth (broth micro dilution) containing dilutions of the antimicrobial agent. The lowest concentration of an antimicrobial agent that will inhibit the visible growth of microorganisms is known as the MIC. In this study agar, dilution method was used to determine the MIC of nalidixic acid and the result was determined using the method that is described in European Society of Clinical Microbiology and Infectious Diseases. In this method 13 isolates of *S.*Typhi and 7 isolates of *S.*Paratyphi were tested

Media

Mueller Hinton

Antimicrobial Agent

Nalidixic acid powder

Solution

Sodium Hydroxide (NaOH)

0.85% normal saline

2.8.1. Preparation of dilution of antimicrobial agents

2.8.1.1. Measurement of solute and Preparation of stock solution

In this study for determination MIC of nalidixic acid, the powder of this antimicrobial agent that was used provided by sigma LTD with a potency of 98%. The amount of required solute was measured on the basis of required stock solution needed to prepare. The required amount of solution and concentration of solute was determined from the data provided by Eucast definitive document (European Society of Clinical Microbiology and Infectious Diseases-2000).

2.8.1.2. Preparation of media

Mueller Hinton Agar (MHA) media was prepared for 18plates (8 plates for *S.*Typhi and 8 plates for *S.*Paratyphi, 2 control plates for *S.*Typhi and *S.*Paratyphi respectively). Each of the plates contained 19ml of media. For each concentration of antimicrobial agent, one plate was used. Under biosafety cabinet 1ml of every concentration of antimicrobial agents was added to 19 ml of autoclaved MHA media after equilibrating the temperature to 50°C. Thus in 8 out of 18 plates, final concentration of antimicrobial agent obtained 256mg/L, 128mg/L 64mg/ L, 32 mg/L, 16 mg/L 8mg/L, 4mg/L, 2mg/L respectively. Other 8 plates were prepared similarly. Besides, two control plates were prepared with 20ml of MHA media only.

2.8.1.3. Preparation of inoculums

From a pure culture of bacteria colonies, 4-5 colonies were picked up and incubated into 2ml or 2000µl Muller-Hinton broth with a sterile loop and the broth was incubated at 37°C for 3-4 hours. The turbidity of broth culture was adjusted to a McFarland 0.5 (McFarland 0.5 contain 10^8CFU/ml). The suspension of organisms was adjusted using 0.85% saline water to get 10^7 CFU/ml suspensions. To prepare this suspension from 2000µl of suspension 400 µl was picked up and diluted with 3600 µl of 0.85% saline water. Thus suspension was adjusted 10^7 CFU/ml.

2.8.1.4. Inoculation and Incubation

Each plate was divided into 7 to 13 sections using the marker (13 strains of *S.*Typhi and 7 strains of *S.* Paratyphi). Each of the section was inoculated with 4 µl suspensions of each strain (10^4 CFU/ml). Under biosafety cabinet the plates of each concentration were inoculated using micropipette. The plates were then incubated overnight at 37°C.

2.8.1.5. Reading Result

After overnight incubation every section of each plate was observed to detect particular susceptible and resistant strain.

Chapter-3
Results

3.1. Isolation and Identification of *S.* Typhi and *S.* Paratyphi from blood

3.1.1. Identification of *S.* Typhi and *S.* Paratyphi

Biochemical Test- Extensive biochemical tests were performed in order to measure the variability of biochemical behavior among the isolates. The detailed biochemical study revealed that about 100 isolates had the biochemical characteristics typical of *S.* Typhi, 21 had a biochemical pattern like *S.* Paratyphi A whereas one of the isolate had a biochemical property like *S.* Paratyphi B.

The isolates showed following characteristic in Salmonella-Shigella (S-S) agar media.

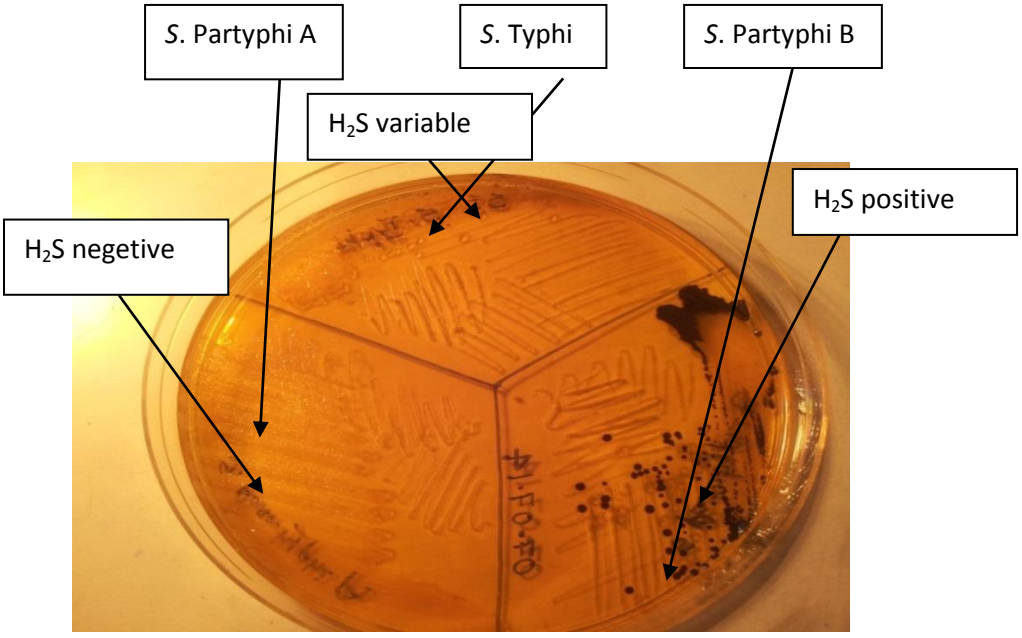

Fig 5: Pure colony of isolates of *S.* Typhi, *S.* Paratyphi A and *S.* Paratyphi B on S-S agar

3.1.2. Biochemical Test

KIA test –result

In Kligler's Iron Agar (KIA**)**test, all isolates produced acid in the butt by fermentation of glucose and alkaline condition in the slant by oxidation which was detected by the yellow color of the butt and pink color of the slant. One hundred isolates of all did not produce

any gas and their hydrogen sulphide production was variable. Gas production was positive for 21 isolates similar like *S.* Paratyphi A but their hydrogen sulphide production was negative, whether only one isolate produced excessive hydrogen sulphide gas that was detected by blackening of half of the KIA media, this isolates formed gas. All of the isolates were detected as facultative anaerobe as they first produced acid in the butt in absence of oxygen and later on by oxidation produced alkali in slant. (Fig-6)

MIU test-result

All isolates were motile and indole negative. Isolates did not produce urease enzyme (Fig-6).

Citrate test – result

Citrate was utilized by only one isolate which was detected by growth and development of a deep blue color reaction within the medium (Fig-6)

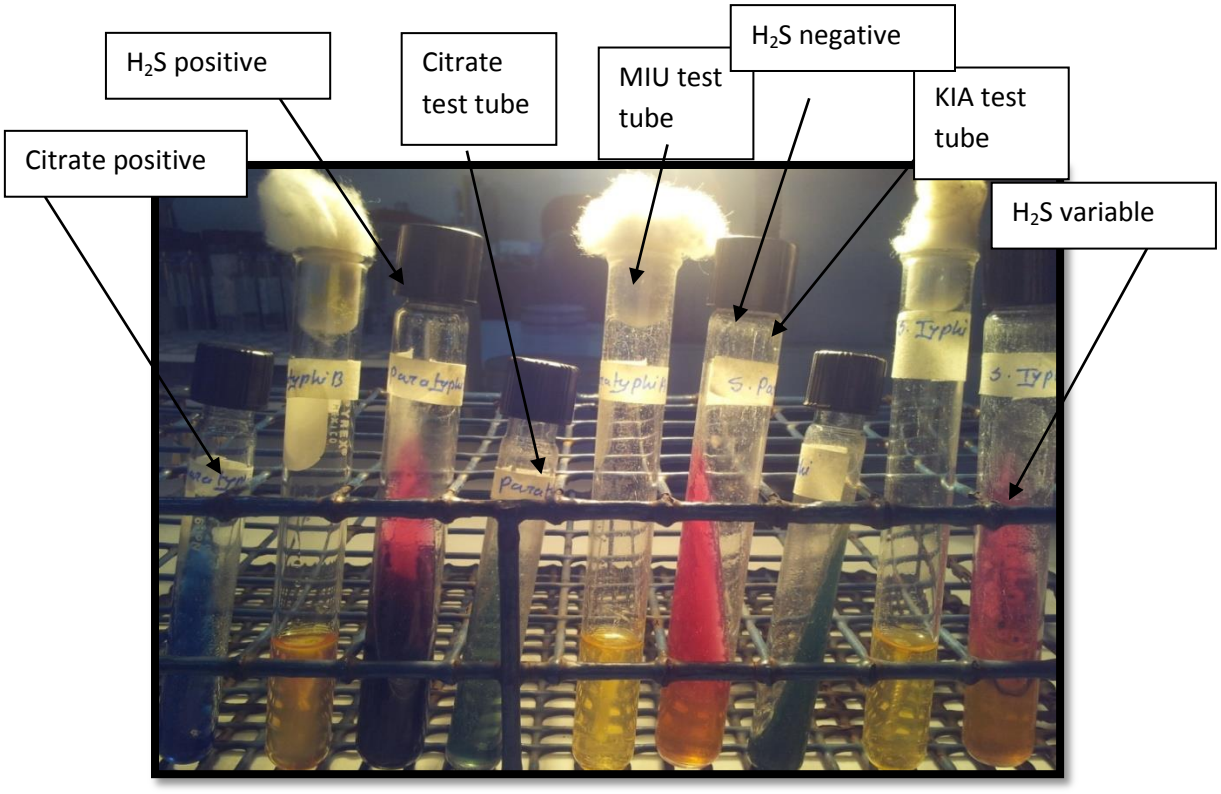

Fig 6: KIA, MIU, and Citrate test for *S.* Typhi, *S.* Paratyphi A and *S.* Paratyphi B (from right to left)

3.1.3. Serological test- result

Serological test was done to identify 100 *S.* Typhi, 21 *S* .Paratyphi A and 1 *S*.Paratyphi B isolates.

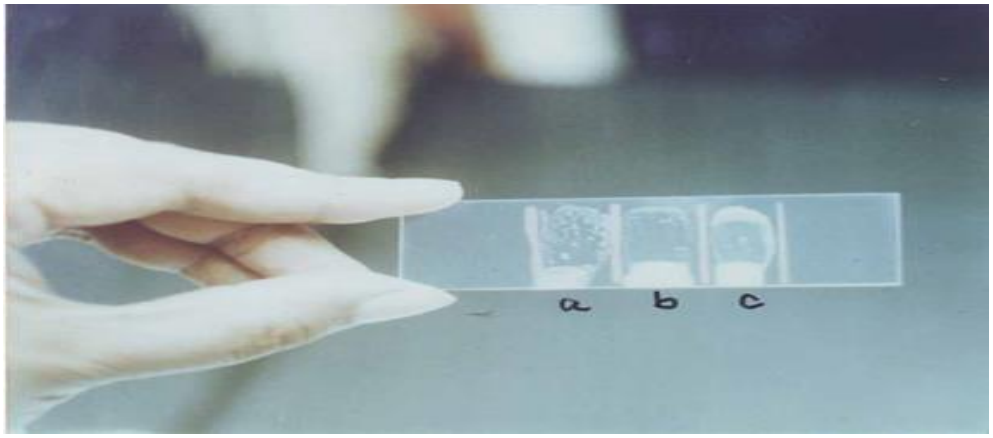

Fig 7: Serological test

3.2. Disc diffusion test results of *S.* Typhi CLSI guideline- 2013

The result of disc diffusion was analyzed by using new CLSI guideline 2013 (Clinical and laboratory Standard Institute-2013) and BSAC-2013 guideline (British Society for Antimicrobial Chemotherapy-2013). In CLSI-2013 guideline the standard zone diameter breakpoint for azithromycin was not available. Among 100 *S.* Typhi isolates, only 22% were resistant to ampicillin and 23% were resistant to chloramphenicol and trimethoprim-sulfamethoxazole. Eighty-seven percent *S.* Typhi was resistant to nalidixic and 83% were intermediate to ciprofloxacin.None of the isolates were resistant to cefixime and ceftriaxone.

Table 8: Frequency Of Antibiotic Resistance among *S.* Typhiisolates from blood culture (N=100) using CLSI guideline-2013

Antimicrobial agents	Percentage of isolates resistant to antibiotics N=100		
	New CLSI-2013		
	Resistant (R)	Intermediate (I)	Susceptible (S)
Ampicillin (Amp)	22%	0%	78%
Chloramphenicol (C)	23%	0%	77%
Azithromycin (Azm)	NA	NA	NA
Nalidixic Acid (NA)	87%	1%	12%
Cefixime (CFM)	0%	0%	100%
Ceftriaxone (CRO)	0%	0%	100%
Trimethoprim-sulfamethoxazole (SXT)	23%	0%	77%
Ciprofloxacin (CIP)	4%	83%	13%

[NA = Not Available]

3.3. Disc diffusion-Result of *S.* Typhi using BSAC guideline- 2013

According to BSAC guidline-2013, disc diffusion results of *S.* Typhi for first line antibiotics were same as found using CLSI-2013.None of the isolates were resistant to cefixime and ceftriaxone. Eighty-four percent *S.* Typhi was resistant to nalidixic acid. Only 16% *S.* Typhi was resistant to azithromycin.

Table 9: Frequency of Antibiotic Resistance among *S.* Typhiisolates from blood culture (N=100) using BSACguideline -2013

Antimicrobial agents	Percentage of isolates resistant to antibiotics N=100		
	BSAC-2013		
	Resistant (R)	Intermediate (I)	Susceptible (S)
Ampicillin (Amp)	22%	0%	78%
Chloramphenicol (C)	23%	0%	77%
Azithromycin (Azm)	16.16%	0%	83.84%
Nalidixic Acid (NA)	84%	0%	16%
Cefixime (CFM)	0%	0%	100%
Ceftriaxone (CRO)	0%	0%	100%
Trimethoprim - sulfamethoxazole (SXT)	23%	0%	77%
Ciprofloxacin (CIP)	NA	NA	NA

[NA= Not Available]

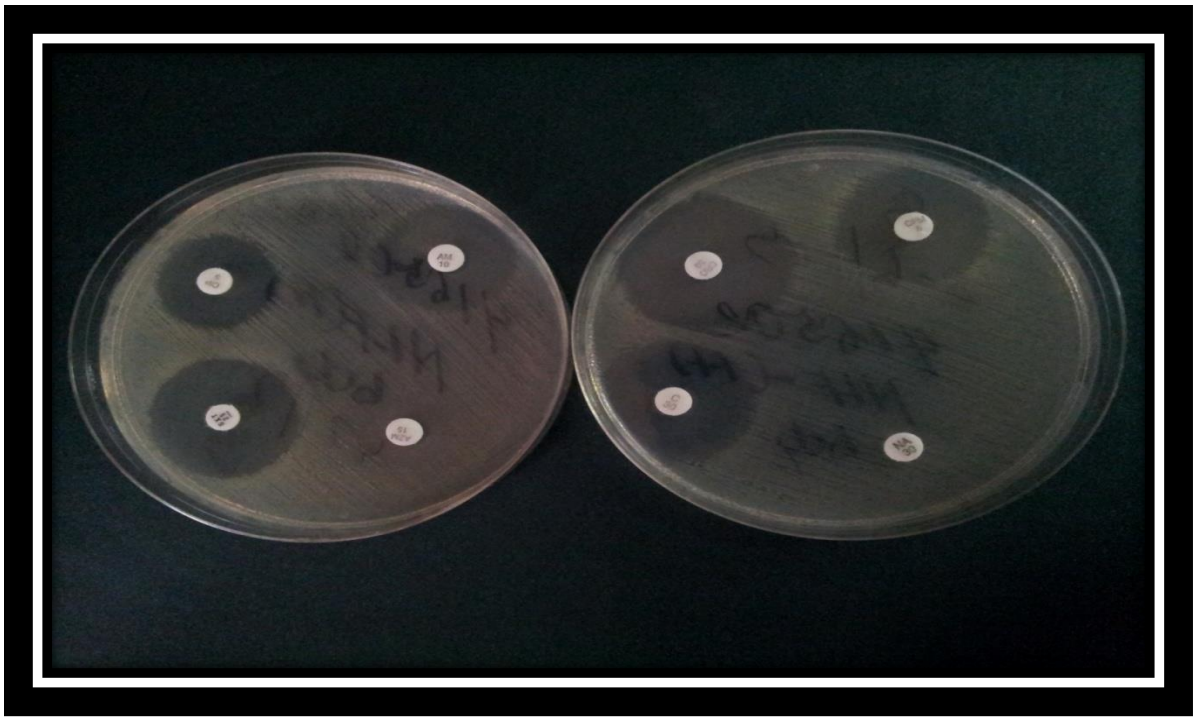

Fig 8: Antibiogram plates of disc diffusion test for *S.*Typhi

Disc diffusion result was analyzed by using CLSI guideline -2013 and BSAC -2013 guideline. A comparative analysis (Table: 10) between both of the result showed that the frequency of ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole-resistant *S.* Typhi was similar in both guidelines. The frequency of cefixime and ceftriaxone-resistant *S.* Typhi was also same using both methods. Standard zone diameter breakpoint for azithromycin was not available in new CLSI guideline-2013. So, the frequency of azithromycin-resistant *S.* Typhi was obtained using BSAC-2013 guideline. Eighty-seven percent of *S.* Typhi was resistant to nalidixic acid according to CLSI guideline-2013 and 84% were resistant to the same antibiotic according to the BSAC-2013 guideline. Such difference was because of the variation of zone diameter breakpoint of nalidixic acid between the two guidelines. Eighty-three percent of *S.*Typhi isolates were intermediate to ciprofloxacin using new CLSI guideline-2013. The breakpoint of ciprofloxacin was available but the disc size was 1µg which was not used in this study. The disc size of ciprofloxacin use in this study was 5 µg for which zone diameter breakpoint was available in CLSI-2013

Table 10: Comparison between CLSI guidelines-2013 and BSAC-2013 guideline in the analysis of disc diffusion result of *S*. Typhi

Antimicrobial agents	Percentage of isolates resistant to antibiotics N =100						
	CLSI-2013				BSAC-2013		
	R	I	S		R	I	S
Ampicillin (Amp)	22%	0%	78%		22%	0%	78%
Chloramphenicol (C)	23%	0%	77%		23%	0%	77%
Azithromycin (Azm)	NA	NA	NA		16.16%	0%	83.84%
Nalidixic Acid (NA)	87%	1%	12%		84%	0%	16%
Cefixime (CFM)	0%	0%	100%		0%	0%	100%
Ceftriaxone (CRO)	0%	0%	100%		0%	0%	100%
Trimethoprim - sulfamethoxazole (SXT)	23%	0%	77%		23%	0%	77%
Ciprofloxacin (CIP)	4%	83%	13%		NA	NA	NA

[NA=Not Available]

3.4 Result of disc diffusion of *S.* Paratyphi using new CLSI-2013 guideline

Using new CLSI-2013, the frequency of antimicrobial resistance among *S.* Paratyphi isolates obtained as follows .Hundred percent *S.* Paratyphi isolates were susceptible to ampicillin, chloramphenicol and trimthoprim- sulfamethoxazole, cefixime and ceftriaxone, whereas 91% were resistant to nalidixic acid and intermediate to ciprofloxacin (Table: 11).

Table 11: Frequency of Antibiotic Resistance among *S.* Paratyphiisolates (N=22) from blood culture using CLSI -2013

Antimicrobial agents	Percentage of isolates resistant to antibiotics N=22		
	CLSI-2013		
	Resistance (R)	Intermediate (I)	Susceptible (S)
Ampicillin (Amp)	0%	0%	100%
Chloramphenicol (C)	0%	0%	100%
Azithromycin (Azm)	NA	NA	NA
Nalidixic Acid (NA)	91%	0%	9%
Cefixime (CFM)	0%	0%	100%
Ceftriaxone (CRO)	0%	0%	100%
Trimethoprim - sulfamethoxazole (SXT)	0%	0%	100%
Ciprofloxacin (CIP)	4%	91%	5%

[NA=Not Available]

3.5 Result of disc diffusion of *S*. Paratyphi using BSAC-2013

According to BSAC-2013 guideline frequency of antibiotic resistance among *S.* Paratyphi is presented in Table: 12. It showed that none of the isolates were resistant to first-line antimicrobials and cefixime and ceftriaxone. This guideline was used to determine the resistant frequency of *S.* Paratyphi to azithromycin. Ninety-one percent of isolates were resistant to azithromycin and nalidixic acid in disc diffusion according to this guideline.

Table 12: Frequency Of Antibiotic Resistance among *S.* Paratyphiisolates from blood culture (N=22)using BSAC-2013

Antimicrobial agents	Percentage of isolates resistant to antibiotics N=22		
	BSAC-2013		
	Resistant (R)	Intermediate (I)	Susceptible (S)
Ampicillin (Amp)	0%	0%	100%
Chloramphenicol (C)	0%	0%	100%
Azithromycin (Azm)	91%	0%	9%
Nalidixic Acid (NA)	91%	0%	9%
Cefixime (CFM)	0%	0%	100%
Ceftriaxone (CRO)	0%	0%	100%
Trimethoprim- sulfamethoxazole (SXT)	0%	0%	100%
Ciprofloxacin (CIP)	NA	NA	NA

[NA=Not Available]

Disc diffusion result of *S.* Paratyphi was analyzed by using CLSI guideline -2013 and BSAC -2013 Guideline. A comparative analysis (Table: 13) between both of the results showed that the frequency of ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole-resistant *S.* Paratyphi was similar in both guidelines. The frequency of cefixime and ceftriaxone-resistant *S.* Paratyphi was also same using both guidelines. Ninety-one percent isolates were resistant to nalidixic acid according to both methods. Standard zone diameter breakpoint for azithromycin was not available in new CLSI guideline 2013. So, the frequency of azithromycin-resistant *S.* Paratyphi was obtained using BSAC -2013 guideline. Ninety-one percent *S.* Paratyphi isolates were intermediate to ciprofloxacin using new CLSI guideline 2013 guideline. In BSAC-2013, disc size of ciprofloxacin was 1µg which was not used in this study. The disc size of ciprofloxacin used in this study was 5 µg for which zone diameter breakpoint was available in CLSI-2013.

Table13: Comparison between CLSI-2013 and BSAC-2013 guideline in analyzing disc diffusion result of *S.* Paratyphi

Antimicrobial Agents	Percentage of isolates resistant to antibiotics N=22					
	CLSI-2013			BSAC-2013		
	(R)	(I)	(S)	(R)	(I)	(S)
Ampicillin (Amp)	0%	0%	100%	0%	0%	100%
Chloramphenicol (C)	0%	0%	100%	0%	0%	100%
Azithromycin (Azm)	NA	NA	NA	91%	0%	9%
Nalidixic Acid (NA)	91%	0%	9%	91%	0%	9%
Cefixime (CFM)	0%	0%	100%	0%	0%	100%
Ceftriaxone (CRO)	0%	0%	100%	0%	0%	100%
Trimethoprim-sulfamethoxazole (SXT)	0%	0%	100%	0%	0%	100%
Ciprofloxacin (CIP)	4%	91%	5.00%	NA	NA	NA

[NA=Not Available]

3.6. Multiple Antimicrobial Resistances among *S*. Typhi

Drug resistant phenotype of *S*. Typhi and *S*. Paratyphi isolates showed in Table 14 and Table: 15 respectively. It revealed that 18%, 5% and 4% *S*. Typhi isolates were resistant to 4 drugs (ampicillin, chloramphenicol, trimethoprim-sulphamethoxazole and nalidixic acid), 3 drugs (chloramphenicol, trimethoprim-sulphamethoxazole and nalidixic acid) and 2 drugs (chloramphenicol, trimethoprim-sulphamethoxazole) and 60% of *S*. Typhi isolates were resistant to only nalidixic acid. Whereas none of the *S*. Paratyphi isolates were multidrug resistant; and 90% were resistant to nalidixic acid only (Table: 15)

Table 14: Pattern of isolates with resistance species to antimicrobial agents among *S*. Typhi (N=100)

Resistance Phenotype	No of isolates	Percentage
A. Resistance to 4 drugs Amp,C,SXT,NA	18	18%
B. Resistance to 3 drugs Amp,C,SXT Amp,C,NA Amp,SXT,NA C, SXT,NA	5	5%
C. Resistance to 2 drugs Amp,C Amp,SXT Amp,NA C,SXT C,NA SXT,NA	4	4%
D. Resistance to at least 1 drug Amp C SXT NA	60	60%
Resistance to all antimicrobial agents (tested)	87	87%
Susceptible to all	12	12%
Nalidixic acid Intermediate	1	1%
Total	100	100%

Table 15: Pattern of isolates with resistance species to antimicrobial agents among *S*. Paratyphi (N=22)

Resistance Phenotype	No of isolates	Percentage
A. Resistance to 4 drugs Amp,C,SXT,NA	0	0%
B. Resistance to 3 drugs Amp,C,SXT Amp,C,NA Amp,SXT,NA C, SXT,NA	0	0%
C. Resistance to 2 drugs Amp,C Amp,SXT Amp,NA C,SXT C,NA SXT,NA	0	0%
D. Resistance to at least 1drug Amp C SXT NA	20	90.9%
Resistance to all antimicrobial agents (tested)	20	90.9%
Susceptible to all	2	9.1%
Total	22	100%

3.7. Result of E-test for Ciprofloxacin of *S*. Typhi and *S*. Paratyphi

Thirteen isolates of *S*. Typhi were tested of which only 2 were resistant to ciprofloxacin whereas others were intermediate to such antimicrobial agent in disc diffusion as well as in E-test (Table: 16). The MIC breakpoint was obtained from CLSI-2013.

Table 16: Comparison of resistance pattern of *S*. Typhi (N=13) to ciprofloxacin by disc diffusion test and E test

Isolates No	Disc diffusion result (mm)	MIC Value (µg/ml)
1	18(R)	2 (R)
2	17(R)	2 (R)
3	30(I)	0.19(I)
4	29.3(I)	0.3(I)
5	29.5(I)	0.38(I)
6	29.5(I)	0.38(I)
7	27.7(I)	0.19(I)
8	30.4(I)	0.194(I)
9	27.3(I)	0.16(I)
10	27.4(I)	0.12(I)
11	25.4(I)	0.25(I)
12	25.6(I)	0.25(I)
13	30 (I)	0.16(I)

[Notes: MIC breakpoint of ciprofloxacin (µg/ml): S= ≤ 0.06, I=0.12-0.5, R= ≥ 1
Disc diffusion breakpoint of ciprofloxacin (mm): R= ≤20, I= 21-30, S = ≥31
R= Resistant, I=Intermediate, S=Susceptible]

On the other side, 14 isolates *S*. Paratyphi were tested. All of which were intermediate to ciprofloxacin in disc diffusion and also in E-test.

Table 17: Comparison of resistance pattern of *S*. Paratyphi (N=14) to ciprofloxacin by disc diffusion test and E test

Isolates No	Disc diffusion result (mm)	MIC Value (µg/ml)
1	27(I)	0.5 (I)
2	30.4(I)	0.5(I)
3	30 (I)	0.23(I)
4	25.3(I)	0.5(I)
5	29.6(I)	0.5(I)
6	24.5(I)	0.5(I)
7	24.5(I)	0.5(I)
8	24.6(I)	0.5(I)
9	26.3(I)	0.5(I)
10	26.3(I)	0.5(I)
11	25.6(I)	0.5(I)
12	29.3(I)	0.5(I)
13	26.3(I)	0.5(I)
14	27 (I)	0.5(I)

[Notes: MIC breakpoint of ciprofloxacin (µg/L): S= ≤ 0.06, I=0.12-0.5, R= ≥1

Disc diffusion breakpoint of ciprofloxacin (mm): R= ≤20, I= 21-30, S = ≥ 31

R= Resistant, I=Intermediate, S=Susceptible]

3.8. Result of E-test for Azithromycin of *S*.Typhi and *S*.Paratyphi

The correlation was not observed between the result ofE-test and disc diffusion test for azithromycin. Ten isolates of *S.* Typhi were resistant (≤18mm) to azithromycin in disc diffusion (according to BSAC-2013) were susceptible to azithromycin in E-test with a value 6-16 mg/L (MIC breakpoint is ≤16 mg/L) (Table: 18). On the other side, 16 azithromycins resistant (in disc diffusion) *S.* Paratyphi (all were *S.* Paratyphi A) isolates were used for E-test showed that 10 out of 16 isolates (all were *S.* Paratyphi A) were susceptible to azithromycin (Table:19).

Table 18: Comparison of resistance pattern of *S.* Typhi (N=10) to azithromycin by disc diffusion and E test

Isolates No	Disc Diffusion (mm)	MIC (mg/L)
1	18.3 (R)	8 (S)
2	16 (R)	8 (S)
3	18.3 (R)	8 (S)
4	17.6 (R)	8 (S)
5	16.3 (R)	6 (S)
6	17.3(R)	6 (S)
7	18.3(R)	16 (S)
8	18.17 (R)	6 (S)
9	18.13 (R)	8(S)
10	18 (R)	6 (S)

[Notes: MIC breakpoint of azithromycin (mg/L): ≤ 16 mg/L

Disc diffusion breakpoint of azithromycin (mm): R= ≤ 18, S= ≥19

R= Resistant, I=Intermediate, S=Susceptible]

Table 19: Comparison of resistance pattern of *S.* Paratyphi (N=16) to azithromycin by disc diffusion and E test

Isolates No	Disc Diffusion (mm)	MIC (mg/L)
1	17 (R)	32(R)
2	15.3(R)	24(R)
3	16 (R)	24(R)
4	16.3(R)	24(R)
5	18 (R)	24(R)
6	16.3(R)	16(S)
7	18.3(R)	16(S)
8	17.3(R)	24(R)
9	16.3(R)	16(S)
10	16.6(R)	16(S)
11	16.6(R)	16(S)
12	17.6(R)	16(S)
13	17.6(R)	16(S)
14	16.3(R)	16(S)
15	17.6(R)	16(S)
16	17 (R)	16(S)

[

Notes: MIC breakpoint of azithromycin (mg/L): ≤ 16 mg/L

Disc diffusion breakpoint of azithromycin (mm): R= ≤ 18, S= ≥19

R= Resistant, I=Intermediate, S=Susceptible]

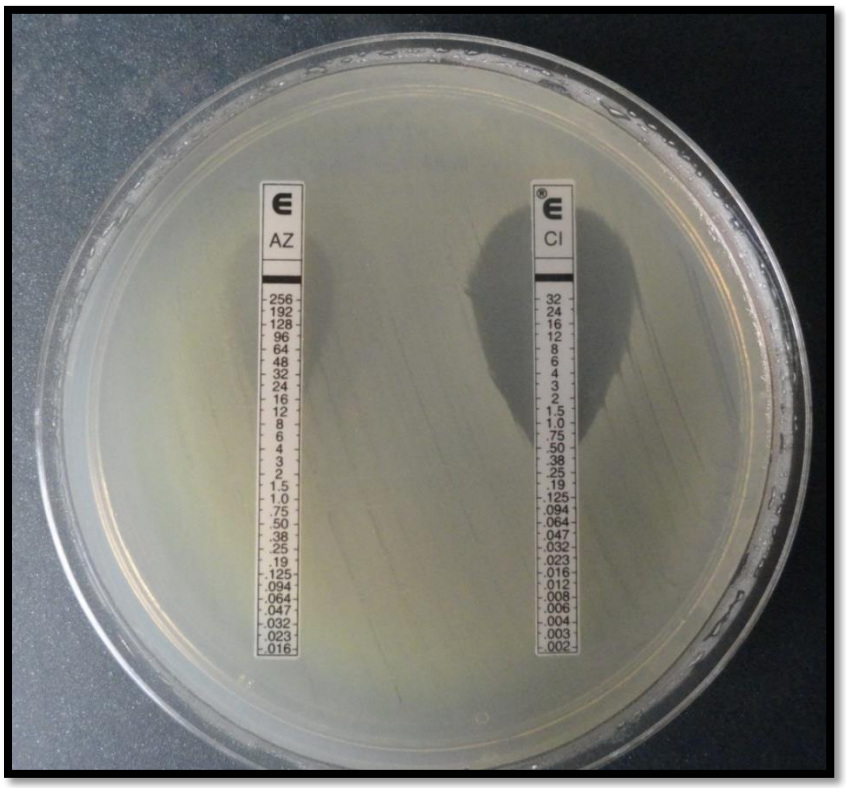

Fig 9: E-test of *S.* Paratyphi for azithromycin and ciprofloxacin

3.9. Result of Agar dilution for nalidixic acid of *S.* Typhi (N=13)

The MIC value of 13 *S.* Typhi isolates (tested) indicated that isolates those were resistant in disc diffusion to nalidixic acid were resistant in agar dilution to such antimicrobial agent. Four out of 13 isolates were susceptible to nalidixic acid in agar dilution as well as in disc diffusion.

Table 20: Agar dilution for nalidixic acid of *S.* Typhi (N=13)

Isolates no	MIC (µg/ml)	Disc Diffusion
1	>256 (R)	6 (R)
2	>256 (R)	6 (R)
3	>256 (R)	6 (R)
4	>256 (R)	6 (R)
5	>256 (R)	6 (R)
6	>256 (R)	6 (R)
7	>256 (R)	6 (R)
8	>256 (R)	6 (R)
9	>256 (R)	6 (R)
10	8 (S)	22.34 (S)
11	8 (S)	19.6 (S)
12	8 (S)	26.34 (S)
13	2 (S)	25 (S)

[Notes]: MIC breakpoint of nalidixic acid (µg/ml): S= ≤16, R=≥32
Disc diffusion breakpoint of nalidixic acid (mm): R= ≤ 13, I= 14-18, S=≥19
R= Resistant, I=Intermediate, S=Susceptible]

Whereas, seven isolates of *S.* Partyphi were tested and all of them were resistant in agar dilution as well as in disc diffusion

Table 21: Agar dilution for nalidixic acid of *S*. Paratyphi (N=7)

Isolates no	MIC (µg/ml)	Disc diffusion (mm)
1	>256 (R)	6 (R)
2	>256 (R)	6 (R)
3	>256 (R)	6 (R)
4	>256 (R)	6 (R)
5	>256 (R)	6 (R)
6	>256 (R)	6 (R)
7	>256 (R)	6 (R)

[Notes: MIC breakpoint of nalidixic acid (µg/ml): S= ≤16, R= ≥32
Disc diffusion breakpoint of nalidixic acid (mm): R= ≤ 13, I= 14-18 S= ≥19
R= Resistant, I=Intermediate, S=Susceptible]

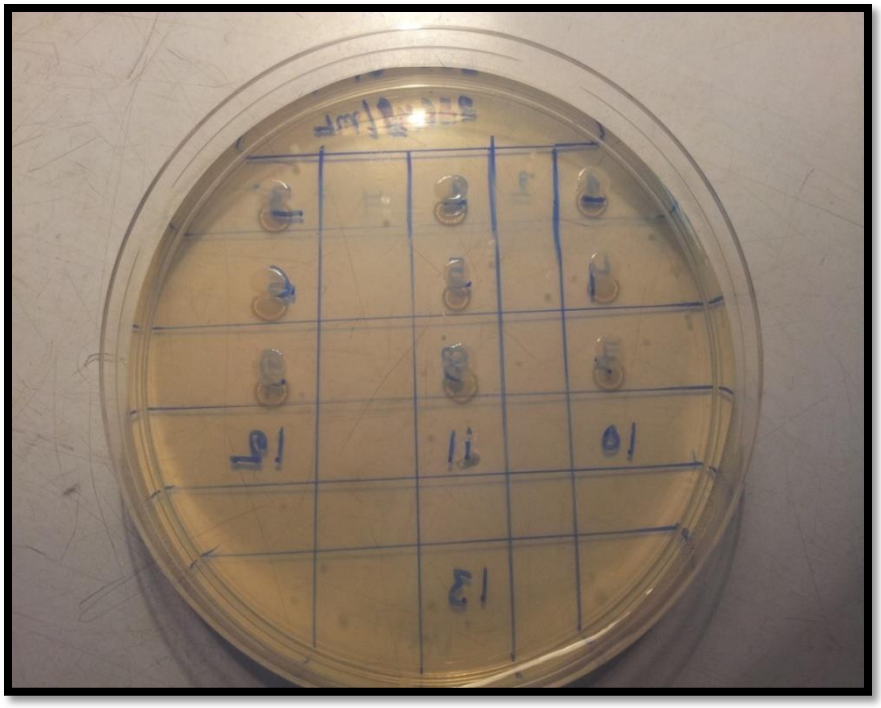

Figure 10: Growth of 9 out of 13 isolates of *S*. Typhi in the plate containing 256 µg/ml of nalidixic acid

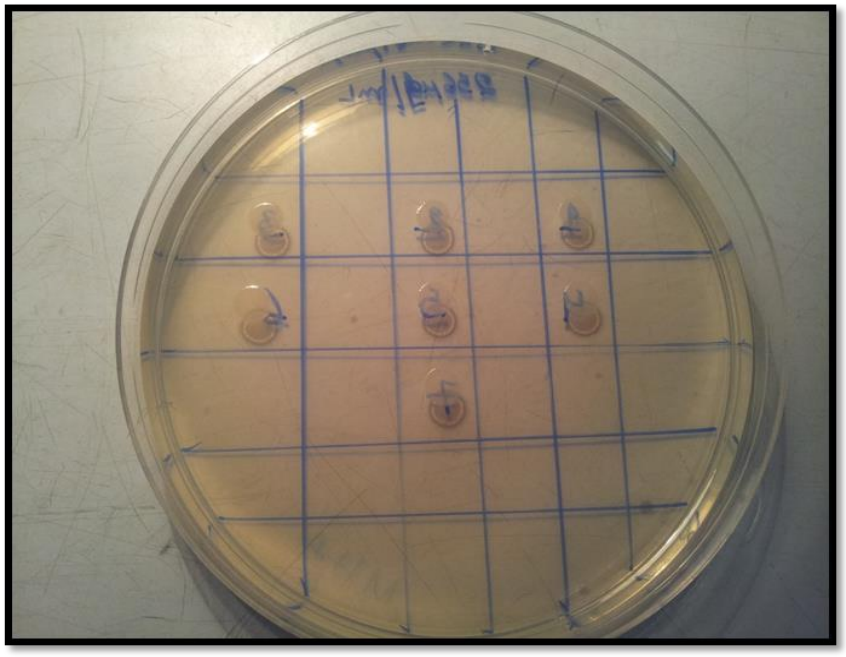

Figure 11: Growth of all the 7 isolates of *S*. Paratyphi A in the plate containing 256 µg/ml of nalidixic acid

3.9. Comparison between the disc diffusion test result of ciprofloxacin and nalidixic acid

A comparison in the disc diffusion test result of nalidixic acid and ciprofloxacin suggest a relationship of resistant pattern between these two antimicrobials among *S.*Typhi and *S.*Paratyphi. It was observed that maximum number of nalidixic acid resistant isolates was intermediate to ciprofloxacin.

Table 22: Comparison in the resistant pattern of *S.* Typhi (N=100) for ciprofloxacin and nalidixic acid

Ciprofloxacin	Nalidixic Acid			Total
	R	I	S	
R	4	0	0	4
I	74	1	8	83
S	9	0	4	13
				Total = 100

[R=resistance, I=Intermediate, S=Susceptible]

Table 23: Comparison in the resistant pattern of *S.* Paratyphi (N=22) for ciprofloxacin and nalidixic acid

Ciprofloxacin	Nalidixic Acid			Total
	R	I	S	
R	0	0	0	0
I	20	0	1	21
S	0	0	1	1
				Total = 22

[R=resistance, I=Intermediate, S=Susceptible]

3.10. Detection of decreased ciprofloxacin susceptibility

A correlation of MIC and disc diffusion results using new CLSI guideline-2013 also showedan association between nalidixic acid resistance and reduced susceptibility to ciprofloxacin among *S*. Typhi and *S*. Paratyphi isolates.

Among 13 *S*. Typhi isolates only 2 were resistant to ciprofloxacin in disc diffusion and in E-test (2 µg/ml) and thesewere also resistant to nalidixic acid in both disc diffusion and in agar dilution test (>256mg/L). Four isolates with reduced susceptibility (intermediate) to ciprofloxacin were susceptible to nalidixic acid in agar dilution with a value ≤16 µg/ml (2-8 µg/ml). Maximum*S*. Typhi isolates (N=7) with decreased ciprofloxacin susceptibility in disc diffusion and E-test was resistant to nalidixic acid in disc diffusion method and in agar dilution method at >256 mg/L. When MIC of ciprofloxacin was compared with the inhibition zone diameter of ciprofloxacin susceptibility zone diameter, 11 out of 13*S*. Typhi isolates had zone diameter of 21-30mm (range of intermediates is 21-30mm) and MICs of 0.12-0.25 µg/mL (intermediate susceptible range of ciprofloxacin is 0.12-0.5 µg/ml), exhibiting a decreased ciprofloxacin susceptibility (Table: 24).

Most of the nalidixic acid resistant*S*. Paratyphi isolates (7 out of 9) showed higher MICs (0.15-0.25 µg/ml) for ciprofloxacin which was within the range of intermediate, whereas isolates those were susceptible to nalidixic acid had MIC range 0.12-0.19 µg/ml. Thus maximum nalidixic acid resistant isolates of *S*. Typhi (in disc diffusion) showed decrease susceptibility to ciprofloxacin with comparatively higher MIC (Table: 25).

Table 24: Ciprofloxacin and nalidixic acid disc diffusion test results as an indicator for ciprofloxacin MICs in *S.* Typhi (N=13) for detecting decreased ciprofloxacin susceptibility.

	Disc diffusion results of		CLSI zone diameter for ciprofloxacin susceptibility	Range of MIC µg/ml Of	
No of strains	NA	CIP		NA	CIP
4	S	I	I (21-30 mm)	2-8 (≤ 16)	0.12-0.19 (0.12-0.5)
2	R	R	R (≤ 20 mm)	≥ 256 (≥ 32)	2 (≥ 1)
7	R	I	I (21-30 mm)	≥ 256 (≥ 32)	0.15-0.25 (0.12-0.5)

Table 25: Ciprofloxacin and nalidixic acid disc diffusion test results as an indicator for ciprofloxacin MICs in *S.* Paratyphi (*S.* Paratyphi A) (N=7) for detecting decreased ciprofloxacin susceptibility

	Disc diffusion results of		**CLSI zone diameter for ciprofloxacin susceptibility**	**Range of MIC µg/ml of**	
No of strains	NA	CIP		NA	CIP
0	S	I	I (21-30 mm)	-	-
0	I	I	I (21-30 mm)	-	-
7	R	I	I (21-30 mm)	≥ 256 (≥ 32)	0.15-0.25 (0.12-0.5)

Chapter-4
Discussion

S. Typhi infection remains a worldwide health problem, *S.* Typhi and *S.* Paratyphi A and *S.* Paratyphi B are serious illness particularly in the developing world including Bangladesh.

In this study, 100 *S.* Typhi, 21 *S.* Paratyphi A and 1 *S.* Paratyphi B were isolated from blood (21 isolates) and stools (1 isolate). Most of the *S.* Typhi isolateswere more resistant to first-line antimicrobials (ampicillin, chloramphenicol and trimethoprim-sulphamethoxazole) than *S.* Paratyphi.During 2004-2009 majority of*S.* Typhi (38%) isolates were simultaneously resistant to all three first line drugs compared to only 1.8% *S.* Paratyphi Aobserved in a study in BIRDERM, Dhaka (Shadia *et al.*, 2011) . In Clinical Microbiology Laboratory of icddr, b from 2006 to 2010, among 765 *S.* Typhistrains, 60%, 61% and 60% strains wereresistant to ampicillin, chloramphenicol and sulphamethoxazole-trimethoprim respectively using CLSI guideline (Azmi *et al.*, 2013). Whereas data of our study revealed a gradual decline of resistance to these first lines antimicrobials .In recent years, such changing trend in the resistance pattern of *S.* Typhi to first line antibiotics have been observed in a study conducted at different hospitals and diagnostic Centre of Dhaka, Rajshahi and Chittagong from 2011-2012 (Mannan, 2014). The study revealed that in all these districts of Bangladesh none of the *S.* Typhi isolates were chloramphenicol resistant; whereas ampicillin resistant isolates were less than 10%. In different region of India *S.* Typhi and *S.* Paratyphi isolates were found variably susceptible to traditional first line antimicrobials. In 2007-2009 in India *S.* Typhi showed susceptibility to ampicillin (67.5%), chloramphenicol (97.5%) and co-trimoxazole(97.5%). Whereas 39%, 95% and 95%*S.*Paratyphi A were susceptibleto ampicillin, chloramphenicol and co-trimoxazole. Another retrospective study from India (January 2008-December 2010) suggested that ampicilin, chloramphenicol and trimethoprim- sulphamethoxazole resistant *S.* Typhi and *S.* Partyphi A were less than 5%. . Our result indicates the decline of resistance of *S.* Typhi and *S.* Paraytphi to ampicillin, chloramphenicol and trimethoprim- sulphamethoxazole.

From late 1987, these first line antibiotics wereused for treating typhoid and paratyphoid fever especially in Asian countries which posed a great medical problem in treatment of enteric fever. Different study in Bangladesh conducted within last few years showed

higher rates of multidrug resistant (MDR, resistant to the first-line recommended drugs such as chloramphenicol, ampicillin and trimethoprim-sulfamethoxazole)*S.* Typhi and *S.* Paratyphi**.** In the early 1990, MDR*S.* Typhistrain was detected in the clinical microbiology laboratory of icddr, b and since then it was isolated at an increasing frequency. In 1990,MDR*S.* Typhi strains were first detected in Bangladesh (rate 8%) which increased in 1994 (44%), declined in 1996 and reemerged again in 2001 (36%) - 2002 (42%) (Rahman*et al.,* 2006).A study conducted in the northern part of Bangladesh from July 2006- June 2007 reported that among 16 *S.* Typhi 10 isolates were MDR (Hassan *et al*., 2011). A study Dhaka, Rajshahi and Chittagong conducted from November 2011 to November 2012 explained that among 70 isolates of *S*. Typhi MDR was 64.28% (Mannan *et al*., 2014). In our study,MDR (multidrug resistant to traditional first line antibiotics, ampicillin, chloramphenicol and trimethoprime-sulphamethoxazole) *S*. Typhi was only 18%, in contrast multidrug resistant *S*. Paratyphi was zero. Previous study, conducted Dhaka Clinical Research and Service Centre (CRSC), of icddr, b during1989-2002, showed that in Bangladesh 74% of *S.* Typhi strain isolated with decreased ciprofloxacin susceptibility were MDR (Rahman*et al.,* 2006). In our study 83% *S*. Typhi isolates had decreased ciprofloxacin susceptibility of which 18 isolates (21.6%) were MDR. Whereas 87% *S.* Paratyphi with decreased ciprofloxacin susceptibility were not MDR. As the use of these first line antibiotics was stopped for the last few years, *S*. Typhi and *S.* Paratyphi susceptible strain to these antimicrobials (ampicillin, chloramphenicol and trimethoprim-suphamethoxazole) have been gradually increasing in our country. Previously the reason behind this multidrug-resistance was loss of high molecular weight self-transmissible multi-resistant plasmid, as these antibiotics were not available in environment for a long time. But recently *Salmonella* genomic island SGI11 has been found in multidrug- resistant *S.*Typhi isolates in Bangladesh, which showed that resistant genes reside in 18 out of 26 multidrug-resistant *S*. Typhi isolates (Chiou *et al*., 2015). In our study decline in multidrug – resistant strain may arise from the loss of self-transmissible IncHI1 plasmid or due to the loss of resistant genes from genomic island. Such gradual reduction of MDR*S.* Typhi and *S*. Paratyphi isolates indicates that, first line of antibiotics can be an effective in the treatment of enteric fever in near future in Bangladesh.

Maximum*S.* Typhi (84-87%) and *S.* Paratyphi (91%) isolates were resistant to nalidixic acid. Even all the MDR isolates wereresistant to nalidixic acid in disc diffusion. A study in Bangladesh during 2006-2007 showed 100% *S.* Typhi isolates wereresistant to nalidixic acid (Hassan *et al.*, 2011). Nalidixic acid resistant *S.* Paratyphi was 75-94% in 2004-2009 in another study. Similar result was obtained during the year 2012 in a study in Dhaka (Akter *et al.,* 2012). In a multi-country study (Bangladesh, Indonesia, Taiwan and Vietnam)during 2007-2009, 38 *S.* Typhi isolates were from Bangladesh of which 82% MDR *S.* Typhi were nalidixic acid resistant and 40% were ciprofloxacin resistant . (Chiou *et al.*, 2014).In 2000, *S.* Typhi isolated from typhoid patients in Bangladesh exhibited nalidixic acid resistance and decreased ciprofloxacin susceptibility which increased sharply in 2002 (Rahman *et al.*, 2006). In following years*S.* Typhi and *S.* Paratyphi were susceptible to ciprofloxacin in Bangladesh, which observed in a study in 2008, where 74 out of 100 *Salmonella* (*S.* Typhi and *S.* Paratyphi) strains was susceptible to ciprofloxacin (kawser *et al.,* 2013). In India prior to 2012, 84% *S.* Typhi isolates were susceptible to ciprofloxacin whereas 85% were susceptible to the same in 2011. But in 2012, 90% of *Salmonella* isolates were resistant to ciprofloxacin with MIC breakpoint <0.06 μg/ml for susceptible strain according to CLSI-2012 (Grirish *et al.*, 2013). In Dhaka, Rajshahi and Chittagong from 2011-2012, a study revealed that ciprofloxacin cannot be potential option for the treatment of typhoid (Shadia *et al.,* 2014). Our findings revealed that most of the *S.* Typhi (83%) and *S.* Paratyphi (91%) were moderately susceptible to ciprofloxacin. Maximum numbers of nalidixic acid resistant isolates were intermediate to ciprofloxacin.A correlation of MIC and disc diffusion result using new CLSI guideline-2013 also showedan association between nalidixic acid resistance and reduced susceptibility to ciprofloxacin among *S.* Typhi and *S.* Paratyphi isolates.Thus *S.* Typhi and *S.* Paratyphi isolates resistant to nalidixic acid is an indicator of decreased susceptibility to ciprofloxacin. Similar findings observed in a study of Clinical Microbiology Laboratory of icddr,b where among 474 nalidixic acid resistant *S.* Typhi isolates 85% (402) were moderately susceptible to ciprofloxacin(range of intermediate 0.064-0.25μg/ml) (Azmi *et al.,* 2013). But E-test result for ciprofloxacin of all isolates (tested) of *S.* Typhi an d *S.* Paratyphi according to BSAC-2013 guideline (May-2013)

showed that all the isolates were resistant to ciprofloxacin as MIC value of ciprofloxacin was < 0.06 µg/ml for susceptible strain in that guideline (Grirish *et al*., 2013).

Resistance to fluroquinolones is evolving in an ominous direction. This resistance to quinolones is caused by amino acid substitution in the quinolone resistance- determining region of DNA gyrase, subunit *gyr*A, *gyr*B or DNA topoisomearse IV (*par*C, *par*E) which are the key target of quinolones. Single point mutation in *gyr*A is said to be responsible for decreased susceptibility to ciprofloxacin whereas combination of 2 or more mutations in *gyr*A, *gyr*B, *par*C, *par*E makes them resistant (Grirish *et al*., 2013).

In the year 2002 to 2003 in Nepal, point mutation in *gyr*A and *par*C was found innalidixic acid resistant *S.* Typhi isolates which were also moderately susceptible to ciprofloxacin (Nobthai *et al*., 2010).It has been found in a study that *S.* Typhi isolates with reduced susceptibility to ciprofloxacin (MICs of 0.12-0.5 µg/ml) and resistant to nalidixic acid (MICs of 128-256 µg/ml) is associated with a mutation in gyrase subunit A (S38F, S83Y or D87N) and enhanced activity of efflux pump for nalidixic acid (Chiou *et al*., 2014). Considering the MIC value of E-test results from both of the guidelines, ciprofloxacin cannot be used as a potential drug for the treatment of enteric fever.

Another finding of our studyshowed that majority (91%)*S.* Paratyphi isolates were much more resistant to azithromycin than *S.* Typhi (16.6%) according to zone diameter breakpoint for azithromycin in BSAC-2013. Using zone diameter breakpoint ≥15 mm(Garg *et al.,* 2013), none of the *S.* Typhi isolates were resistant to azithromycin but 3/22 *S.* Paratyphi isolates were resistant to such antibiotic. Azithromycin resistant *Salmonella enterica* serotype Paratyphi A that caused treatment failure was first reported in a patient from Pakistan hospitalized in U.K. In this report MIC of azithromycin was 64 mg/L. Thiswas the first clinical case of azithromycin-resistant *S.* Paratyphi A (Aoife*et al.,* 2010). The azithromycin resistant isolates may emerged via several way - *Escherichia coli* may be a reservoir for macrolide resistant genes and may azithromycin resistance in *Salmonella* through plasmid exchange (Nguyen. *et al.,* 2009). Mutations in 23S rRNA close to the sites of methylation have also been associated with resistance to the macrolide group of antibiotics in a range of organisms.

Unique finding of this study is that,isolates of *S.*Typhi those were resistant to azithromycin in disc diffusion using zone diameter breakpoint from BSAC guideline -

2013 were susceptible to such antimicrobial in E-test with MIC value ≤16 mg/L. But correlation between the disc diffusion result and E-test result was observed when zone diameter breakpoint for azithromycin (≥15 mm) was used from the article of Garg *et al.* in Indian journal of microbiology 2013.

On the other side, 16 isolates of *S.* Paratyphi were resistant to azithromycin according to the zone diameter breakpoint mentioned in BSAC-2013. These isolates were susceptible to such antimicrobial when zone diameter breakpoint of azithromycin was ≥15 mm (from an articleof Garg *et al.* of Indian journal of microbiology 2013). In our study, Eleven out of 16 isolates of *S.* Paratyphi were susceptible to azithromycin in E-test with a range of ≤16 mg/L. Thus using BSAC guideline for both disc diffusion test and E-test, correlation was not observed that much. Such variation between disc diffusion and E- test was observed among *S*. Paratyphi A isolates in a study in India, carried out during 2012-2013, where 4 out of 25 isolates of *S.* Paratyphi A were resistant to azithromycin in disc diffusion using zone diameter ≥15 mm among those resistant isolates only one was susceptible to such antimicrobial agent in E-test at 16 µg/ml (MIC breakpoint was ≤16 µg/ml) (Garg *et al*., 2013).

In determination of azithromycin resistance among *S*. Typhi and *S.* Paratyphi by disc diffusion test zone diameter breakpoint for azithromycin mentioned in the article by Garg *et al*., 2013in Indian journal of microbiology-2013 (≥15 mm) is more accurate than BSAC- 2013 guideline. There is no evidence that MIC's are more relevant than susceptibility category results but MIC's have the power to predict efficacy in vivo. As E-test is much more reliable and quantitative method than disc diffusion for determination of antimicrobial resistant patterns so, azithromycin resistant strains should be determined by E-test.

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Conclusion

S. Typhi is more predominant than *S.* Paratyphi in Bangladesh and itis more resistant to first line of antimicrobials (ampicillin, chloramphenicol and trimethoprim-sulphamethoxazole) than *S.* Paratyphi.*S.* Typhi and *S.* Paratyphi susceptible to firstline antimicrobials(ampicillin, chloramphenicol and trimethoprim-sulfamethoxazol) are reemerging, it can be expected that these long term unused drugs can be used for the treatment of typhoid and paratyphoid in near future.The role of cefixime and ceftriaxone can be appreciated as these are highly effective in removing intracellular *Salmonella*. For the treatment of typhoid and paratyphoid fever cefixime and ceftriaxone can be used regularly in our country. Nalidixic acid is noteffective for treatment of typhoid and paratyphoid.Nalidixic acid resistance can be used to predict decreased ciprofloxacin susceptibility of *S.* Typhi and *S.* Paratyphi.As susceptibility to ciprofloxacin is decreasing, it cannot be used as a potential drug for treatment of typhoid.Azithromycin is more effective to treat typhoid than paratyphoidAzithromycin can be usedfor typhoid and paratyphoid patients, based upon MIC determination by E-test

Chapter-6
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Appendix

Appendix-I

MacConkey Agar	
Ingridients	**Amount (g/L)**
Peptic digest of animal tissue	1.5
Casein enzymic hydrolysate	1.5
Pancreatic digest of gelatin	17
Lactose	10
Bile salt	1.5
Crystal violate	0.001
Neutral Red	0..03
Agar	15

Mueller Hinton Agar	
Ingredients	**Amount (g/L)**
Beef Extract	2
Acid Hydrolysate of Casein	17.5
Starch	1.5
Agar	17
Distilled Water	
Final pH 7.3 ± 0.1 at 25ºC	

Salmonella-Shigella Agar	
Ingredients	**Amount (g/L)**
Lactose	10
Bile Salts	8.5
Sodium Citrate	8.5
Sodium Thiosulfate	8.5

Kligler's Iron Agar (KIA)	
Ingredients	**Amount (g/L)**
Polypeptone	20
Dextrose	1
Ferric Ammonium Citrate	0.5
Agar	15
Lactose	10
Sodium Chloride	5
Sodium Thiosulfate	0.5
Phenol Red	0.025
Final pH 7.4 $\pm$ 0.2	

Motility Indole Urea (MIU) Media	
Ingredients	**Amount (g/L)**
enzymic hydrolysate	10
Dextrose	1
Sodium chloride	5
Phenol red 0.010	0.01
Agar 2.0	2

Citrate Agar	
Ingredients	**Amount (g/L)**
Sodium Chloride (NaCl)	5.0
Sodium Citrate (dehydrate)	2.0
Ammonium Dihydrogen Phosphate	1.0
Dipotassium Phosphate	1.0
Magnesium Sulfate (heptahydrate)	0.2
Bromothymol Blue	0.08
Agar	15.0
Deionized water = 1,000 ml	
Final pH 6.9 +/- 0.2 at 25 degrees C.	

McFarland Standard No. 0.5	
Ingredients	**Amount (g/L)**
1.0% Barium chloride (ml)	0.05
1.0% Sulfuric acid (ml)	9.95
Approx. cell density (1X10^8 CFU/mL)	1.5
% Transmittance*	74.3
Absorbance*	0.08 to 0.1

Appendix-II

0.5 McFarland

0.5 McFarland turbidity standard provides an optical density comparable to the density of a bacterial suspension 1.5x 10^8 colony forming units (CFU/ml). 0.5 McFarland standard is commercially available.

Mueller Hinton

Suspend 38 g of the powder in 1 liter of distilled or deionized water. Mix well. Heat until completely dissolved. Sterilize in autoclave at 121°C for 15 minutes. Cool to 45-50°C. Aseptically dispense in Petri dishes

MacConkey Agar

Prepare the medium per label directions. Inoculate and incubate at 35 ± 2°C for 18-24 hours (incubate E. coli ATCC 25922 for 40-48 hours). For E. coli ATCC 8739, inoculate in duplicate and incubate one plate at 30-35°C for 18-24 hours and the other plate at 35-37°C for 18-72 hours

The Salmonella antiserum

This was used for serological test. Vi is a heat-sensitive envelope antigen usually found in fresh isolates of *S*. Typhi .

The antigens used in the test are “H” and “O” antigens of *Salmonella* Typhi and “H” antigen of *S.* Paratyphi. The paratyphoid “O” antigen are not employed as they cross react with typhoid “O” antigen due to the sharing of factor 12. “O” antigen is a somatic antigen and “H” antigen is flagellar antigen.

Nalidixic acid Stck Solution Preparation

0.144g Sigma nalidixic acid powder (98% potency) was added with 1 ml NaoH and adjust volume to 10 ml bydistilled water.

Two fold Serial Dilution

A two-fold dilution reduces the concentration of a solution by a factor of two that is reduces the original concentration by one half. A series of two-fold dilutions is described as two-fold serial dilutions.

Preparation of dilution agents using Eucast definitive document 3.1

Antimicrobial concentration (mg/L)in stock solution	Volume stock solution (ml)	Volume distilled water (ml)	Antimicrobial concentration obtained (mg/L)	Final concentration in medium after addition of 19 ml of agar
10240	1	0	10240	512
10240	1	1	5120	246
10240	1	3	2560	128
2560	1	1	1280	64
2560	1	3	640	32
2560	1	7	320	16
320	1	1	160	8
320	1	3	80	4
320	1	7	40	2

Nalidixic acid powder solution was prepared as follows:

Weight of Powder (mg) = Volume of solvent (ml) × Concentration (mg/L)/ Potency of powder (mg/g)

Weight of Solvent (mL) = Weight of powder (mg) × / Potency of powder (mg/g) / Concentration (mg/L)

Appendix-III

Laboratory Apparatus

- Media were taken from Difco & BBL USA
- Nalidixic acid powder from sigma USA
- Seimens Refrigerator was used for keeping culture palte
- Eppendorf tubes and micropipette tips were taken from Eppendorf and Sigma,
- Petridishes used in the experiments were provided by either Sterilin or Gibco.
- Screw capped tubes and other glass wares were taken from Pyrex® Labware,USA.