In vitro effect of new antibiotics against Clinical isolates of Salmonella enterica subspecies enterica

Thesis Submitted to
Baqai Medical University Karachi
For the fulfillment of the requirements
for the degree of
Doctor of Philosophy (Microbiology)

By
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March 2017

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IN THE NAME OF ALLAH, THE MOST COMPASSIONATE THE MOST MERCIFUL
In vitro effect of new antibiotics against Clinical isolates of *Salmonella enterica* subspecies enterica

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Doctor of Philosophy (Microbiology)

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2017

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DEDICATION

“To my parents”
ACKNOWLEDGEMENTS
I wish to acknowledge my supervisor, Professor Dr. Khursheed Ali Khan for his supervision, guidance, assistance, keen interest and above all giving me his valuable time, suggestions and attention during my research work.

I also wish to express my gratitude to Professor Dr. Shahid Ahmed Abbasi of Armed Forces Institute of Pathology for his kind cooperation, research assistance, valuable suggestions and for putting all the facilities of his department at my disposal. He also utilized his good office for the procurement of needed material, without which this work was not possible.

I also acknowledge the help and valuable suggestions given by Brigadier Dr. Nadir Ali in every step from application in university to thesis preparation.

I also wish to express my gratitude to Professor Dr. Farooq Ahmed Khan and all my colleagues and friends who have extended their cooperation, valuable suggestions and assistance.

Special thanks to Mr. Sakhawat for preparation of media and Mr. Yasin for computer work.

Special thanks to my sons and wife for their kindness, moral support and encouragement during practical work and in preparing the thesis.
I appreciate the cooperation of junior staff of microbiology department of Armed Forces Institute of Pathology.

Graceful acknowledgement is made to all my colleagues and friends whose moral support remained constant source of inspiration throughout laboratory work.

I highly appreciate the partial monetary assistance provided by the administration of Armed Forces Institute of Pathology.
Dedication

“To my teachers”
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Abstract

**Background:** Typhoid fever is caused by Salmonella enterica serovar Typhi (S, Typhi, T is capital because Typhi is serotype and not species) a gram negative bacterium (1). It continues to be a global health problem with over 21.6 million cases and more than 200,000 deaths occurring annually (2;3). Most of these deaths occur in Asia (4). Salmonella enterica serotype Paratyphi is traditionally associated with relatively milder illness as compared to Salmonella enterica serotype Typhi.

**Aim & Objectives:** The aim of this study was to determine in vitro MIC patterns of various therapeutic alternatives available for the treatment of enteric fever and non-typhoidal salmonellae in an endemic region reporting a recent increase in ciprofloxacin resistance.

**Study design:** It is a cross sectional research carried out at Armed Forces Institute of Pathology Rawalpindi. Research was conducted from June 2011 to May 2013.

**Materials and Methods:** Samples of blood, bone marrow, pus, urine, stool and fluids were collected from suspected cases of salmonella infections. Standard protocols were observed during collection and transportation. Culture was applied on Bactec 9050 special and/or standard media. Suspected salmonella colonies were tested by API 20E and confirmed by serology. The isolates were also tested for resistance to antibiotics ampicillin, cotrimoxazole, ciprofloxacin, ceftriaxone, doripenem, imipenem, ertapenem, aztreonam, moxifloxacin, cefpirome, cefepime, gatifloxacin, and chloramphenicol by Kirby-Bauer disc diffusion method (5). MIC (Minimum Inhibitory concentration) was done on MDR and ciprofloxacin intermediate or resistant cases by E-strips.

**Results:** 316 isolates of Salmonella were recovered from 2230 specimens. Resistance by disk diffusion technique was determined, for Salmonella Typhi
against Ampicillin 72%, Chloramphenicol 62%, Cotrimoxazole 41.2%, ceftriaxone 2.4%, ciprofloxacin 8%, cefpodoxime 4.7%, doripenem 2.3%, ertapenem 3.5%, aztreonam 3.5%, and moxifloxacin 3.5%. No resistance was noted for imipenem, cefepime and gatifloxacin. Resistance noted in Salmonella Paratyphi A was ampicillin 60%, chloramphenicol 40%, cotrimoxazole 38%, ceftriaxone 7.9%, ciprofloxacin 8%, cefpodoxime 7.9%, imipenem and ertapenem 2.6%, aztreonam 1.3%, moxifloxacin 6.6%, and gatifloxacin 1.3%. No resistance was noted for doripenem and cefepime. For non-typhoidal salmonellae resistance was noted only against cotrimoxazole 66.6% and ciprofloxacin 7%. For S. Typhi imipenem MIC90 was 0.38 and MIC50 was 0.25. For cefpirome MIC90 was 0.64 and MIC50 was 0.09. For aztreonam MIC90 was 0.12 and MIC50 was 0.09. For cefpodoxime MIC90 was 0.75 and MIC50 was 0.38. For azithromycin these values were 16.0 and 7.0 and for tigecycline they were 0.25 and 0.09
Introduction

Salmonella infection causes significant mortality worldwide. Incidence of Salmonella infections is particularly high in Asia. Disease may be limited to gastrointestinal tract or systemic and is acquired by faeco-oral route. Hence, the infection is also an indicator of poor personal or environmental hygiene. Because of the same reason, these infections are common in under-developed and developing countries. Infections with salmonellae can result in various clinical presentations like enteric fever, gastroenteritis, septicemia with or without suppurative lesion and carrier state (6). Typhoid fever is relatively uncommon in developed countries, where it is found in immigrants or after recent travel to developing countries especially India, Pakistan, Bangladesh, Mexico and Philippines. Typhoid fever is endemic in developing countries, more so in Indian subcontinent (4). Salmonella infections especially those involving blood stream have high mortality (30%). This can be reduced to about 1% with appropriate use of antibiotics (7;8). Salmonella enterica serotype Typhi, Paratyphi A, B and C cause typhoid fever, while non-typhoidal salmonellae (NTS) that have more than 2500 serotypes, cause gastroenteritis and invasive infections like meningitis and osteomyelitis in immunocompromised patients and children (9). Name of the Salmonella serotype is traditionally derived from the place it was first isolated. Serotypes are based on outer lipopolysaccharide somatic O antigen, surface Vi antigen which is capsular polysaccharide and flagellar H antigen which is a protein. Vi (virulence) antigen is present on Salmonella typhi and paratyphi C and many strains of S. Dublin. It is
antiphagocytic and prevents immune mediated killing (10). Paratyphoid fever by *Salmonella* Paratyphi especially Paratyphi A, is considered emerging disease because its incidence has increased dramatically during last two decades, causing more asymptomatic infections than Salmonella Typhi (11). *Salmonella enterica* is mostly acquired directly or indirectly through human feces by faeco-oral route from the diseased person or a carrier. While non-typhoidal salmonellae are acquired through consumption of contaminated food primarily of animal origin. In Pakistan, where these infections are endemic, they are a major cause of morbidity and mortality. Animal source of non-typhoidal salmonella may be symptomatic or asymptomatic depending on the animal and the serovar of *Salmonella enterica*.

Salmonellae can grow over a wide temperature range from 7°C to 48°C. They can grow in aerobic as well as anaerobic conditions on ordinary media. Generally the salmonellae ferment glucose, mannitol, maltose and sorbitol. They do not ferment sucrose or salicine and are negative for indole and urease.

Threat of growing resistance to antibiotics is of grave concern to human health. Resistant strains also lead to prolong illness and more rate of complications (12). Resistance of Salmonella spp. to chloramphenicol, cotrimoxazole and ampicillin developed in 1980s. This led to increasing use of fluoroquinolones. Gradually resistance to them also developed. Resistance to fluoroquinolones is common in *Salmonella Choleraesuis* (13).

Development of resistance is usually attributed to indiscriminate use of antibiotics. Hence, there is need for judicious use of antibiotics with the proper
route and dose. Properly identifying the pathogen and applying its antibiotic sensitivity is important as it ensures early treatment of infection without aggravating the disease and prevents development of resistance.

High prevalence of antibiotic resistant salmonella poses public health concern since they lead to treatment failure. Multi-drug resistant (MDR) strains (resistant to chloramphenicol, ampicillin and cotrimoxazole) are very common. Resistance to 3rd generation cephalosporins is beginning to emerge. Due to development of resistance, therapeutic options for treatment of typhoid and other salmonella infections are getting limited. Outbreaks of MDR Salmonella typhi may be difficult to manage and the results can be devastating especially in developing countries where resources are already limited. Outbreaks have been reported throughout the world especially in south-east Asia, Indian subcontinent, Africa and South America (14;15). An outbreak of MDR S.typhi in late 1990s in Tajikistan caused more than 24,000 infections (16). Hence, there is dire need to explore new avenues for treatment of resistant Salmonellae. In this research we would analyze in vitro effect of new drugs, not usually used for treatment of Salmonella infections. This study will help improve the long-term efficacy of antibiotics.

One of the important causes of food-borne diseases is Salmonella spp. Most of the non-typhoidal Salmonella infections are self-limiting requiring only symptomatic treatment like re-hydration.
Review of Literature
Chapter 1

1.1 Salmonella

There are more than 2,400 species of Salmonella. *Salmonella typhi* and *paratyphi* (T and P are sometimes written capital because Typhi and Paratyphi are serotypes and not species) A, B, C have been isolated only from humans while other salmonellae have been recovered from animals as well (17;18). The different subspecies are *enterica* (1416 serotypes), salamae (477 serotypes), arizonae (94 serotypes) diarezonae (317 serotypes), houtenae (66 serotypes), indica (10 serotypes) and *Salmonella bongori* (19 serotypes) (19). In mice, *Salmonella typhimurium* and *Salmonella enteritidis* have been said to produce disease similar to humans. *Salmonella typhi* has Vi polysaccharide capsule that increases its potential for infectivity and severity of the disease, but is not necessary for infection (20).

1.1.1 Importance

Typhoid fever is caused by *Salmonella enterica* serovar Typhi (*S. typhi*) a gram negative motile bacterium (1). It continues to be a global health problem with over 21.6 million cases and at least 250,000 deaths occurring annually. (2) Almost 80% of the cases and deaths are in Asia, the rest occur mainly in Africa and Latin America (21). The problem is mainly due to invasive infections and mortality without treatment in such cases is around 30%. However, with treatment, the mortality is reduced to around 0.5% (22). The overall mortality with Multi drug resistant typhoid fever MDRTF epidemics is 7 to 16% and is much
higher than 2% seen in susceptible typhoid fever (23). Children with MDRTF (Multi-drug Resistant Typhoid Fever) are more toxic and at a higher risk of developing complications than those with susceptible S. typhi strains (24). There is not only the problem of resistance in Salmonella, but there is also a potential of epidemic outbreaks in these cases, which may be difficult to control. An outbreak in the potable water system in a rural area of South Africa caused an outbreak of typhoid, causing illness in nearly 4000 persons and many deaths (25). Salmonella is lodged in gall bladder for years and remains an important source of spread (26). Salmonella infection can spread through blood stream and can cause suppuration at any distant site. Such localized suppurative infections are common in haemoglobinopathies like sickle cell anaemia (27).

1.1.2 Salmonella – The bacterium

Salmonellae measure 2-3 μm by 0.4 - 0.6 μm. Except the serotype gallinarum and pullorum all of them are motile with peritrichous flagella. Except S. typhi all of them produce gas on sugar fermentation. Ninety nine % of Salmonellae are non-lactose fermenters. Rest 1% require special attention in diagnosis. Besides serology other methods used to separate and identify the types of salmonellae are pulsed-field gel electrophoresis, molecular typing and polymerase chain reaction. It is difficult to differentiate Salmonella paratyphi A from other Salmonellae on biochemical basis. However, it can be identified by serological technique.
1.1.3 Incidence

In developing countries such as India, the disease occurs with an incidence ranging from 102 to 219 per 100,000 of the population (28). According to World Health Organization the incidence of typhoid fever for South East Asia caused by *Salmonella typhi* is 110/100,000 persons per year (29). In Asia, Pakistan has the highest incidence of *Salmonella typhi* and *paratyphi* A (30). Salmonella infection is more common in pre-school children and infants than in adults (31). About one quarter to one third of paediatric typhoid fever cases are under five years of age and about 6 to 21% are under two years of age (32). *Salmonella paratyphi* A is now being encountered in many cases of blood stream infections especially in Asia (33). The infectious dose of *Salmonella typhi* is $10^6$ organisms but varies with gastric acidity. The risk of infection increases as the number of bacteria ingested increases. In Pakistan, factors that have contributed towards increased incidence of typhoidal diseases include eating ice-cream from vendors, drinking contaminated water at worksite and eating food from vendors. *Salmonella typhi* alone is said to cause 12-21 million infections annually and about 700,000 deaths (34;35). The incidence of Salmonella infection in AIDS patients is 20-100 times more than in the general population (36).

1.1.4 Non-typhoidal salmonellae

Non-typhoidal salmonellae are mostly acquired through ingestion of contaminated food of animal origin especially undercooked meat, eggs, poultry
products or dairy products contaminated with animal waste. *Salmonella enteritidis* and *Salmonella typhimurium* have been reported to be more important causes of blood stream infection than *Salmonella typhi* and *paratyphi* in Africa (37). *Salmonella enteritidis* has been associated with eating turkey and chicken (38). *Salmonella enteritidis* is now the commonest Salmonella isolated from humans in Europe (39). Because of increased resistance of Campylobacter and *Salmonella enteritidis* to nalidixic acid, FDA had banned used of fluoroquinolones in poultry feed in 2005. S. *enteritidis* is mostly associated with food poisoning. However, rare complications are spinal meningitis, septicemia and subcutaneous abscess (40). Its reservoir is the poultry and its meat and eggs are said to be its important mode of spread to humans (41).

There are more than 2500 serotypes of *Salmonella enterica* but serotype *typhimurium* is number one cause of non-typhoidal salmonellosis worldwide (42). It is mostly associated with consumption of food contaminated with waste from animal origin. Ciprofloxacin remains the drug of choice for severe *Salmonella typhimurium* infections in adults (43). Restraint should be exercised in its use to prevent resistance. In United States non-typhoidal salmonellae cause 15000 admissions and more than 400 deaths annually (44). *Salmonella Choleraesuis* causes infection mainly in pigs. It also rarely causes gastroenteritis in humans, after contact with animals, especially in immunocompromised patients (45). *Salmonella Choleraesuis* is commonly involved in metastatic infections. There is evidence of genotypically similar Salmonella Choleraesuis in pigs and humans, hence transmission (46). Multi-drug resistance (resistance to cotrimoxazole,
ampicillin and chloramphenicol) has been reported in non-typhoidal salmonellae (47;48). Hence, the treatment options for these MDR strains are fluoroquinolones or third generation cephalosporins. However, generally the gastroenteritis caused by them is self-limiting and treatment is not required. Moreover, the duration of fever and diarrhea is not significantly affected by antibiotics. Hence, in case of NTS, antibiotic resistance is not important in immunocompetent. However, immunocompromised patients are prone to systemic infections, which require antibiotic therapy. These are the cases, in which, antibiotic resistance makes impact in therapy. Antibiotic therapy entails the risk of side effects, increased antibiotic resistance and increased relapse rate. There is also a danger of prolonging carrier state while treating them with antibiotics (49). Fluid and electrolyte replacement is the mainstay of therapy. In neonates or in people with chronic disease who are at risk for septicemia or disseminated abscess, antibiotic therapy is indicated.

Rare cases of extra-intestinal spread, causing meningitis, osteomyelitis and bacteremia have been reported (50). Antibiotic therapy is however, indicated in immunocompromised, elderly and children or in systemic infections (51). Ampicillin, cotrimoxazole and chloramphenicol have traditionally been used to treat these infections. However, reports of resistance to these antibiotics is disturbing (52). This led to the use of ciprofloxacin to treat non-typhoidal salmonellae. Initially it was thought that resistance to quinolones will not develop because MICs of Salmonellae were very low and because tissue concentrations
achieved were high. But, resistance to ciprofloxacin has also been reported since 1990 (53;54).

In developed countries the invasive infections due to non-typhoidal salmonella is rarely seen, due to better sanitary conditions. However, they are endemic in sub-Saharan Africa (55). Routine antibiotic sensitivity of non-typhoidal salmonellae is not-required and is to be discouraged as it may prompt clinicians to unnecessarily treat these cases.

About 2-8% of NTS lead to bacteremia and out of these 8%, about 7% cause localized infections. The extraintestinal infections include hepatic or splenic abscess, meningitis, brain abscess, pneumonia, lung abscess, empyema, cystitis, pyelonephritis, osteomyelitis and septic arthritis (56).

Another common non-typhoidal salmonella is *Salmonella* Virchow. It is transmitted through poultry especially chicken (57).

**1.1.5 Pathogenesis**

Mostly the salmonella infections are acquired by faeco-oral rout by ingestion of food or water. The infective dose varies widely depending on the species and is generally $10^4$ to $10^6$ bacteria. In contrast, the infective dose for Shigella sp. Is small, usually 10-100 only. Once the Salmonella *typhi* and *paratyphi* are in the intestine they penetrate the mucosa and traverse the peyer's patches. They are then phagocytosed by macrophages but survive inside them. Survival and growth of the bacterium within the phagosome is a striking feature. Although in enteric fever there is mononuclear infiltration into the intestinal mucosa, in non-typhoidal salmonellae there is infiltration of polymorphs into large as well as small intestine.
Convalescent patients may continue to shed bacteria in urine or faeces even after clinical cure. Sometimes asymptomatic persons may be harboring salmonella knowingly or unknowingly. They may continue to excrete salmonella for weeks or months or even for life-time.
Chapter 2

1.2 Future Trends

Genome sequence of *Salmonella typhi* and *paratyphi* A denotes that both bacteria have similar origin, are strictly human pathogens and can be controlled if measures are taken seriously (58). New drugs preferably oral, and cheap are required to address the problem of resistance. Vaccine effective against *Salmonella paratyphi* A, that is useful for children, is under development.

1.2.1 Vaccines/Prevention against Salmonella

An attack of typhoid fever does not provide long-lasting immunity from future episodes of the same illness. Early treatment can also reduce development of immunity (59). Typhoid vaccine should be administered at least four weeks after full recovery from illness (60). Two safe and effective vaccines are available: the live attenuated oral vaccine Ty21a, and injectable Vi polysaccharide vaccine (61). Protection begins seven days after Vi polysaccharide vaccine with maximum protection attained after 28 days. It confers 55% to 72% immunity (62).

No vaccine against typhoid fever is available commercially for children under two years of age. Ty21A should not be given under 6 years (63). However, a new vaccine that is basically Vi antigen conjugated to a carrier protein that is safe for children also is under development. Since MDRTF is being reported in infants and toddlers, an effective and safe vaccine effective against *S. typhi* and *paratyphi* A in children under two years of age is needed and is still undergoing clinical trials (61). Typhoid vaccine should be introduced into the national immunization programme in typhoid-endemic areas (61). As the number of
infections due to *Salmonella paratyphi* A is increasing, a potent vaccine effective against it is urgently needed. The relative increase in paratyphi A cases may be due to decrease in *Salmonella typhi* cases due to vaccination.

About 1-5% of enteric fever cases become carriers. The carrier rate is more in ladies, especially those with gallstones or gallbladder disease. Carriers should be detected by stool culture especially in food handlers. Hand washing before food handling, proper cooking of meat, eggs and poultry and pasteurization of milk are measures other than the vaccination. Carriers are treated for 4-6 weeks with oral ampicillin, co-trimoxazole or ciprofloxacin. In cases where there is some anatomic abnormality like kidney or gall stones, surgical intervention is also required. All cases of enteric fever should be monitored for carriage and treated accordingly, if indicated.

### 1.2.2 Breakpoints

Before starting clinical trials of new antibiotics, their in vitro efficacy should be measured against the disease causing bugs. Next step is the measurement of breakpoints with the help of clinical correlations of the in vitro efficacy. The in vitro effects are then categorized into Sensitive, Intermediate and Susceptible. In USA it is done by FDA or CLSI. In Europe the task is traditionally with EUCAST. This study will be the first step towards establishment of breakpoints for *Salmonella* spp. for several new drugs.
Chapter 3

1.3 Antibiotic resistance in Salmonella

1.3.1 Historical Perspective Initially, the name typhoid fever was given because of its similarity with typhus fever. In 1800s, the term typhoid fever was defined because of the involvement of intestine, peyer’s patches and mesenteric lymph nodes. In 1869 the term enteric fever was coined basically because it mainly involves intestines (64).

In 1948, when chloramphenicol was discovered, it was commonly used and was the most effective drug for typhoid fever (65). Within two years, due to its extensive and indiscriminate use, chloramphenicol-resistant *Salmonella typhi* isolates were reported from England (65). Till start of 1970s chloramphenicol remained the mainstay of treatment for enteric fever (66). Outbreaks of chloramphenicol-resistant strains were reported from Mexico and India in 1972 (67;68). Similar reports were published from Vietnam in 1973 and from Korea in 1977 (69;70). Although there were intermittent reports of resistance, the efficacy of chloramphenicol remained reasonable until 1989, when there was rapid appearance of resistance. These strains became resistant to ampicillin also. Co-trimoxazole remained effective in treating these resistant strains until 1975, when resistance to it was reported in France. In 1989 multi-drug resistant strains, called MDR, resistant to chloramphenicol, ampicillin and co-trimoxazole appeared in India and Pakistan (71). Strains of *Salmonella typhi* resistant to all three first line drugs were reported in 1980s (23;72). The epidemic of MDRTF
forced paediatricians in the world to use ciprofloxacin, although there were concerns regarding its safety in children (23;34;73). Luckily, later studies proved them to be safe, effective and cheap (74).

Salmonella typhi strains resistant to fluoroquinolones were reported in 1992 from United Kingdom (72). Similar resistance was then reported from other countries (75-78). After the development of quinolone resistance, third generation cephalosporins is becoming popular as treatment but sporadic cases of resistance to them have also been reported (79).

Strains previously resistant to first-line drugs (co-trimoxazole, chloramphenicol, and ampicillin) are now showing decreasing resistance (62;80;81). The probable reason is the withdrawal of selective pressure (80).

In Kenya, till 1992, all the Salmonella isolates from blood cultures were susceptible to conventional antibiotics. MDR Salmonella was first reported in 1992. It increased to around 50% in 1997 and to 80% in 2008 (82).

1.3.2 Multidrug-resistane

Multidrug-resistant typhoid fever (MDRTF) is defined as typhoid fever caused by Salmonella typhi strains which are resistant to all the three first-line recommended drugs for treatment, i.e., chloramphenicol, ampicillin, and co-trimoxazole (TMP-SMX) (24;63;75;83;84). It was initially reported from Pakistan, India and Middle East but then quickly spread to other areas of the world. In last two decades, multidrug resistant (MDR) S. typhi strains have emerged and spread worldwide resulting in high rates of morbidity and mortality (63;79;85;86). Countries from Asia which have reported MDRTF include Pakistan, 1996 (14),
India 1988 (24), Indonesia 2009 (87), Malaysia 1991 (88), Nepal 2005 (89), Bangladesh 1994 (90), and Kuwait 1996 (91). MDRTF has also been reported from Africa like Egypt in 2006 (92), South Africa 1992 (2), Nigeria 2005(93) and Kenya 2000 (94). A study in Bangalore India showed MDRTF to be as high as 95% (95). A prospective study, done at three years intervals found that MDRTF incidence in Delhi increased from 34% in 1999 to 66% in 2005 (84). An outbreak of S. typhi MDR strain in Tajikistan in 1990 caused 24000 infections (16). Similar resistance has been reported from few advanced countries like Italy in 2000(96), Britain 1990 (14), and United States (97). Majority of these cases were from travellers returning from areas of high endemicity or where outbreaks had occurred.

The therapeutic alternatives available for use against ciprofloxacin-resistant enteric fever isolates in an endemic area are limited. It has been observed in some studies that as compared to the children infected by sensitive Salmonella typhi strains, children with MDRTF are sicker and more toxic (24;75;98). There are reports that few Salmonella are now resistant to both quinolones and third generation cephalosporins (99;100). It makes it mandatory, to search for new antibiotics effective against these resistant strains.

1.3.3 Outbreaks of Salmonella

An outbreak of resistant Salmonella typhi fever was reported in Mumbai in 1990 (101). In New Delhi similar outbreak was observed the same year (102). In both these outbreaks S. typhi strain was sensitive to ciprofloxacin. Outbreaks of MDR
*Salmonella typhi* appeared in Middle East and South Asia and then spread to Africa and Southeast Asia (7;103).

### 1.3.4 Resistance to Chloramphenicol

Use of chloramphenicol is limited because of its toxicity. Aplastic anaemia is rare but can occur after oral or intravenous administration. Gray baby syndrome and bone marrow suppression are other complications that can be avoided by appropriate dose and monitoring drug levels. Chloramphenicol was the pioneer drug in treatment of typhoid fever and reduced the death rate for typhoid fever from 20% to 1% and interval of fever from 14-28 days to 3-5 days (104). The concept of antibiotic recycling envisages the re-use of chloramphenicol in isolates resistant to other antibiotics (105). High rate of chronic carriers, bone marrow depression and high relapse rate hamper its use. Resistance of *Salmonella* to chloramphenicol began in India in 1961.

### 1.3.5 Resistance to Ciprofloxacin/Quinolones

Ciprofloxacin was considered to be the first choice for treatment of multidrug resistant typhoid fever because it had high cure rate and very low carrier or relapse rate (106). Its use started in late 1980s when *Salmonella* resistant to cotrimoxazole, penicillin and ampicillin emerged (107). It has special property to be concentrated ten times more intracellularly than extracellularly (15). Ofloxacin shares with ciprofloxacin, the anti-salmonella activity as well as intracellular concentration (108). Till 1990 the drug of choice for typhoid fever has been chloramphenicol, but then due to increased resistance, it was gradually replaced by ciprofloxacin (109). Ciprofloxacin resistance in *Salmonella typhi* in many
countries in Asia including Pakistan was almost absent till 2004 (110). Resistance to ciprofloxacin then, increased over the years (84;111;112). Widespread use of ciprofloxacin, for typhoid fever, for other infections, and in animal feed slowly led to increased MICs of *Salmonella enterica* serotype Typhi to ciprofloxacin, threatening its curative value (113;114). First case of therapeutic failure due to fluoroquinolone in Typhoid fever in Pakistan was reported in 1993 (115). In 2003, the Clinical Laboratory Standards Institute (CLSI) included testing for resistance to nalidixic acid (nalidixic acid-resistant *S.typhi* or NARST) as an indicator of reduced fluoroquinolone susceptibility in *Salmonella* spp. (105). It was noted that in NARST strains response to short course treatment was not adequate. Treatment failures were frequent with standard dosage schedules. Higher doses of ciprofloxacin or ofloxacin were suggested in these cases. Nalidixic acid resistance increased tremendously between 2003 and 2005 and has remained steady at around 80% since 2005. A study in 2008 confirmed that nalidixic acid resistance was greater than 50% both in Pakistan and India (4). Ciprofloxacin resistant cases were detected in relatively greater numbers from India and Pakistan where this disease is endemic or epidemic (116). It is suggested that to check reduced susceptibility to ciprofloxacin MIC is better indicator than nalidixic acid resistance (105). In one study strains sensitive to nalidixic acid but with increased MIC to ciprofloxacin were found and use of nalidixic acid as surrogate marker for ciprofloxacin resistance became doubtful. Revised breakpoints for ciprofloxacin were then
suggested (117). In one study, decreased ciprofloxacin susceptibility was associated with point mutations in the gyrA genes at codons 83 & 87 (118).

If there is no defervescence in 7 days after treatment with ciprofloxacin, then treatment failure is implicated (119). Outbreaks of isolates showing reduced susceptibility to fluoroquinolones (Nalidixic acid resistant typhoid) appeared in early 1990s in Vietnam (120) and Tajikistan (77) and then spread to other countries of Southeast Asia including India and Pakistan (121-124), Nepal (125) and Kuwait (126). Treatment of such cases with fluoroquinolones usually resulted in delayed response or treatment failure. Fluoroquinolone resistance these days is common in Bangladesh (127) and India (128).

In resource poor countries, increasing rate of isolation of multidrug resistant Salmonella with high MICs to fluoroquinolones remains a major health concern. High level ciprofloxacin resistant strains of Salmonella paratyphi A were reported from Japan in 2002 (129) and later from Bangladesh in 2006 (130). Isolated high level resistance to ciprofloxacin was noted in India in 2004 (131). Ciprofloxacin resistance in typhoid salmonella was rising quickly since 2010 (58).

The use of ciprofloxacin and other quinolones in veterinary e.g. for prophylaxis and in growth supplements has increased resistance in salmonella. The use of quinolones in animals raised for human-consumption, which started in 1990s, has been said to be one of the main causes of resistance in salmonella against quinolones (132). This development of resistance is used as an argument against use of fluoroquinolones in animal feeds.
At molecular level, single point mutation at QRDR level confers resistance at gyerA gene that results in modified DNA-gyrase. It results in low level resistance to quinolones (133). Additional mutations are required for ciprofloxacin resistance (132). Antibiotic efflux also plays a role in resistance but alone, is insufficient to cause resistance (134).

### 1.3.6 Resistance to Azithromycin

Azithromycin is said to be effective in nalidixic acid resistant and multidrug resistant cases but is still under evaluation (135). It has a special property of being concentrated in macrophages and neutrophils about 100 times more than in serum. Furthermore, it has the advantage of having long half-life reducing dosage schedule and improving compliance. With increasing use of ciprofloxacin and third generation cephalosporins, resistance is likely to spread against them as well. It has been estimated to be around 1% in cases with reduced ciprofloxacin susceptibility(47). Lack of interpretive criteria like antibiotic zones and MICs of azithromycin for Salmonella in CLSI makes it difficult to report in microbiology lab reports. The British Society of Antimicrobial Chemotherapy (BSAC) guidelines however recommend that an isolate with MIC for Azithromycin ≤16 µg/Ml is to be considered susceptible. Same was the criteria in our study.

Azithromycin has been used clinically in treatment of typhoid fevers and found successful (136-140). In 2009, azithromycin was found to be 100% sensitive by E-test and 83% sensitive by disk diffusion technique, against Salmonella and Shigella (141). E-test is approved by Food and Drug Administration USA (142). In one study *Salmonella Choleraesuis* was found to be resistant to azithromycin
Azithromycin resistance in *Salmonella paratyphi* A has already been reported but remains rare (143).

### 1.3.7 Resistance to Ceftriaxone

These days third generation cephalosporins are the mainstay of treatment in typhoid fever especially in ciprofloxacin (quinolone) resistant isolates. Third generation cephalosporins are considered inferior to fluoroquinolones as far as defervescence time, relapse and clinical outcome are concerned (79). Ceftriaxone treated cases have defervescence time of more than 7 days and 10-20% chances of treatment failure and faecal carriage of 2-3% (144). Reports of resistance to ceftriaxone have come up recently from India (21;84) and from Bangladesh (145). If this resistance spreads, it would leave very few options for treatment since most of them are resistant to ciprofloxacin as well. Mechanism of resistance is either ESBL or AmpC. Despite in vitro sensitivity, first and second generation cephalosporins, tetracyclines and aminoglycosides are not effective in vivo.

### 1.3.8 Factors for resistance

In developing countries, local production of antimicrobials with questionable quality and potency control, coupled with poor compliance of patients to costly antimicrobials adds to the threat of antimicrobial resistance (146). Antimicrobials are frequently prescribed for infections of viral etiology such as diarrhea, common cold and cough, which otherwise can be resolved by the defense system of the body. Stress is on treatment and not on finding the etiology or
proper diagnosis. This leads to patients being treated with broad spectrum antibiotics, which results in the emergence of MDR organisms (147).

1.3.9 Risks for resistance

Approximately 40% of the antibiotics manufactured in United States are used in animal feed. The reason is to prevent infectious diseases in farm animals. These infections, could lead to huge economic losses for farmers and ranchers. The problem is that when antibiotic are fed to an animal, the antibiotics kill indigenous microflora that are susceptible to the antibiotics. But, the bacteria resistant to the antibiotics survive. Having less competition now, for space and nutrition, these drug resistant organisms multiply and become the predominant organisms of the animal’s indigenous microflora. These drug resistant organisms are then transmitted in the animal’s feces or food products (e.g. eggs, milk, meat) obtained from the animal. Many multidrug-resistant Salmonella strains, strains that cause disease in animals and humans, developed in this manner. The use of antibiotic–containing animal feed is quite controversial. Microbiologist concerned about ever-increasing number of drug-resistant bacteria are currently attempting to eliminate or drastically reduce the practice of adding antibiotics to animal feed (148). Plasmid mediated resistance is leading to rapid emergence of resistance in bacteria. With the passage of time, highly resistant bacteria are likely to emerge.
Chapter 4

1.4 Mechanisms of drug resistance in Salmonellae

There are two main mechanisms of drug resistance in salmonellae

1.4.1 Plasmid mediated

Plasmids are extra-chromosomal, self-replicating circular pieces of DNA which can carry and transfer multiple resistance genes between bacteria (149). Risk factors for the development of resistance in Salmonella typhi include overuse, misuse and inappropriate antibiotic prescribing practices (23;150;151). Patient and time pressures and diagnostic uncertainties lead to irrational use of antibiotics (146). R-plasmid mediated antibiotic resistance of enteric bacteria also referred to as multiple antibiotic resistance is known since its discovery in Japan in 1959. R-plasmid is an extrachromosomal element which carries genes determining resistance to one or more antibiotics. All the resistances borne by an R plasmid can be transmitted from one bacterium to another by cell contact or conjugation. The problem of multiple antibiotic resistance has now acquired worldwide significance.

1.4.2 Chromosomal DNA mediated resistance

Quinolones act at different places in gram positive and gram negative organisms. In gram negative it acts at DNA gyrase and in gram positive it acts at topoisomerase IV. Due to the uncontrolled use of fluoroquinolones, chromosomal-mediated drug resistance against them has emerged as a result of selective pressure on bacterial population. It has been attributed to a single point
mutation in the quinolone resistance determining region (QRDR) of the topoisomerase gene gyre A, which encodes DNA gyrase. However, parC topoisomerase gene mutation also plays role (15;152;153). Decreased permeability and active efflux of antibiotics are other mechanisms that may be involved (153).

1.4.3 Gatifloxacin

Gatifloxacin is an oral fluoroquinolone effective against Salmonellae. Its mechanism of action differs from other fluoroquinolones because it binds at a different site (154). However, it was not included in our study because of its reduced use mainly due to its potential side effect, dysglycemia in diabetics and elderly which may limit its widespread use.
Chapter 5

1.5 Multi drug resistant Typhoid Fever

1.5.1 Clinical Features

Enteric (typhoid) fever is a systemic disease characterized by high grade fever, constipation or diarrhea and abdominal pain and caused by dissemination of the bacteria S. *typhi* or S. *paratyphi*. The disease name is derived from its clinical similarity to typhus. However, in the early 1800s, typhoid fever was clearly defined pathologically as an exceptional illness due to its association with mesenteric lymph nodes and enlarged Peyer’s patches. In 1869, because of the anatomic site of infection, the term enteric fever was planned as an alternative name to differentiate typhoid fever from typhus. However, till today, the two names are used interchangeably.

1.5.1.1 Importance

Patients with MDRTF have longer duration of fever defervescence (8± 5) days as compared to those with drug-susceptible typhoid fever (5.7 ± 4) days (155). Therapeutic choices also become limited in these cases. Quinolones have remained the mainstay of treatment, but resistance to them also emerged rapidly.

1.5.2 Laboratory Detection

Clot cultures have not been found to be of superior sensitivity as compared to blood cultures in several clinical studies and hence is not recommended (156). Factors which result in failure to isolate organisms from blood culture are inadequate laboratory media, prior use of antibiotics, inadequate quantity of
blood added, time of blood collection and incubation condition (75;98;156). Bone marrow culture is better than blood culture, for(156).

Flagellin gene (fliC-d) for Salmonella *typhi* detection is used for the isolation of enteric fever pathogens. It is because of larger number of bacteria in bone marrow, that may be protected from effects of antibiotics (157;158). The sensitivity of stool cultures is about 33% and of urine is around 9%. Specific gene can be detected by PCR for diagnosis (159). Multiplex PCR assay is useful in detecting clinically relevant antibiotic resistance genes. Nested PCR can detect specific gene sequence of Salmonella *typhi* and may become a new gold standard (160;161).

Serological tests do not give any information regarding antibiotic susceptibility and hence are not useful in the diagnosis of MDRTF (156). They have poor sensitivity and specificity for diagnosis of Salmonella infections as well

### 1.5.3 Therapy

Some important criteria for the selection of antibiotics in the treatment of MDRTF in the developing countries are susceptibility patterns, prevalence of antimicrobial resistance and the cost. As per WHO guidelines, either third generation cephalosporins or fluoroquinolones can be used in MDRTF, depending on the sensitivity of *S. typhi* strains to quinolones (62). In quinolone sensitive MDR strains, quinolones are considered better because of lower cost, shorter duration of therapy, availability of oral preparation and higher cure rates (156). In cases of quinolone resistance, third-generation cephalosporins are recommended as treatment (62). Amongst oral cephalosporins, cefixime and cefpodoxime are
generally used, while cefotaxime and ceftriaxone are given in two or three parenteral doses (62). These alternative regimens have several disadvantages such as expense, the intravenous route of administration, prolonged defervescence time and treatment failure (130;162).

1.5.4 MDR carriers

Ciprofloxacin (15mg/Kg/day in two divided doses) for four to six weeks is used for the treatment of carriers of quinolone-sensitive multi-resistant strains (63). They remain a potential threat to the community, especially the gall bladder carriers. They keep on excreting the bacteria in feces for many years
1.6 Material and Methods

1.6.1 Study design:

It is a cross sectional research carried out at Department of Microbiology Armed Forces Institute of Pathology Rawalpindi. Research was conducted during June 2011 to May 2013 after approval of the research ethic committee of the institute.

1.6.2 Collection of Clinical material

Proper collection of blood was ensured especially with regard to adequate amount of blood added in blood culture bottle (10 ml for adults and 1-6 ml for kids as per age). Due attention was given to disinfection of skin also. Blood cultures from different wards of the hospital received in the laboratory were incubated in BACTEC 9050 blood culture system. Other specimens like stool, pus & urine were also collected according to the standard protocols (163).

1.6.3 Isolation of etiological agent from clinical material

Positive blood culture bottles were sub cultured on blood and MacConkey agar, after gram stain findings. Only one isolate per patient was included in the study. For stool specimens, enrichment was done in Selenite broth.

1.6.4 Identification of etiologic agent

a) Cultural characteristics

Non-lactose fermenting colonies growing on MacConkey agar or red/transparent colonies on XLD agar were identified by standard biochemical and serological tests (164). All Salmonellae isolated from clinical specimens at AFIP and Army medical college Rawalpindi from June 2011 to June 2013 were
included in the study. Samples were taken from CMH Peshawar as well. Only one isolate per patient (e.g. first strain after admission) was included in the study. Track record showed that age of the patients ranged from less than 1 to 75 years of age (median age 19 years). The male to female ratio was 9:1.

b) Microscopic morphology

Gram stain was performed on nonlactose fermenting colonies from MacConkey agar or XLD agar. Oxidase negative, Gram negative rods were dealt with further, and their motility was observed.

c) Biochemical reaction

The isolates were identified using phenotypic colony characteristics and confirmed by biochemical reactions with API 20 E (bioMerieux SA, Marcy l’Etoile, France).

d) Serotyping

Serotyping was done with specific antisera using polyclonal and monoclonal O, H and Vi antisera (Bio-Rad, Marnes-la-Coquette, France) according to the Kauffmann-White classification scheme (165). *Salmonella enterica* serotype Typhi was suspected when the isolate was nonlactose fermenter with little hydrogen sulphide production. It was confirmed when it showed agglutination with serogroup D, somatic antigen 09 and flagellar antigen Hd. Polysaccharide Vi antigen was also tested. *Salmonella paratyphi* A was suspected when the gram negative rod was positive for glucose, arabinose and ODC but negative for LDC, citrate, urease, H$_2$S and indole tests. Confirmation was done on serology.
1.6.5 Determination of resistance of isolates to conventional antibiotics

The isolates were also tested for resistance to conventional antibiotics ampicillin, cotrimoxazole, ciprofloxacin, ceftriaxone, doripenem, imipenem, ertapenem, aztreonam, moxifloxacin, cefpirome, cefepime, gatifloxacin, and chloramphenicol by Kirby-Bauer disc diffusion method. Inoculum equivalent to 0.5 McFarland turbidity was used.

The disks of antimicrobial drugs used were chloramphenicol (30 µg), cotrimoxazole (1.25/23.75 µg), ampicillin (10 µg), ciprofloxacin (5 µg), and ceftriaxone (30 µg) doripenem (10 µg), imipenem (10 µg), ertapenem (10 µg), aztreonam (30 µg), moxifloxacin (?? µg), cefpirome (30 µg), cefepime (30 µg), gatifloxacin (5 µg), chloramphenicol (30 µg) and nalidixic acid (30 µg). All disks were of Oxoid company. The inoculated agar plates containing the suitable antibiotic discs were incubated for 16-18 hrs at 36°C and inhibitory zone diameters obtained around the antibiotic discs were measured. The results were interpreted following those for Enterobacteriaceae or salmonella in Clinical Laboratory Standards Institutes guidelines (166).

1.6.6 MIC determination

All isolates that were MDR or were intermediate or resistant to ciprofloxacin on disk diffusion were subjected to MIC test using E-test strip (AB Biodisk, Solna, Sweden). The Etest was first validated with broth dilution MIC using cation adjusted Muller–Hinton broth for imipenem, aztreonam and cefpodoxime. The same 0.5 McFarland organism suspension of the isolates was used with Mueller-Hinton agar (Oxoid, Hampshire, UK) and incubated under similar conditions, and
according to the manufacturer’s instructions. The antibiotics tested for MICs were imipenem, cefpirome, aztreonam, cefpodoxime, azithromycin and tigecycline. *Escherichia coli* ATCC 25922 was used as control for the disk diffusion and MIC testing. The results were interpreted following Clinical Laboratory Standards Institutes guidelines (167). Isolates that were resistant to Ampicillin, cotrimoxazole and Chloramphenicol were declared MDR (multi-drug resistant) isolates. Verification studies of E-strips were carried out as and when required. Based on recommendations of EUCAST (www.eucast.org) and one previous study (168) the cutoff for Azithromycin was taken to be ≥32 µg/ml. Isolates were preserved at -45 / -60°C in nutrient agar with glycerol. To check the relationship between zone diameter in mm and MIC in mg/L, the two variables in our study, we used the Statistical Package for Social Sciences (SPSS) version 15 (IBM Chicago, Illinois, USA).

As human subjects were not directly involved and no intervention was made, hence formal permission by ethical review committee was not sought.
Results & Discussion
1.7 Results

A total of 316 isolates were recovered from 2230 specimens. Out of 316 isolates 186 (59%) isolates were from blood culture, 63 (20%) isolates from stool, 21 (6.7%) from urine, 41 (13%) isolates from pus and 4 (1.3%) were from fluids. They belonged to 12 different districts, all from north/north-west of the country but majority were from Rawalpindi. Highest number of culture positive cases 135 (51%) were between 8 and 17 years. Total number of cases less than 8 years of age were 22 (8.3%) while total cases more than 17 years age were 108 (41%). Gender was known for 210 (79%) isolates. Out of these 143 (54)% were males and the rest were females.

Table 1: Frequency and percentage of species of Salmonellae isolated.

<table>
<thead>
<tr>
<th>Organism isolated</th>
<th>Frequency</th>
<th>Percentage isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella typhi</em></td>
<td>128</td>
<td>40.5</td>
</tr>
<tr>
<td><em>Salmonella paratyphi A</em></td>
<td>111</td>
<td>35.1</td>
</tr>
<tr>
<td><em>Salmonella paratyphi B</em></td>
<td>16</td>
<td>5.1</td>
</tr>
<tr>
<td><em>Salmonella enteritidis</em></td>
<td>26</td>
<td>8.2</td>
</tr>
<tr>
<td><em>Salmonella infantis</em></td>
<td>18</td>
<td>5.7</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td>17</td>
<td>5.4</td>
</tr>
<tr>
<td><strong>Total Isolates</strong></td>
<td><strong>316</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>

Of 128 isolates of *Salmonella enterica* serovar Typhi (S. *typhi*) 6 (4.7 %) demonstrated resistance to ciprofloxacin (5µg), 86 (66.6 %) were intermediate and 36 (28.2%) isolates were sensitive on screening using the Kirby-Bauer disc
diffusion method as shown in Table 2. Out of 111 isolates of *Salmonella enterica* serotype paratyphi A, 70 (63%) were intermediate, 34 (30.6%) were sensitive while 7 (6.3%) were resistant to ciprofloxacin using the Kirby-Bauer disc diffusion method. All 26 isolates of *Salmonella enteritidis* (100%) were sensitive to ciprofloxacin. Out of 18 isolates of *Salmonella infantis* 16 were sensitive while 4 were intermediate.

For chloramphenicol resistance out of 128 isolates of *Salmonella enterica* serovar Typhi (S. *typhi*) 53 (41.6 %) demonstrated resistance to chloramphenicol (30 µg). Out of 111 isolates of *Salmonella enterica* serotype Paratyphi A, 66 (59%) isolates were sensitive, 44 (40%) isolates were resistant while only one was intermediate using the Kirby-Bauer disc diffusion method.

For cotrimoxazole resistance out of 128 isolates of *Salmonella enterica* serovar Typhi (S. *typhi*) 49 (38%) demonstrated resistance to cotrimoxazole (30 µg). Out of 111 isolates of *Salmonella enterica* serotype Paratyphi A, 68 (61%) isolates were sensitive, 42 (38%) isolates were resistant while only one was intermediate using the Kirby-Bauer disc diffusion method as shown in Figure 1.

For ceftriaxone resistance out of 128 isolates of *Salmonella enterica* serovar Typhi (S. *typhi*) only four (3 %) demonstrated resistance to ceftriaxone (30 µg). Out of 111 isolates of *Salmonella enterica* serotype Paratyphi A, 9 (8.3 %) isolates were resistant while all the rest were sensitive. No resistance was noted in non-typhoidal salmonellae (Table 2).

For cefpodoxime resistance out of 128 isolates of *Salmonella enterica* serovar Typhi (S. *typhi*) only 6 (4.7 %) demonstrated resistance to cefpodoxime
(10 µg) while 4 (3.1%) isolates were intermediate, rest were sensitive. Out of 111 isolates of Salmonella enterica serotype Paratyphi A, 6 isolates (5.5 %) were resistant, while 8 (8.8%) were intermediate. Rest were sensitive.

For doripenem resistance, out of 128 isolates of Salmonella enterica serovar Typhi (S. typhi) only 3 (2.3 %) demonstrated resistance to doripenem (10 µg) while only one isolate was intermediate, rest were sensitive. Out of 111 isolates of Salmonella enterica serotype Paratyphi A, only one isolate was intermediate rest all were sensitive.

For imipenem resistance out of 128 isolates of Salmonella enterica serovar Typhi (S. typhi) 17 (13.3 %) isolates demonstrated intermediate resistance to imipenem (10 µg) while rest were sensitive. Out of 111 isolates of Salmonella enterica serotype Paratyphi A, only 3 (2.8%) isolate were resistant while 8 (7.2 %) showed intermediate resistance, rest all were sensitive. Out of 26, six isolates (21.4%) of Salmonella enteritidis showed intermediate resistance. Other non-typhoidal salmonellae were sensitive.

For aztreonam resistance out of 128 isolates of Salmonella enterica serovar Typhi (S. typhi) only 5 (3.9 %) demonstrated resistance to aztreonam (30 µg) while 11 isolate (8.6%) were intermediate, rest were sensitive. Out of 111 isolates of Salmonella enterica serotype Paratyphi A, 8 (7.2%) isolate were resistant while 3 (2.8%) were intermediate rest all were sensitive. Out of 18, two isolates of Salmonella infantis were resistant. Rest of non-typhoidal salmonellae were sensitive.
For Cefepime resistance out of 128 isolates of *Salmonella enterica* serovar Typhi (*S. typhi*) only 2 (1.5 %) demonstrated resistance to cefepime (30 µg) while rest were sensitive. Out of 111 isolates of Salmonella *enterica* serotype Paratyphi A, only one (0.9%) isolate was resistant, rest all were sensitive (Figure 2). All non-typhoidal salmonellae were sensitive (Non-typhoid salmonellae Table 2).

For gatifloxacin resistance out of 128 isolates of *Salmonella enterica* serovar Typhi (*S. typhi*) only one (0.8 %) demonstrated resistance to gatifloxacin (5 µg) while rest were sensitive. Out of 111 isolates of *Salmonella enterica* serotype paratyphi A, only 2 (1.8%) isolates were resistant, rest all were sensitive as shown in figure 2. All non-typhoidal salmonellae were sensitive.

For azithromycin, breakpoints were available from BSAC for *Salmonella enterica* serovar Typhi (*S. Typhi*) only. Out of 128 isolates 120 (93.7%) were resistant, only eight (6.2%) were sensitive.

All isolates that were either MDR (resistant to ampicillin, chloramphenicol and co-trimoxazole) or were ciprofloxacin intermediate or resistant on disk diffusion technique were subjected to MIC test for the antibiotics for which E-strips were available. E-strip or Etest are the based on the diffusion of a stable concentration gradient of antibiotic from a plastic strip onto the agar medium.
As far as the MIC is concerned, in MIC for imipenem all isolates were sensitive.

For aztreonam MIC, in *Salmonella typhi* out of 120 isolates 6 (5%) isolates were intermediate while 3 (2.5%) were resistant. In *Salmonella enterica* serotype Paratyphi A (*Salmonella paratyphi A*) out of 96 isolates 6 (6.2%) were resistant and 18 (18.7%) were intermediate. (Figure 4 & 5)

For cefpodoxime MIC, in *Salmonella typhi* out of 120 isolates only 5 (4.1%) were resistant. In *Salmonella enterica* serotype Paratyphi A (*Salmonella paratyphi A*) out of 96 isolates 9 (9.3%) were resistant.

For azithromycin MIC (169) in *Salmonella enterica* serotype Typhi out of 120 isolates only 12 (10%) were resistant. In *Salmonella enterica* serotype Paratyphi A (*Salmonella paratyphi A*) out of 96 isolates 6 (6.2%) were resistant.

For Cefpirome and Tigecycline no MIC breakpoints were available in CLSI. Interpretation of tigecycline MIC results was determined according to the recommendations of the United States Food and Drug Administration (U. S. FDA) given in the package insert for treating Enterobacteriaceae (susceptible, \( \leq 2 \) g/ml; 4 = Intermediate, resistant = 8 g/ml) and those recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (susceptible = 1 g/ml; resistant, 2 g/ml). According to both criteria all the isolates were sensitive.

For Cefpirome no previous breakpoints could be found. The range of MIC was from 0.047 to 0.75. for both *S. typhi* and *S. paratyphi A*. 
Table 2. Resistance percentage of Salmonellae to various antibiotics by Disk diffusion method.  
I= Intermediate, R= Resistant, S= Sensitive

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>S. typhi n = 128</th>
<th>S. paratyphi A n = 111</th>
<th>NTS Non-typhoid Salmonellae n = 61</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>Ampicillin 30µg</td>
<td>12%</td>
<td>72%</td>
<td>16%</td>
</tr>
<tr>
<td>Chloramphenicol 30µg</td>
<td>0%</td>
<td>42%</td>
<td>58%</td>
</tr>
<tr>
<td>Cotrimoxazole 30µg</td>
<td>1.4%</td>
<td>38%</td>
<td>60.6%</td>
</tr>
<tr>
<td>Ceftriaxone 30µg</td>
<td>0%</td>
<td>3%</td>
<td>97%</td>
</tr>
<tr>
<td>Ciprofloxacin 5µg</td>
<td>67%</td>
<td>5%</td>
<td>28%</td>
</tr>
<tr>
<td>Cefpodoxime 10µg</td>
<td>3.1%</td>
<td>4.7%</td>
<td>92.2%</td>
</tr>
<tr>
<td>Doripenem 10µg</td>
<td>1.2%</td>
<td>2.3%</td>
<td>96.5%</td>
</tr>
<tr>
<td>Imipenem 10µg</td>
<td>13.3%</td>
<td>0%</td>
<td>86.7%</td>
</tr>
<tr>
<td>Ertapenem 10µg</td>
<td>3.5%</td>
<td>3.5%</td>
<td>93%</td>
</tr>
<tr>
<td>Aztreonam 30µg</td>
<td>8.6%</td>
<td>3.9%</td>
<td>87.5%</td>
</tr>
<tr>
<td>Moxifloxacin 10µg</td>
<td>14.1%</td>
<td>3.5%</td>
<td>82.4%</td>
</tr>
<tr>
<td>Cefepime 30µg</td>
<td>0%</td>
<td>1.5%</td>
<td>98.5%</td>
</tr>
<tr>
<td>Gatifloxacin 5µg</td>
<td>0%</td>
<td>0.8%</td>
<td>99.2%</td>
</tr>
</tbody>
</table>

Of the 56 multidrug resistant isolates (resistant to ampicillin, chloramphenicol and co-trimoxazole), 50 (89.2 %) were *Salmonella enterica*
serotype Typhi \( (S. \text{typhi}) \) and 6 \( (10.7\%) \) were \textit{Salmonella enterica} serotype Paratyphi \( (S. \text{paratyphi}) \) A. Amongst \( S. \text{typhi} \) 39% were MDR, while 5.4% of \( S. \text{paratyphi} \) A were MDR. (Table 2)

**Figure 1.** Percent Susceptibility pattern of \( S. \text{typhi} \) isolates by disk diffusion technique. \( (n = 128) \)
**Figure 2.** Percent Susceptibility pattern of *S. paratyphi* isolates by disk diffusion technique. (n = 111)
Figure 3. Percent susceptibility pattern of Non-Typhoidal Salmonellae isolates by disk diffusion technique, (n = 61) as in Table 2.
### Table 3: MIC of Antibiotics for *Salmonella typhi*:

<table>
<thead>
<tr>
<th>MIC value (µg/ml)</th>
<th>No. of isolates for each antimicrobial in <em>Salmonella typhi</em> n = 120</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Imipenem</td>
</tr>
<tr>
<td>≤ 0.125</td>
<td>28</td>
</tr>
<tr>
<td>≤0.25</td>
<td>47</td>
</tr>
<tr>
<td>≤0.5</td>
<td>45</td>
</tr>
<tr>
<td>≤1</td>
<td>0</td>
</tr>
<tr>
<td>≤2</td>
<td>0</td>
</tr>
<tr>
<td>≤4</td>
<td>0</td>
</tr>
<tr>
<td>≤8</td>
<td>0</td>
</tr>
<tr>
<td>≤16</td>
<td>0</td>
</tr>
<tr>
<td>≤32</td>
<td>--</td>
</tr>
<tr>
<td>≤64</td>
<td>0</td>
</tr>
<tr>
<td>Resistance</td>
<td>0</td>
</tr>
<tr>
<td>MIC90</td>
<td>0.38</td>
</tr>
<tr>
<td>MIC50</td>
<td>0.25</td>
</tr>
<tr>
<td>Range</td>
<td>0.04-0.5</td>
</tr>
</tbody>
</table>
Figure 4. MIC distribution of antibiotics for *Salmonella typhi*. Y-axis shows the number of isolates.

Table 4: MICs of antibiotics for *Salmonella. paratyphi* A.

<table>
<thead>
<tr>
<th>MIC value</th>
<th>Imipenem</th>
<th>Cefpirome</th>
<th>Cefpodoxime</th>
<th>Aztreonam</th>
<th>Tigecycline</th>
<th>Azithromycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤0.064</td>
<td>0</td>
<td>9</td>
<td>0</td>
<td>12</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>≤0.094</td>
<td>0</td>
<td>47</td>
<td>0</td>
<td>27</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>≤0.125</td>
<td>3</td>
<td>28</td>
<td>0</td>
<td>16</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>≤0.25</td>
<td>48</td>
<td>3</td>
<td>0</td>
<td>8</td>
<td>48</td>
<td>1</td>
</tr>
<tr>
<td>≤0.5</td>
<td>30</td>
<td>3</td>
<td>5</td>
<td>0</td>
<td>39</td>
<td>0</td>
</tr>
<tr>
<td>≤1</td>
<td>15</td>
<td>3</td>
<td>63</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>≤2</td>
<td>0</td>
<td>3</td>
<td>22</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>≤4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td></td>
</tr>
<tr>
<td>≤8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>≤16</td>
<td>3</td>
<td>18</td>
<td>3</td>
<td>3</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>≤32</td>
<td>3</td>
<td>3</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤64</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤128</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤256</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>96</td>
<td>96</td>
<td>96</td>
<td>96</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>MIC50</td>
<td>0.25</td>
<td>0.094</td>
<td>1</td>
<td>0.125</td>
<td>0.25</td>
<td>8</td>
</tr>
<tr>
<td>MIC90</td>
<td>0.75</td>
<td>0.5</td>
<td>2</td>
<td>12</td>
<td>0.38</td>
<td>16</td>
</tr>
<tr>
<td>MIC</td>
<td>0.19 – 0.06-2</td>
<td>0.5-32</td>
<td>0.047-32</td>
<td>0.094- 0.016-24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resistance</td>
<td>0</td>
<td>--</td>
<td>--</td>
<td>20</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

**Figure 5.** MIC of antibiotics for *Salmonella paratyphi* A. Y-axis shows the number of isolates as in Table 4.
**Table 5**: Frequency of Salmonella species isolated from clinical material.

<table>
<thead>
<tr>
<th>Clinical material</th>
<th>S. typhi</th>
<th>S. paratyphi A</th>
<th>S. paratyphi B</th>
<th>S. enteritidis</th>
<th>S. infantis</th>
<th>S. typhimurium</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>92</td>
<td>86</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>187</td>
</tr>
<tr>
<td>Pus</td>
<td>24</td>
<td>15</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>41</td>
</tr>
<tr>
<td>Stool</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>26</td>
<td>18</td>
<td>17</td>
<td>63</td>
</tr>
<tr>
<td>Urine</td>
<td>9</td>
<td>7</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>Fluids</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>128</td>
<td>111</td>
<td>16</td>
<td>26</td>
<td>18</td>
<td>17</td>
<td>316</td>
</tr>
</tbody>
</table>
Amongst all the serotypes maximum resistance was noted in *Salmonella typhi*. It was 38% resistant to chloramphenicol, 72% resistant to ampicillin, and 41.2% resistant to cotrimoxazole. *Salmonella paratyphi A* was found to be less resistant. It was 40% resistant to chloramphenicol, 60% resistant to ampicillin, and 38% resistant to cotrimoxazole. It was only 8% resistant to ciprofloxacin. However, 63% of the isolates were intermediate.

Amongst the non-typhi serotypes, the most common isolate was *Salmonella enteritidis* followed by *Salmonella infantis*. Amongst non-typhoidal
Salmonellae none was MDR. However, cotrimoxazole resistance was present in 66 % (n= 18) of the isolates of *Salmonella infantis*, and 50% (n= 17) of the isolates of *Salmonella typhimurium*. All the isolates of *Salmonella enteritidis*, one isolate of *Salmonella typhimurium* and one isolate of *Salmonella infantis* were sensitive to all the antibiotics tested.

The Pearson’s correlation between the two variables was found to be significant (-0.79, p-value < 0.001) and showed a negative correlation between the zone size and MIC. Also a simple linear regression was used to test whether the zone size can predict MIC. The test was significant at alpha-level of 0.05 (F = 70.4, p < 0.001). R squared = 0.62; therefore, 62% of the variance in MIC can be explained by differences in zone sizes.
Figure. 7. *Salmonella Paratyphi* A reactions with API 10 S
Figure. 8. *Salmonella typhi*, reactions with API 10 S
Figure 9. *Salmonella choleraesuis*, reactions with API 20 E
Figure. 10. *Salmonella enteritidis*, reactions with API 20 E
Figure 11. *Salmonella typhi*, reactions with API 20 E
Figure. 12. *Salmonella typhimurium*, reactions with API 20 E
Figure 13. MDR *Salmonella typhi*, Resistant to Ciprofloxacin and Ceftriaxone
Figure. 14. MIC of MDR Salmonella against Imipenem
Figure 15. MIC of MDR Salmonella against Azithromycin
Figure 16. MIC of MDR Salmonella against Cefpirome
Figure 17. MIC of MDR Salmonella against Aztreonam
Figure 18. MIC of MDR Salmonella against Tigecycline
1.8 Discussion

In this study we tried to find solutions to the emerging problem of resistance in *Salmonella enterica*. Since study was based in laboratories of tertiary care hospitals, hence the isolates were a mixture of extraintestinal and intestinal specimens. Our lab has heavy workload with 40,579 specimens received for culture during the study period. Salmonella *enterica* serotype Typhi (*Salmonella typhi*) and Salmonella *enterica* serotype Paratyphi (*Salmonella paratyphi*) remained the principal isolates. First we subjected all the isolates to conventional and extended spectrum antibiotics by disk diffusion technique. Then we selected the isolates which were resistant to conventional drugs (ampicillin, co-trimoxazole and chloramphenicol) and all isolates that were resistant or intermediate to ciprofloxacin, and performed MICs for newer, non-conventional antibiotics. Ciprofloxacin has been the most commonly used drug against *Salmonella enterica* in recent past. Shanahan et al isolated 21 isolates of *Salmonella typhi* from blood culture at Christian Medical College India and found that eleven isolates were resistant to chloramphenicol (MIC 256mg/L), trimethoprim (64 mg/L) and amoxicillin (>128 mg/L). Four of the isolates were resistant to each of these agents except for amoxicillin. Six isolates were sensitive to all the antibiotics tested. All the isolates were sensitive to imipenem (170).

Azithromycin has the advantage of being available in oral preparation and can be given safely to children, MIC90 for azithromycin in *Salmonella enterica* serotype Typhi (*Salmonella typhi*) in our isolates was 16 µg/ml while the earlier studies have reported MIC in the range of 4-16 microgram/ml (136;171;172) and
MIC90 in the study from Karachi was 8 microgram/ml (171). In a previous in vitro study, azithromycin had an MIC range of 4–16 µg/ml against *S. typhi* (Girgis et al., 1999). Azithromycin is said to have less chances of relapse and causes few side effects (172). At present breakpoints of Azithromycin versus enterobacteriaceae are not available in CLSI. Eucast has mentioned the MIC for *Salmonella typhi* as 16 mg/L. In BSAC breakpoints are given for *S. typhi* but our isolates according to BSAC criteria (18≤ Resistant) were mostly resistant in vitro. It is unlikely to be the case as azithromycin is being used successfully in our setup. It has been shown to have high cure rate and lesser defervescence time as compared to quinolones (136). This success may be due to high intracellular concentration of the drug and hence difference in vitro and in vivo effect of azithromycin. As compared to ceftriaxone, azithromycin is said to have similar clinical outcome but reduced relapse rate (137;172). No resistance of azithromycin has been reported in UK for *Salmonella typhi* and *Salmonella paratyphi* A till 2008 (140). Resistance of *Salmonella paratyphi* A to azithromycin and treatment failure has already been reported (143). It requires large clinical trials to prove its efficacy and to establish breakpoints. Resistance to azithromycin is likely to increase in near future because it is available over-the-counter in developing countries and has oral formulation. It is widely used for respiratory tract infections as well. Most antibiotic sensitivity standards including CLSI and EUCAST do not mention breakpoint for azithromycin against Salmonella. In this study we have tested azithromycin MIC in 216 salmonella isolates. It would pave the way for breakpoint determination for azithromycin after
clinical correlation. However, azithromycin is already being used successfully at many places worldwide (136;137;172;173). Salmonella paratyphi A cannot be prevented by vaccination as well.

Because of rising resistance to ciprofloxacin, and nalidixic acid it is no more an ideal alternative as shown by increased MIC to 8 µg/ml (171). Ciprofloxacin resistant strains have risen sharply in last 3 years. In 1999, thirty seven isolates of Salmonella were examined and all were sensitive to ciprofloxacin (174). It may be due to excessive use of ciprofloxacin to treat typhoid fevers that has selected out resistant strains. Pefloxacin is one of the options for treatment of Salmonella enterica systemic infections. It has been studied by Unal et al and found to have MIC in the range of 0.06 to 1 µg/ml, with MIC90 of 0.05 µg/ml (175). We could not include pefloxacin in our study as its E-strip was not available.

For imipenem and tigecycline, the difference in MIC90 and MIC50 was minimal. Amongst the antibiotics tested against, S. typhi and S. paratyphi A, tigecycline had the lowest MIC90 and MIC50 levels. In an earlier study no resistance to ceftriaxone was noted in Salmonella enterica serotype Typhi as well as Salmonella enterica serotype Paratyphi (176). However, resistance to ceftriaxone has been reported due to plasmid mediated cephalosporinases and extended spectrum beta-lactamases (177). Hence, testing Salmonella isolates to ceftriaxone remains mandatory. In one study by CDC, ceftriaxone resistance in non-typhoidal Salmonellae was 2.8% while there was none in Salmonella typhi and paratyphi (178). In another study from Ireland, the most common isolate
amongst non-typhoidal salmonellae in a hospital lab was *Salmonella enteritidis* followed by *Salmonella typhimurium* (179). In the same study, 12% of *Salmonella typhimurium* and 2% of *Salmonella enteritidis* were found to be multi-drug resistant. Resistant strains of *Salmonella enteritidis* have been rare in UK as well (57). Worldwide, *Salmonella Infantis* has been ranking in top ten non-typhoidal salmonellae and its resistance to antibiotics has also been rarely reported (180). Ceftriaxone resistance has been reported in *Salmonella typhimurium* in previous studies (179;181) but was not detected in our isolates. Resistance in *Salmonella typhimurium* to ceftriaxone was found to be 2-6% in a study from Taiwan. The overall resistance to ciprofloxacin was found to be 1% and to ceftriaxone 0.5% (46). Ceftriaxone resistance in nalidixic acid resistant NTS increased fivefold in USA from 1996 to 2005 (from 0.5% to 2.4%). In a study from China, *Salmonella typhimurium* was found to be resistant to Ciprofloxacin in 31 out of 44 isolates. All these isolates were resistant to 8-11 additional drugs as well (182). While in our study ciprofloxacin resistance in non-typhoidal salmonella was 7% and 67% were intermediate. This relatively low level of resistance may be due to lesser use of ciprofloxacin in livestock in our setup. Our country is basically agricultural country and livestock has ample natural food to eat. Since livestock is not exposed to antibiotics, hence antibiotic resistance has not developed. The reason that NTS isolates were relatively less in number may be due to low incidence in our set up. Traditionally in this area raw food is not consumed and all the food is well-cooked. Low levels of resistance to third generation cephalosporins in our non-
typhoidal Salmonellae may also be due to lesser use of antibiotics in animal feeds in this region.

In non-typhoidal salmonellae in UK, drug resistance was not known till 1960s. Then, due to increasing drug resistance especially in *S. typhimurium*, the use of drugs in animal feed was restricted. In 1994, 78% of Salmonella *typhimurium* isolates were resistant to at least one antibiotic and 61% were resistant to two antibiotics (183)

In our study none of the isolates of *S. Typhi* showed a high azithromycin MIC (64 µg/ml) and the MIC90 was only 16 µg/ml. Hence this drug has a potential for therapeutic use, being oral antibiotic as well. Few drugs, like azithromycin are concentrated manifold intracellularly and hence may show better clinical cure rate. Thus, there is speculation that intracellular MICs may not be represented fully by the currently available *in vitro* MIC testing methods. As this was a laboratory based study and the patients were not easily accessible, the therapeutic efficacy of these drugs was not possible. Furthermore, over-the-counter availability of effective drugs like quinolones and cephalosporins hampered such a move. However, in cases of therapeutic failure or relapse with these drugs, large scale randomized clinical trials of the new in *vitro* effective drugs is warranted. Since *Salmonella typhi* and *paratyphi A* are pathogenic only in humans, the trial in animals would remain dubious.

WHO in 2003 had recommended Fluoroquinolones or ceftriaxone as treatment for uncomplicated typhoid (62). Now after about ten years the situation
is entirely different. Fluoroquinolone resistance should be identified early, and these drugs should be used judiciously.

In this study trials of treatment of typhoid fever with azithromycin, imipenem, tigecycline, cefpirome, cefepime, cefpodoxime, gatifloxacin and aztreonam has been suggested, based on their in vitro activity against *Salmonella enterica* serovar Typhi and Paratyphi A. In a previous study MIC range of Cefpirome for *Salmonella* species was 0.094 to 0.91 but in our study it was 0.10 to 1.5 for *Salmonella typhi* and 0.06 to 1.6 for *Salmonella paratyphi* A (184;185).

Cefpodoxime penetrates in bile is 15% more than serum and can be given orally. Imipenem can be given only parenterally and its peak serum concentration is 40 µg/ml, bile penetration is minimal. Aztreonam peak serum concentration is 90 µg/ml and bile penetration is 2 to 4 times serum concentration. Azithromycin peak serum concentration is 3.6 µg/ml and bile penetration is high. Cefpirome can only be given parenterally. Its maximum serum concentration is 119 µg/ml. Tigecycline maximum serum concentration is 0.63 µg/ml and bile penetration is 138% as compared to serum (186).

Due to frequent power outages in our country, we frequently faced problems like incubator failure resulting in no growth on culture plates or requirement of repetition of biochemical tests. Many isolates could not be saved properly due to the same reason. There was poor yield of Salmonellae in many culture results. One of the likely reasons is the easy availability and widespread use of antibiotics by the patients early in disease. Contamination rate in blood
cultures remained around 5%. In Pakistan and other developing countries, due to paucity of resources typhoid fever is frequently diagnosed on clinical grounds. Isolation by culture is required for definite diagnosis and also for antibiotic sensitivity.

We found close relationship between MICs and zone diameters of the antibiotics. Disk diffusion technique is economical and MICs are not affordable in most setups of Pakistan.

Since we used revised breakpoints for ciprofloxacin sensitivity of Salmonella isolates hence nalidixic acid was not used as a surrogate marker. However, in a study from Nepal 76% of the isolates of Salmonella typhi were resistant to nalidixic acid (187). MIC of Salmonella for ciprofloxacin by E-strips has been shown to correlate well with agar dilution technique (188;189).

Antibiotics are used irrationally and are available over-the-counter in developing countries including Pakistan. Quinolones are available in oral form and are relatively cheap, hence they are used for any form of high grade fever in the developing world, presuming it to be typhoid fever. In developing countries like Pakistan, use of ciprofloxacin for Salmonella is widespread. Main reason is its oral effectiveness but another reason is that the clinicians are ignorant of the NAR (Nalidixic Acid Resistant) strains. With the rise of NARS and fall in MDR isolates, there is a chance of considering recycling the conventional antityphoid drugs. Randomized large scale clinical trials are required on new antibiotics before they can be recommended for treatment as such.
One of the trends seen was the increase in the relative proportion of *Salmonella paratyphi* A as compared to *Salmonella typhi* in isolates from blood culture, 55% *Salmonella typhi* and 45% *Salmonella paratyphi* A.

In this study we have given MICs of various antibiotics against *Salmonella* species, We expect that these MICs would be utilized by renowned antimicrobial susceptibility testing agencies like CLSI and EUCAST in establishing breakpoints for these antibiotics against *Salmonella*.

From our study it is inferred that conventional anti-typhoid drugs are not effective in clinical isolates especially in case of *Salmonella typhi* and *paratyphi* A. The study would help in formulating empiric therapy for salmonella infections in developing as well as developed countries. Strong collaboration is desired between clinicians and microbiologists for treatment of bacterial diseases and judicious use of antibiotics to avoid the development of drug resistance.

Tigecycline, aztreonam and cefpirome have shown low MICs against both *Salmonella enterica* serotype Typhi and *Salmonella enterica* serotype Paratyphi A. Their efficacy however must be proven by clinical trials. Except azithromycin, the other drugs tested are not available in oral form. Tigecycline is notorious for development of resistance during therapy also. Since imipenem, cefpirome and tigecycline are effective against anaerobes also they can be used successfully in mixed anaerobe and Salmonella infections like intra-abdominal infections.

In a previous study from Karachi none of the isolates were resistant to ceftriaxone (171), however in our study 3-8% isolates of *Salmonella typhi* and *Salmonella paratyphi* A were resistant. This shows emerging resistance to
cephalosporins and need to explore for alternatives. MIC90 of 178 isolates was compared by Cooper et al and found it to be 0.06µg/ml in 2001. It increased to 0.25 in 2005 (135). Rising and alarming trend has been noted in our study. High cost, requirement of parenteral administration and poor intracellular penetration makes ceftriaxone a difficult therapeutic option. It highlights the need to monitor emergence of resistance in typhoid salmonellae for third generation cephalosporins and to search for more alternatives.

In Pakistan MDR cases were 70 % in 1996 (34) while in another study they were 30% in 2010 (171). In our study MDR cases were 39% in Salmonella enterica serotype typhi while they were 5.4 % in Salmonella enterica serotype Paratyphi A . This declining trend may be due to loss of resistant plasmid or due to new susceptible strains. In a study from Nepal the MDR strains in Salmonella typhi were 26.5% (187). Molecular analysis is required, in future studies, to throw light on this aspect. However, the acquisition of ESBL in MDR cases would be a disaster, compromising the utility of third generation cephaloporins in these cases. ESBL has been detected in Salmonella enteritidis (190). There is one report of ESBL in Salmonella typhi from Philippines as well (191). Since ESBL genes are mostly located on plasmids, further spread of this resistance genes is expected.

Studies comparing association of MICs of new drugs (Tigecycline, cefpodoxime, azithromycin, imipenem, cefpirome, aztreonam, cefepime, gatifloxacin and doripenem) with treatment failures are required before these drugs are marketed for clinical use in typhoid. It would be required to establish
MIC breakpoints for these drugs as well. Tissue concentration achieved, side effects and intracellular penetration would be the main deciding factors in therapeutic response.

There were few (total 61 only) non-typhoidal salmonellae isolated in our study. The reason is likely to be less number of immunocompromised patients in our patient population, in which they are common (55). Moreover Pakistan remains a low prevalence country as far as HIV is concerned (45). In a previous study, Non-typhoidal Salmonellae showed high frequency of MDR and low level resistance to ciprofloxacin and azithromycin (47). However, our isolates had intermediate sensitivity to ciprofloxacin but were mostly sensitive to third generation cephalosporins. Salmonella enterica rate (29) as well as resistance is high in south & south-east Asia (192).

From our study it is difficult to infer the true burden or incidence of the disease as patient referral and population denominator were not specifically chosen. However, typhoid remains endemic in our area.

Poor hygiene, lack of education, poor access to potable water and redundant sewage systems are the factors contributing to Typhoid in our country.

Gatifloxacin is another option for treatment of typhoid. It has been studied in Nepal (193) and Vietnam (194). Main impediments in its use are its side effects especially on glucose metabolism in the elderly. Gatifloxacin is not available in united states and many other countries. Cheap and affordable antibiotics are required in developing/poor countries.
Our catchment area has peculiar problems. Being a third world country, Pakistan is a poor resource country. Most of the clinicians have to rely on clinical picture of the patient. Clinical laboratories are scarce and are not well equipped. Microbiology setups are the worst affected as there is no urgent demand from the clinicians as well. Quality control and training of laboratory technicians are the worst affected. Hence, the resistance pattern of Salmonella is poorly characterized.

In Pakistan and other developing and underdeveloped countries, medical microbiology is a neglected field. In the few labs that exist, MICs are not performed, but disk diffusion is commonly used. Lack of facilities and quality control is contributing to the increased resistance in Salmonella and other bacteria.

There is a reported case of *Salmonella typhi* that was resistant to quinolones and ceftriaxone and was successfully treated with aztreonam and meropenem (195). It had gyrA mutation and was TEM-1 positive. TEM-1 is β-lactamase but is not ESBL. In another study on mice, aztreonam was seen not only to treat Salmonella infections but could also eradicate it completely from reticuloendothelial system and was more effective in eliminating it from bloodstream than ceftazidime (196;197).

In a study from Korea combination of cefotaxime and ciprofloxacin was found to have synergistic effect in vitro against *Salmonella paratyphi* (198). In another study combination of ampicillin with chloramphenicol was found to be useful against serious *Salmonella* infections, if chloramphenicol was bactericidal (199). Such useful combinations need to be explored further to deal with resistant Salmonella cases.
1.9 Summary of findings

This research work was carried out to investigate in vitro effect of new antibiotics on various species involved in Salmonella infections in population residing in 12 different districts of north and north-west of Pakistan including majority of the cases from Rawalpindi.

Out of 2230 various clinical specimens 316 cultures of various salmonella species were isolated and identified. These cultures included *Salmonella typhi* 128 (41%), *Salmonella paratyphi* A 111 (35%), *Salmonella paratyphi* B 16 (5%) *Salmonella enteritidis* 26 (8%), *Salmonella infantis* 18 (6%), and *Salmonella typhimurium* 17 (5%).

These cultures were recovered from 186 (59%) blood specimens, 63 (20%) from stool, 21 (6.7%) from urine, 41 (13%) from pus and 4 (1.3%) from fluids.

Antibiotic susceptibility by disk diffusion showed 56 MDR cases. Amongst *Salmonella typhi* 39% were MDR, while in *Salmonella paratyphi* A 5.4% were MDR. In *Salmonella typhi* by MIC only 2 % were resistant to Aztreonam while 20% of *Salmonella paratyphi* were resistant.

None of the *Salmonella typhi* were resistant to imipenem or cefpodoxime. Non-typhoidal Salmonellae were 66% resistant to co-trimoxazole but were all sensitive to ampicillin, chloramphenicol and ceftriaxone.
1.10 Conclusions

1. Imipenem, azithromycin, tigecycline, aztreonam, cefpodoxime and cefpirome are potential therapeutic agents for resistant salmonella infections.

2. Azithromycin should be used with caution as MICs are higher in vitro. However, intracellular increased concentration in vivo, may prove to be a good therapeutic option.

3. *Salmonella typhi* and *paratyphi* A are the predominant causes of Enteric fever in Northern Pakistan. Present laboratory guidelines for detecting fluoroquinolone resistance in typhoid salmonellae need revision.

4. Imipenem and cefepime are good options in resistant cases to be treated in healthcare settings.

5. Gatifloxacin is also potential therapeutic options.

6. Isolates with high resistance to ceftriaxone are a grave concern and stresses upon continuous lab surveillance and sensitivity testing to guide the clinicians.
1.11 Recommendations

1. Clinical correlation studies need to be performed to know the therapeutic efficacy of azithromycin, tigecycline, imipenem, cefpodoxime, aztreonam, gatifloxacin, doripenem, cefepime and cefpirome.

2. Since Salmonella is resistant to many antibiotics the clinicians should keep themselves aware of the changing trends in antimicrobial resistance in this emerging superbug, to ensure adequate treatment for this life threatening disease.

3. Self-prescriptions, sale and prescription of antibiotics over-the-counter are the factors that lead to development of the multi-drug resistant organisms. People should be educated on the proper use of antibiotics. Legislations restricting over-the-counter sale and dispensing of antibiotics, need to be developed and enforced in Pakistan.

4. Since transmission of *Salmonella typhi* is faeco-oral and humans are the only source of infection, resistance in these organisms is most likely associated with human antibiotic use. Prudent antibiotic use is mandatory to avoid development of resistance. Water and sanitation improvement along with health education are required. Although, it requires development of appropriate infrastructure and investment and hence is a distant objective for poor countries like Pakistan.

5. New, simple and effective techniques are required for rapid diagnosis of typhoid and other salmonellae in resource poor countries. Cost of current
diagnostic modalities needs to be reduced especially for developing/poor countries.

6. Preventive measures against typhoid fever, improvements in hygiene, ongoing surveillance and education on sensible use of antibiotics in human and veterinary medicine are needed to control typhoid cases. There is a need for stronger collaboration between physicians and microbiologists/laboratory for correct choice of antibiotics against bacterial diseases like typhoid to curb the menace of resistance.

7. Studies comparing association of MICs of new drugs (Tigecycline, cefpodoxime, azithromycin, Imipenem, cefpirome, aztreonam) with treatment failures, in patients, are required before these drugs are marketed for clinical use in typhoid. Pharmacokinetic measurements would be required in future studies.

8. There is a need to monitor the pattern of antibiotic resistance of Typhoid salmonellae especially in endemic areas. Use of antibiotics in animal feeds needs to be curbed to avoid selection of resistance in salmonellae.

9. Treatment guidelines for Salmonella spp. need to be revised and implemented in true spirits.

10. All Salmonellae cultured should be notified to public health authorities. Investigation should be carried out to know the source of the disease.

11. Typhoid vaccine should be included in national immunization programme in typhoid endemic areas.
1.12 **Summary**

1. Resistance of Salmonella species to antibiotics has gained importance in last couple of decades. MDR cases (that are resistant to ampicillin, chloramphenicol and co-trimoxazole) are on the rise. Resistance to ceftriaxone has also been reported. There is a dire need to search for more antibiotics clinically effective against this lethal bug. In Asia, Pakistan has one of the highest incidences of *Salmonella typhi* and *paratyphi A*.

2. Ciprofloxacin has remained the drug of choice in the recent past but resistance to this drug is on the rise since 1993.

3. It is a cross sectional study carried out at Armed Forces Institute of Pathology Rawalpindi Pakistan. Research was conducted from May 2011 to June 2013. Six new drugs tested by MIC against Salmonella species have proved to be promising choices for treatment in future. Four new drugs tested by disk diffusion also merit consideration in treatment.

4. Azithromycin is being used in the treatment of Typhoid resistant cases. Our study shows it to have higher MICs as compared to other antibiotics tested. However it may be effective in vivo, due to its higher intracellular concentration.
Reference List


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