BACTERIA CAUSING ENTERIC FEVER IN HUMANS IN GADARIF STATE

By

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A Thesis Submitted in Partial Fulfilment Of The Requirements For Master Degree In Microbiology

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Feb, 2006
Dedication

To my friends for their patience, understanding and support throughout this effort............and to my parents for preparing me for the challenges.
Preface

This work has been carried out in the department of Microbiology, Faculty of Veterinary Medicine, University of Khartoum under the supervision and guidance of Professor Mohammed Taha Shigidi.
Acknowledgments

To begin with, my gratitude and praise are due to Almighty Allah, the Beneficent and the Merciful for the precious gift of health and the capability to accomplish this work.

I am deeply indebted to my supervisor Professor Mohammed Taha Shigidi for his serious guidance, close supervision and continuous encouragement.

My special thanks are due to Prof. Eldirdiri Elgaili, Dean Faculty of Medicine, University of Gadarif, for his kind sponsorship. I would like also to thank the registrar, the teaching staff, employees and workers of the faculty of Medicine. My deep appreciation to my friend Yassir Fadul for his unlimited assistance and support.

I warmly thank the Executive Administration of Health Insurance, Gadarif State, represented by the Medical Laboratory Administration. Sincere gratitude is extended to my intimate friend Osman Ibrahim Eldaw and Ammar Abdelmonem for their appreciable efforts.

I convey heartily thanks to my dear colleagues Yagoub Hamadtallah, Khalid Abusalif, Moneer Fathi, Mohammed Yagoub and Mohammed Elfatih for the efforts exerted to push this work forward and further to Dr. Ali Elwakeel for technical assistance and precious advice.
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Abstract

This study was carried out in Gadarif State, Eastern Sudan, during the period from December 2004 to July 2005. The study was designed to isolate and identify bacteria that cause enteric fever in humans in Gadarif State, and to determine the antibiograms of the isolated bacteria to the commonly used antibiotics. The study involved sick individuals in the community, which included different ages and genders.

From 71 patients, who were diagnosed as suffering from enteric fever and had significant titres for Widal test, faecal and blood samples were collected. Specimens from 24 patients were dropped because they did not give growth of significant bacteria. Isolates from the remaining 47 patients were further characterised and their susceptibility to various antibiotics was determined.

The main bacteria of enteric fever in Gadarif State was *S. paratyphi* B, while organisms such as *Proteus* and *Pseudomonas spp*, were isolated from some specimens.

Fourteen of *S. paratyphi* B isolates showed resistance to ciprofloxacin while the other nine isolates showed multidrug-resistance. All isolates were highly susceptible to chloramphenicol.

In this study it was observed that the disease was more common among females than males. This was perhaps due to lack of household
and personal hygiene in poor communities, beside the development of
carriers. Women are more exposed to compost, waste, food and
contaminated water supplies in Gadarif State.
THE RESEARCH

The research was conducted in the state of South Darfur, in the governorates of South Darfur and in the studies. This is a project from December 2004 to 2005.

The purpose of this research is to determine the characteristics of the study area and the governorates of the study and to conduct the necessary examinations. The project was conducted from December 2004 to 2005.

The study included the ages of different parts of the population, the health of the individuals, and the health of the population in general. The study included the ages of different parts of the population, the health of the individuals, and the health of the population in general.

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CHAPTER ONE

1. Introduction

Salmonellae acquired by the oral route are often pathogenic for humans and animals. They may be transmitted from animals and animal products to humans, where they cause enteritis, systemic infection, and enteric fever (Brooks, Bult, Morse, 1998). Interestingly, humans are the sole reservoir for *Salmonella typhi* which causes the most dangerous form of salmonellosis, typhoid fever, in man.

Enteric fever is an acute infectious disease characterized by abdominal symptoms and enlargement of spleen. It may be presented with the history of prolonged pyrexia of obscure origin (Elfaki, 1987). Enteric fever (typhoid) is classically caused by *Salmonella enterica serotype typhi*, but a similar syndrome may be caused with *Salmonella paratyphi* A,B and C (Chandel et al., 2000).

following the ingestion of contaminated food or water the bacteria penetrate the intestinal mucosa to gain entry to the lymphatics and blood stream, where they produce an initial bacterimic phase, exhibiting systemic symptoms that last for about a week. The organisms then appear in the alimentary tract, possibly via bile secretion (MacSween and Whaley, 1992). Bile is a good culture medium for the bacteria, which multiply abundantly in the gall bladder and are discharged continuously
into the intestine where they involve the peyer’s patches and lymphoid follicles of the ileum (Ananthanarayan and Paniker, 1997). Transient nephritis was diagnosed clinically and biochemically in Sudanese patients suffering from typhoid fever in Gezira area (Musa, Salih and Abu Asha, 1981).

*Salmonella paratyphi*, is also an exclusively human health problem and its clinical and pathological features are very similar to those of typhoid fever, in almost all cases except it is, milder in degree and largely confined to the distal ileum. Here, too, the carrier state provides the reservoir of infection (McSween and Whaley, 1992).

*Salmonella enterica serotype typhi* is endemic in developing countries in Africa, south and Central America, and the Indian subcontinent, with an estimated incidence of 33 million cases each year. By contrast, in developed countries such as United Kingdom or the USA, the incidence is much lower, and most cases are in travellers returning from endemic areas (Threlfall and Ward, 2001).

*S.typhi*, *S.paratyphi A* and *S.paratyphi B* are the common cause of typhoid and paratyphoid in Sudan (Khan, 1962) and *S.paratyphi C* has
never been reported from man in this country, but it was isolated from lizards (Krik, 1966). *S. typhi* was isolated from medical students of the University of Khartoum (Yousif and Salim, 1966).

Diagnosis of enteric fever by Widal test, need to be judged in association with knowledge of normal O and H agglutinins titres in local population. In Sudan, a titre of 1:160 or more for O somatic antigen is considered as evidence of typhoid fever (Elfaki, 1987).

1.1 Objectives:

The main objectives of this study were:

1- To determine the type of bacteria causing enteric fever in humans living in areas in the vicinity of Gadarif town.

2- To compare Widal test with isolation procedures in detecting the disease.

3- To determine the susceptibility of isolated bacteria to antibiotics commonly used for treating enteric fever.
2. LITERATURE REVIEW

2.1 History of enteric fever:

The term enteric fever includes typhoid caused by *Salmonella typhi* and paratyphoid fever caused by *Salmonella paratyphi* A, B and C.

Typhoid fever was once prevalent all over the world and was not well demarcated from other prolonged fevers. (Bretonneau, 1826), who identified the intestinal lesions, presented a detailed study of the disease (Youmans, Paterson and Sommers, 1986). The name typhoid was given by Lois (1829), to distinguish it from typhus fever. (Budd, 1856) pointed out that the disease was transmitted through the excreta of patients. (Eberth, 1880), described the typhoid bacillus and (Gaffy, 1884), isolated it in pure
culture. (Metchnickoff and Besredica, 1900),
confirmed its causative role by infecting apes
experimentally by injecting the organism (Hyde,
1995).

*Salmonella paratyphi* A was isolated by Gwyn
(1898); *S. paratyphi* B (*S. schottmüller*) by Achared
and Bensuade,(1896), and *S. paratyphi* C (*S.
hirschfeldi*) by Uhlenhuth and Hubener (1908), from
cases resembling typhoid fever (Ananthanarayan and
Paniker, 1997).

2.2 The Aetiology of enteric fever:

Enteric fever is a clinical syndrome produced
classically by *Salmonella typhi* and, also at times, by
*Salmonella paratyphi* A, *S. schottmüller*, (formerly)
*paratyphi* B and *S. hirschfeldi*, (formerly) *paratyphi* C.
*Salmonella* serotypes other than those mentioned
above can occasionally produce a clinical feature of
enteric fever. Enteric fever produced by *Salmonella typhi* is called typhoid fever and that produced by *Salmonella* serotypes other than *S. typhi* is termed paratyphoid fever. The main typical cause of enteric fever is *S. typhi*, whereas other similar but not typical ones are *Salmonella paratyphi* organisms.

2.2.1 *Salmonella typhi*:

*Salmonella typhi* is pathogenic only for man, causing typhoid (enteric) fever and is transmitted by water or food contaminated by human excreta.

The organism does not grow on Simmons’s citrate medium or on minimal defined medium and requires tryptophane as a growth factor. It does not produce gas from glucose or other sugars (Krieg and Holt, 1984).

2.2.2 *Salmonella paratyphi* A:
Salmonella paratyphi A causes a typhoid–like infection. It is pathogenic only for man. The majority of strains do not produce hydrogen sulphide, and in this respect S. paratyphi A is unlike most other salmonellae (Krieg and Holt, 1984).

2.2.3 Salmonella schottmülleri (formerly paratyphi B):

The organism causes enteric fever in man and very rarely infects animals. It produces a slime layer when grown on medium containing 0.5% glucose and 0.2 M sodium phosphate solution at pH 7 (Krieg and Holt, 1984).

2.2.4 Salmonella paratyphi C:

Salmonella paratyphi C causes mainly septicaemia, and also causes paratyphoid fever.

Infection with paratyphi C is usually more serious than those of S. paratyphi A and S. paratyphi B. Virulent and multi resistant strains of S. paratyphi
C are common in the Indian subcontinent (Ochei and Kolhatkar, 2000).

2.3 Classification of *Salmonella* Species:

*Salmonellae* can be classified according to biochemical, serological and genetical analyses.

2.3.1 Biochemical classification:

The *salmonellae* contain a single genus, *Salmonella*, and are named after the American microbiologist, D.E. Salmon. On the basis of biochemical reactions, Kauffmann proposed that *Salmonella* be classified into six subgenera (Paniker and Vilma, 1997).

- Subgenus 1:

  It is the largest and medically the most important group that contains all the species commonly
causing human and animal infections. It includes most of the serotypes *S. typhi*, *S. cholerae-suis*, *S. paratyphi* and *S. galinarum*.

- Subgenus 2:

  This subgenus contains mostly species isolated from reptiles for example *S. salmae*.

- Subgenus 3:

  Subgenus 3 contains bacilli, formerly designated as “*Arizona*”, originally isolated from lizards but subsequently found in reptiles, birds, domestic animals and human beings. Many of them are prompt lactose fermenters and are subdivided into:

  *Salmonella* subgroup 3a *S. arizona*

  *Salmonella* subgroup 3b: *S. diarzona*
Subgenus 4:

Those strains are rarely encountered and may be considered as atypical members of subgenus 2, e.g. *S. houtenae*.

Subgenus 5:

An example of this subgenus is *S. bongori*.

Subgenus 6:

An example of this subgenus is *S. chloaresuis subspecies indic* (Koneman *et al.*, 1997; Paniker and Vilma, 1997).

2.3.2 Serological classification:

(Kauffmann-White classification)

*Salmonella subcommittee* (1934), proposed that serology was the ultimate criterion in the classification of *Salmonella* (Barrow and Feltham, 1993). Kauffmann-White scheme for classification was first developed in 1934 and it classifies salmonellae into different O groups or O serotypes, each of which contains a number of serotypes.
possessing a common O antigen not found in other O groups. The O groups, first defined, were designated by capital letters A to Z and those discovered later by the number (51-67) of the characteristic O antigen (David, Bishop and Mass 1989).

Beginning July, 1983 the Centre for Disease Control (CDC) changed the method for reporting Salmonella serotypes; so that all organisms identified as salmonellae were reported as genus and serotype, omitting reference to species (Koneman et al., 1997).

Within each group, the differentiation of serotypes is carried out by the identification of phase 1 and 2 flagellar antigens.

The Kauffmann-White scheme gave species status to each serotype; the genus Salmonella is subdivided into more than 2300 serotypes containing different combinations of antigens (Collee et al., 1996). Complete antigen analysis is not a routine
procedure for clinical laboratories, but reference laboratories are available in most countries (Barrow and Feltham, 1993).

2.3.3 Classification by Deoxyribonucleic Acid (DNA) hybridization:

Modern taxonomical techniques, especially DNA studies, have shown that all the members of the genus Salmonella and the former genus Arizona are so closely related that they should all be considered as belonging to a single species, in a genetic, phylogenetic and evolutionary sense (David et al., 1989)

DNA hybridization studies have shown all salmonellae to be genetically identical (Cheesbrough, 2000). A new species named Salmonella enterica has been coined to include all salmonellae. S. enterica is classified into seven subspecies based on DNA
reassociation tests (Ananthanarayan and Paniker, 1997), each with its own phenotypic characteristic and history (Cheesbrough, 2000). These subspecies are named *enterica, salamae, arizona, diarizoneae, houtenae, longori* and *indica*. Subspecies *enterica* corresponds to the former subgenus 1 (Paniker and Vilma, 1997).

This genetically based new classification system has not yet been widely adopted by medical microbiologist (Cheesbrough, 2000). Deoxyribonucleic Acid (DNA) classification and nomenclature, while being taxonomically correct, would be too complicated for use in clinical bacteriology. For example, the taxonomically correct name for typhoid bacillus would be “*Salmonella enterica*, subspecies *enterica*, serotype *typhi*“. Therefore, the old practice of referring to clinically important *Salmonella* serotypes by the species name continues in clinical
bacteriology. Important pathogens such as S. typhi, S. paratyphi A and B can further be typed for epidemiological purposes by phage susceptibility, biochemical properties, and bacteriocin production (Stratton et al., 2001).

2.4 Morphology of Salmonella Species:

Salmonellae are Gram–negative straight rods, 0.7-15 x 2.0-5.0 µm, conferring the general definition of the family Enterobacteriaceae, (Krieg and Holt, 1984). With the exception of S. pullorum–gallinarum, all salmonellae are actively motile (Krieg and Holt, 1984; Barrow and Feltham, 1993; Cheesbrough, 2000). They are motile with peritrichous flagellae (Collee et al., 1996; Paniker and Vilma, 1997). They are non-sporing and with the exception of S. typhi are non-capsulated (Collee et al., 1996; Cheesbrough, 2000).

2.5 Cultural characteristics of Salmonella Species:
Salmonellae are aerobic and facultatively anaerobic bacteria (Barrow and Feltham, 1993). They grow readily on simple media with a pH 6-8 and at temperatures of 15 - 41C (optimum 37C) (Collee et al., 1996). Many stains are prototrophic, i.e. capable of growing on glucose– ammonium minimal medium, but some strains are auxotrophic and require minimal medium with one or more amino acids or vitamins, e.g. Cystine or nicotinamide; most S. typhi strains require tryptophan (Barrow and Feltham, 1993).

On nutrient agar and blood agar colonies are large, 2-3mm in diameter, low convex, smooth, grey-white, moist, circular with entire edge, thus resembling the colonies of many other enterobacteria (Collee et al., 1996).

In liquid media such as peptone water and nutrient broth most strains give abundant growth with uniform turbidity. A thin surface pellicle usually
forms on prolonged incubation. Rough (R) variants, which have hydrophobic surface and tend to agglutinate, produce a granular deposit and sometimes a thin pellicle (Collee et al., 1996).

On differential and selective solid media, MacConkey agar, colonies are colourless as lactose is not fermented.

Colonies of salmonellae on Salmonella-shigella agar medium are similar to, or smaller in size than, those on MacConkey agar. They are pale, colourless, smooth, shiny and translucent. They have a black centre and sometimes they are surrounded by a zone of clear medium (Collee et al., 1996).

On brilliant green bismuth sulphite agar (BBSA) S. typhi and paratyphi B form black colonies with metallic sheen. Many strains of paratyphi B form large mucoid colonies, or colonies surrounded by a thick mucoid or slime layer (Collee et al., 1996).
On Kligler iron agar (KIA) salmonellae produce a yellow butt and red–pink slope due to the fermentation of glucose only. Cracks and bubbles in the medium indicate gas production from glucose fermentation. Gas is produced by *S. paratyphi*, (Cheesbrough, 2000).

On selenite F-broth medium there is a change of colour from colourless to red.

In brilliant green bile broth salmonellae change the green colour to grey.

2.6 Biochemical reaction of Salmonella Species:

The enteric fever group can be separated biochemically from other enterobacteria (Koneman, *et al.*, 1997). Salmonellae ferment glucose, mannitol and maltose forming acid and gas. An important exception is *S. typhi*, which is anaerogenic (Krieg and Holt, 1984; Barrow and Feltham, 1993). Sucrose,
salacin and lactose are usually not fermented. The bacteria negative for oxidase, urease and indole production.

Hydrogen sulphide is usually produced on Kligler iron agar (KIA), S.typhi differs from most other salmonellae in producing only small amounts of hydrogen sulphide. S. paratyphi A does not produce hydrogen sulphide. Citrate is usually utilized as a sole carbon source, but S. typhi is usually an important exception as it does not grow on Simon’s citrate medium.

Salmonellae are positive for nitrate reduction and methyl red (MR) tests, but are negative for Voges Proskauer (VP) test, (Krieg and Holt, 1984; Barrow and Feltham, 1993).

2.7 Identification of *Salmonella* Species:
The identification of *Salmonella* species can be carried out through biochemical investigation. Then the corresponding serotype is determined by the specific serotyping using the agglutination test. The recently developed genetic methods using deoxyribonucleic acid (DNA), provide important tools for accurate specification (Graham, *et al.*, 1999).

2.8 Antigenic structure of the *Salmonella* species:

While salmonellae are initially detected by biochemical characteristics, group and species are identified by antigenic analysis (Brook, *et al.*, 1998). *Salmonella*, like other bacteria, has somatic (O) antigens, which are lipopolysaccharide components of the cell wall, and flagellar (H) antigens, which are proteins (Mandell, Hook and Douglas, 1985; Collee, *et al*. 1996; Brook, *et al.*, 1998).

There are about 60 (O) antigens, and many H antigens, each of which is designated by number and
letter (Mandell, et al., 1985; Paniker and Vilma, 1997; Brooks, et al., 1998). Certain serotypes (e.g. S. typhi) have a capsular or virulence (Vi) antigen composed of homopolymer of N-acetyl glucosaminuronic acid (Mandell, et al., 1985). The presence of Vi antigen on the cell surface may block agglutination by anti-O serum (Mandell, et al., 1985). Many strains of S. typhi fail to agglutinate with the anti-O serum when freshly isolated. This is due to the presence of surface antigen, enveloping the O antigen (Ananthnarayan and Paniker, 1997). (Mandell, et al., 1985), who first described this antigen, believed that it was related to virulence and gave it the name Vi antigen. It is analogous to the K antigens of coliforms and is heat labile. Bacilli, bearing this antigen are inagglutinable with the O anti serum yet become agglutinable after boiling or heating at 60°C for one hour. It is also destroyed by 1.0 N HCL and 0.5 N NaOH. It is unaffected by alcohol or 0.2% formol
(Ananthanarayan and Paniker, 1997). Almost all recently isolated strains of *S. typhi* form Vi antigen as a covering layer outside their cell wall (Collee, *et al.*, 1996). The Vi antigen is lost in serial subculture. Other surface antigens include the capsular or K antigens, the slime (mucus) or M antigen and fimbrial or F antigen. Such antigens may cause difficulty in the serological identification of bacteria, either by masking the O antigens so that the bacteria are inagglutinable by O antibodies or by causing non-specific cross-reaction due to their presence in unrelated bacteria (Cheesbrough, 2000).

2.8.1 R antigens:

In smooth to rough mutation The O antigens of *Salmonellae* are lost and new R antigens are revealed at the bacterial surface (Collee, *et al.*, 1996). The antigens are the same in the R variant of different *Salmonellae* serotypes, though different from The R
antigens of other enterobacteria, for they are autoagglutinable and lack serotype specificity. They are unsuitable for serological test (David et al., 1989).

The smooth–to–rough variation is associated with change in colony morphology and loss of the O antigen of virulence. The colony becomes large, rough, and irregular. Suspension in saline is autoagglutinable. Conversion into R forms occurs by mutation. R forms may be common in laboratory strains maintained by serial subcultivation (David et al., 1989).

Mucoid colonies, associated with the development of a new mucoid or M antigen, have been described with S. paratyphi B and some other species (Ananthanarayan and Paniker, 1997).

2.9 History of the Salmonella species:
The genus *Salmonella* consists of bacilli that reside innately in the intestines of a large number of vertebrates and infect human beings, leading to enteric fever, gastroenteritis and septicaemia.

The most important member of genus is *S. typhi*, the causative agent of typhoid fever. Typhoid bacillus was first observed by Eberth (1880), in the mesenteric lymph nodes and spleen of fatal cases of typhoid fever and was isolated by Gaffy (1884). It came to be known as *Eberth-Gaffy bacillus* or *Eberthella typhi*. Salon and Smith (1885), described a bacillus, which was believed to cause hog cholera. This bacillus, later called *S. cholerae-suis*, was the first of a series of similar organisms to be isolated from animals and human beings in the genus *Salmonella*. It was subsequently realized that the typhoid bacillus also belongs to this group, despite minor biochemical
differences and it was reassigned the name *S. typhi*, the genus *Eberthella* having been abolished.

*Salmonella* currently comprises over 2000 serotypes or species, all of which are potentially pathogenic (Ananthanarayan and Paniker, 1997).

2.10 Transmission of causative agent of typhoid to host:

Human carriers provide the source of infection, there being no animal reservoir (MacSween and Whaley, 1992). Infection occurs through the ingestion of the organism in contaminated hands. *Salmonella typhi* is mainly water-borne, whilst *S. paratyphi* is mainly food-borne (Cheesbrough, 2000).

2.11 Pathology and pathophysiology of typhoid fever:

The main pathological changes are found in the gastrointestinal tract. The peyer’s patches show hyperplasia during the first week of typhoid, necrosis during the second week and ulceration during the third week.

Healing takes place without scarring during the fourth week. The ulcer is oval in the long axis of the lower ileum. There is exudate on the peritoneal surface. Separation of the sloughs may lead to haemorrhage and perforation. The incidence of ulcer bears no relationship to the
clinical severity of the infection. The liver shows a cloudy swelling and typhoid nodules, which are small lesions consisting of collection of macrophages and lymphocytes with or without central necrosis (El-faki, 1987).

2.12 Pathogenicity of the aetiologic agent of typhoid fever:

2.12.1 Pathogenesis

The development of the disease after ingestion of Salmonella is influenced by the number and the virulence of the organisms and by multiple host factors (El-faki, 1987).

A large number of Salmonella must be swallowed in most instances to produce disease in healthy human beings. Limited studies with several serotypes, including S. typhi, show that, in general, 10^6-10^9 organisms must be ingested to produce symptomatic infection. However, in the event of infection with usually virulent organisms or in patients with reduced resistance, symptomatic infection may result from extremely small inocula. It is also known that an asymptomatic infection can be established with 10 to 100 times fewer organisms than are needed to produce symptomatic infection (Khan, et al., 1998).

Ingested organisms pass from the mouth to the stomach. In the past it was thought that S. typhi gained access to the body through the tonsils or pharyngeal lymphatics. However, volunteers gargling large
inocula of *S. typhi*, (Quails strain) have failed to acquire the disease, and invasion through the pharynx is thought not to occur (Youmans, *et al.*, 1986).

In the stomach salmonellae are exposed to gastric acid and low pH, which reduces the number of the viable organism. Most salmonellae are killed rapidly at pH 2, which is readily achieved in the normal stomach. Viable bacilli that survive then pass into small intestine, where the organism is further reduced in number or eliminated entirely. The antimicrobial activity observed in the small bowel is related, at least in part, to the normal microbial flora of the intestine, which elaborates short-chain fatty acids and perhaps other substances capable of killing or inhibiting growth of *Salmonella*. *Salmonella* that survives the antibacterial mechanisms in the stomach and upper small bowel may multiply in the small intestine (Brooks *et al.*, 1998).

Multiplication of salmonellae in the intestinal tract may be asymptomatic, associated only with transient excretion of organisms in stool, or symptomatic, associated with clinical manifestations of enterocolitis (acute gastroenteritis), enteric fever, or bacteraemia (Ochei and Kolhatkar, 2000).

Local factors in the stomach and upper intestinal tract are important determinants of the disease. Factors that neutralize the low pH
of the stomach or decrease the time the pathogen is exposed to stomach acid diminish local bactericidal action and increase the probability that an infectious inoculum will reach the small intestine. It has been suggested that ingestion of organisms in food allows for long exposure to gastric acid, thereby necessitating the presence of relatively larger inoculum to produce disease, while water and other liquids, which have a fast gastric transient time, may be less heavily contaminated and still cause disease (Youmans, et al., 1986).

The small intestine provides other protective mechanisms through motility and normal flora. Alteration of intestinal flora by antibiotics such as streptomycin markedly reduces the size of the inoculum required to produce Salmonella infection in animals and man and prolongs the convalescent carrier state (Mandell, et al., 1985). Antimicrobial drugs affect not only the infecting microorganisms but also susceptible members of normal flora of the body. An imbalance is thus created that, in itself, may lead to disease (Brooks et al., 1998).

Age is an important determinant of the disease produced by salmonellae. The influence of age on incidence may be attributed to the immunity of humoral and cellular immune mechanism, diminished antibacterial action of the normal intestinal flora, high frequency of faecal oral contamination, or other factors. In some instances, increasing
resistance with age is related to immunity consequent to previous exposure to the organism, even though the disease has not been imposed (Khan, et al., 1998).

Typhoid fever also occurs with increased frequency amongst the young in areas of world such as Mexico, Chile, India, Southeast Asia, and parts of Africa where the disease is endemic. In these areas, adults have acquired substantial resistance through repeated exposure to the organism. However, in more developed areas where enteric fever is not endemic, there is opportunity of repeated subclinical infection, and the incidence is not age-related (Sood, et al., 1999).

Patients with impaired cellular humoral immune mechanisms are at increased risk for the development of salmonellosis. Impairment of host defenses caused by malnutrition, malignancy, or therapeutic measures such as corticosteroid or immunosuppressive therapy also predisposes to infection and disease (Sarasombath, et al., 1987). The relative importance of change in intestinal flora, motility, pH, is unknown and remains under scrutiny. The pathogenesis of enteric fever has been extensively studied. The site at which S. typhi penetrates the intestinal barrier in man has not been defined with certainty. Jejunal biopsy from infected humans revealed inflammation, suggesting the
possibility that the jejunum would be the site of penetration in some patients (Younans, et al., 1986).

Many studies have been directed to the cause of prolonged fever and toxic symptoms of enteric fever. For years, circulating endotoxins and lipopolysaccharides, the components of the bacterial cell wall, were implicated. Injections of an endotoxin into healthy volunteers produced symptoms of the disease: headache, fever, abdominal pain, and malaise, and moreover laboratory findings (anaemia, leucopenia) remarkably similar to findings in the same volunteers during actual S. typhi infections (Mandell, et al., 1985).

2.13 Clinical manifestations of enteric fever:

The clinical course may vary from mild undifferentiated pyrexia to a rapidly fatal fulminating disease. The onset is usually gradual, with headache, malaise, anorexia, a coated tongue and abdominal discomfort with either constipation or diarrhoea. The typical features are step-ladder pyrexia, with toxaemia. Hepatomegaly is also common. Rose spots appear on the skin during the second or third week of enteric fever (MacSween and Whaley, 1992).

2.14 Diagnosis of enteric fever:
2.14.1 Widal test:

Detection of anti *S. typhi* antibodies in patients is a useful diagnostic aid. Amongst the various methods developed over the years for this purpose the Widal test, based on bacterial agglutination, has remained the most widely used, even though it is neither specific nor sensitive. It is popularly used due to the fact that it is simple to use and inexpensive (Lim, *et al.*, 1998).

This test for the serological diagnosis of enteric fever was much used in the past when effective selective media were not available and it was more difficult to isolate the causal, *Salmonella* species, from the patient’s blood or faeces (Collee, *et al.*, 1996). In developing countries, where typhoid fever is endemic and where there are few microbiology laboratories to provide diagnosis by culture, the search continues forth for non-cultural techniques that provide rapid and reliable diagnosis (Jesudason, *et al.*, 1998).

2.14.2 Interpretation of Widal test results:

The value of Widal test in diagnosing enteric fever in endemic areas remains controversial. Some express the view that the test lacks standardization and adequate sensitivity to be clinically useful, while others consider the test to have a diagnostic value when judged in association with clinical findings and knowledge of the normal O and H
agglutinins titre in local population. Of shared concern, however, is now necessary to avoid misuse and misinterpretation of Widal test (Cheesbrough, 2000). In typhoid fever the H titre is elevated earlier and more frequently than the O titre and antibodies rise during the second week of illness. It appears that H titre is more useful than O titre. Antibiotic treatment did not affect the rise of antibody titre in typhoid fever (Abraham, et al., 1981). In Sudan, a titre of 1:160 or more for O somatic antigen is considered as evidence of typhoid fever (Elfaki, 1987).

The features significantly associated with a final diagnosis of typhoid fever were: pre-admission duration of fever (longer than or =7 days), Hepatomegaly, leucopenia due to absolute neutropenia with relative lymphocytosis, although the sensitivity, specificity and predictive value of any of these features can not be used reliably to distinguish typhoid fever from other non typhoidal febrile illnesses (Khan, et al., 1998).

### 2.14.3 Misguiding O and H titres:

The registration of O and H titres can often indicate previous *Salmonella* infections, chronic salmonellosis associated with schistosomai infection, vaccination with typhoid vaccine, current infection with other than *Salmonella* species, chronic liver disease
associated with raised globulin levels and disorders such as rheumatoid arthritis, rheumatic fever, multiple myeloma or a nephritic syndrome besides ulcerative colitis (Johnson, et al., 1996).

2.15 Toxin production:

All members of the family enterobacteriaceae possess an endotoxin. This toxin is responsible for endotoxic shock in Gram-negative bacterial septicaemia (Ochei and Kolhatkar, 2000). Salmonella typhi contains gluc-lipo-protein complexes. The endotoxin is obtained by extracting the bacterial emulsion with trichloroacetic acid. This endotoxin is thermo stable; survives at a temperature of 120 C for 30 minutes, and is characterized by a highly specific precipitin reaction and pronounced toxic and antigenic properties. Investigations have shown the presence of exotoxic substances in S. typhi, which are inactivated by light, air and heat (80 C), as well as enterotropic toxin phosphatase and pyrogenic substances (Pyatkin and Krivochein, 1987).

2.16 Immunity:

Infections with S. typhi or paratyphi usually confer a certain degree of immunity. Reinfections may occur but are often milder than the first infection. Circulatory antibodies to O and Vi antigens are related to resistance to infection and disease. However, relapses may occur in 2-3 weeks after recovery in spite of the antibodies. Secretory IgA (SIgA)
antibodies may prevent attachment of *Salmonella* to intestinal epithelium (Brooks, *et al.*, 1998). It can persist in the gut for about 48 weeks. Thus, the immunities as a whole can persist beyond one year after the onset of illness, unless there are persistent booster stimulation *S. typhi* bacilli that exist in the environment, where, then, the immunities may be lifelong (Sarasombath, *et al.*, 1989). The microorganism is an example of facultative intracellular parasite, surviving well within macrophages and requiring cell-mediated immunity for control (Youmans *et al.*, 1986). After the onset of illness, the cell-mediated immunity persists for 16 weeks (Sarasombath, *et al.*, 1987).

Immunity acquired after typhoid fever and paratyphoids is relatively stable but relapses and reinfections sometimes occur. Antibiotics, used as therapeutic agents, inhibit the immunogenic activity toward the pathogens, which change rapidly and lose their O and Vi antigens (Pyatkin and Krivochein, 1987).

### 2.16.1 Antibodies in enteric fever:

*Salmonella* causing enteric fever stimulates the formation of three types of agglutinins, which are of use in diagnosis. They are H, O and Vi. The H antibody, which is produced in response to stimulation by flagellar antigens, appears towards the end of the first week of the disease, and usually reaches the highest titre. It persists longer than the
others after recovery, sometimes for many years, and its formation may again be stimulated, non-specifically, in subsequent febrile illnesses.

Agglutination is rapid and the agglutinated bacilli form large fluffy clumps. There is no evidence that H antibody is protective or helps to combat the disease (Stokes and Ridgway, 1983; Stokes, Ridgway and Wren, 1993).

Formation of O antibody is stimulated by O somatic antigens. It also appears in the first week of illness and seldom rises above a titre of about 1:640 (Curtis, Edwards A, Kapil A 1983; Stokes, et al., 1993). Anti O polysaccharide chain antibody titres are lower at the first week and increase up to the third week of the infection (Mastroianni, et al., 1989).

After recovery the titre falls and it is seldom demonstrable a year later. Production is not easily stimulated, non-specifically, in subsequent illnesses. Agglutination occurs more slowly and the clumps of the bacilli are small and dense (Stokes and Ridgway, 1983).

After the onset of illness, immunoglobulins; IgG, IgM, anti O and H agglutinins persist for two years, 16 weeks, 16 weeks and 36 weeks respectively (Sarasombath, et al., 1987).

The Vi antigen, also, a somatic antigen, is possessed by Salmonella typhi, S. paratyphi C and other coliforms, including certain
strains of *E. coli*. Typhoid and paratyphoid bacilli, which possess large quantities of this antigen often, give rise to severe disease (Stokes *et al.*, 1993).

Occasionally, only Vi antibody can be investigated but that can happen in rare cases. However, in most cases there is no need to perform the Vi agglutination test. The antibody is probably of value in combating infections of Vi strains.

In the majority of patients H and O agglutinins can be easily demonstrated. Occasionally only one of the agglutinins is found, and very occasionally no specific antibody is demonstrable in enteric fever (Stokes and Ridgway, 1983; Stokes, *et al.*, 1993).

### 2.17 Complications of typhoid fever:

Complication to typhoid fever that can be classified as secondary to toxaemia are myocarditis, hyperpyrexia, hepatic and bone marrow damage and those secondary to local gastrointestinal lesions are haemorrhage and perforation (Mandell, *et al.*, 1985). Some degree of bronchitis or bronchopneumonia is frequently found. Some develop psychosis, abscesses, nephritis, haemolytic anaemia, venous thrombosis and peripheral neuritis beside other complications. Nephritis was reported during typhoid fever in patients, who had previous Schistosomiasis mansoni infections in Gezira irrigated scheme, Sudan.
(Musa, Saleh, Abu Asha 1981). It is also known that a chronic form of salmonellosis can exist in patients with schitosomaisis as reported from a study in Brazil (Hagashi, et al., 1975; Saleh et al 1975). Osteomyelitis is a rare sequel (Ananthanarayan and Paniker, 1997).

Fatalities still occur occasionally, probably in less than 1% of the patients receiving appropriate antimicrobial and supportive therapy (Mandell, et al., 1985).

2.17.1 Relapses:

Relapses, a recurrence of manifestation of typhoid after initial clinical response, occur in about 8-12% of patients who have not received antimicrobial therapy. Antimicrobial therapy may increase the rate of relapse occurrence. In several studies, the relapse rate was found to be doubled in patients receiving chloramphenicol therapy for 2 weeks. Ampicillin probably does not affect the rate of relapse. Studies of patients given trimethoprim-sulphamethoxole (co-trimoxazole), indicate that the rate of relapse may be relatively less than the patients treated with chloramphenicol (Pyatkin and Krivochein, 1987).

Convalescence is slow; in about 5-10% of cases, relapses occur during convalescence. The relapse rate is higher in patients treated with chloramphenicol (15-20%) (Ananthanarayan and Paniker, 1997).

2.18 Epidemiology:
In the vast majority of cases, human beings acquire *Salmonella* by the ingestion of contaminated food or water. Direct faecal-oral spread can occur, particularly in children. Transmission by the airborne route is thought to occur rarely (Cheesbrough, 2000).

A few *Salmonella* serotypes are highly host adapted and tend to be virtually “species specific”. For example, the only known reservoir for *S. typhi* is man, and infection with the organism strongly implies direct or indirect exposure to a human source (Mandell, *et al.*, 1985).

Typhoid fever has been virtually eliminated from the advanced countries during the last several decades mainly as a result of improvements in water supply and sanitation but it continues to be endemic in the poor nations of the world (Thong *et al.*, 1998). It is endemic in developing countries, in Africa, South Central America and the Indian subcontinent. By contrast, in developed countries incidence is much lower (Threlfall and Ward, 2001). The control of paratyphoid fever has not been successful. The distribution of paratyphoid bacilli shows marked geographical differences. *S. paratyphi A* is prevalent in India and other Asian countries, Eastern Europe and South America, *S. paratyphi B* in Western Europe, Britain and north America; and *S. paratyphi C* in eastern Europe and Guyana (Rowe, *et al.*, 1997).
Carriers occur with paratyphoid bacilli also. While *S. paratyphi* A occurs in human beings, *S. paratyphi* B can infect animals such as dogs or cows, which may act as sources of human disease (Cheesbrough, 2000). Typhoid occurs into two epidemiological types. The first is endemic residual typhoid that occurs through the year, though seasonal variation may sometimes be apparent. The second is epidemic typhoid, which may occur in endemic or non-endemic areas irrespective of season (Johnson, *et al.*, 1996).

Typhoid epidemics are water, milk or food-borne. Water-borne epidemics, once so common, have become rare due to better control of drinking water. Milk-borne epidemics also have become more rare due to the general application of pasteurization. Food-borne outbreaks may be due to contamination of tinned stuffs, vegetable or shellfish which are eaten raw or uncooked (Rowe, *et al.*, 1997).

### 2.18.1 *Salmonella enterica* serotype *typhi* outbreaks:

After extensive outbreaks of typhoid fever caused by *S. enterica* serotype *typhi* which occurred in Mexico in the early and mid 1970s, it was reported that epidemic strains were resistant to chloramphenicol (Anderson and Smith, 1972; Paniker and Vilma, 1972).
2.18.2 *Salmonella paratyphi* A outbreaks:

Outbreaks of enteric fever associated with *S. paratyphi* A have rarely been reported in India (Thong, *et al.*, 1998; Sood, *et al.*, 1999). In 1998, the incidence of enteric fever, caused by drug-resistant *S. paratyphi* A, abruptly increased in the New Delhi region. In the first 6 months of treatment in 1999, 32% of isolates were found resistant to both chloramphenicol and co-trimoxazole and another 13% resistant to more than two other antibiotics. *Salmonella paratyphi* A, which caused 1 to 15% of the enteric fever cases in India, has been increasing since 1996 (Sood, *et al.*, 1999). The studies proved that some of the isolates from the New Delhi region had a decreased susceptibility to ciprofloxacin, the drug of choice for enteric fever in India (Chandel, *et al.*, 2000).

2.18.3 *Salmonella paratyphi* B outbreaks:

Incidence of *Salmonella paratyphi* B has generally increased since 1998; the spike in *S. paratyphi* B in the third and fourth quarters of 1999 can be attributed to an outbreak in British Columbia, Alberta, and Saskatchewan caused by contaminated alfalfa sprouts (Stratton, *et al.*, 2000).
In addition to this large outbreak, additional outbreaks were reported between 1998 and 2002. However each outbreak was small, and most involved fewer than six patients (Mulvey, et al., 2004). Antimicrobial susceptibility testing was performed on all strains by using the disk-diffusion method as described by the National Committee for Clinical Laboratories Standards (NCCLS). Susceptibilities were determined for ampicillin (A), chloramphenicol (C), ciprofloxacin (Cp), streptomycin (S), sulfamethoxazole (Su), tetracycline (T), and trimethoprim (Tm). Some isolates displayed resistance to five commonly used antibiotics (ACSSu and T) (Mulvey, et al., 2004). The emergence of multi-resistant \textit{S. paratyphi} B was documented recently in Netherlands and Scotland (van Pelt, et al., 2003; Brown, et al., 2003).

\subsection*{2.18.4 Chronic carrier state:}

Enteric fever occurs at all ages but is probably most common in the 5-20 year age group. The age incidence is related to the endemicity of the disease and the level of sanitation (Cheesbrough, 2000). The faeces of persons who have unsuspected subclinical disease or are carriers are more important as a source of contamination than those of frank clinical cases that are promptly isolated; e.g. when carriers work as food handlers they are “shedding” the organism (Brooks, et al., 1998). The source of infection, a patient, or far more frequently, a carrier
patient, who continues to shed typhoid bacilli in faeces weeks to three months after clinical cure is called a “convalescent carrier“. Those who shed the bacilli for more than three months but less than a year are called “temporary carriers” and those who shed the bacilli for over a year are called “chronic carriers”. In other words, persons who continue to excrete organisms more than a year after initial discovery of organisms in stool are designated chronic carriers (Mandell, et al., 1985).

The development of the carrier state is more common in women and in the older age groups (over 40 years). Some persons may become carriers, following a past infection, symptomless excretors. The shedding of the bacilli is usually intermittent in this case (Paniker and Vilma, 1997).

Food handlers or cooks who become carriers are particularly dangerous. The best known of such typhoid carriers was Mary Mallon (Typhoid Mary), a New York cook who, over a period of 15 years, caused at least seven outbreaks affecting over 200 persons,(Curtis, et al 1983).

Despite recovery from the acute phase of the disease, some individuals continue to excrete the organism in the faeces or less often in urine (MacSween and Whaley, 1992). Excretion of organisms in stool after enteric fever persists for variable periods of time, usually a few

Incidence of development of chronic enteric carrier state after typhoid fever increases with age (Mandell, et al., 1985). In most cases the carrier state is due to persistence of gallbladder infections. Typhoid cholecystitis is probably initiated during the bacterimic phase, and represents the reservoir from which all cases are directly or indirectly acquired (MacSween and Whaley, 1992). In most carriers, the organism persists in the gallbladder, particularly if gallstones are present, and in the biliary tract (Brooks, et al., 1998).

Patients with Schistosoma haematobium involvement, in the urinary tract, have a propensity to become chronic urinary carriers after infection. Urinary carriers may continue to excrete large numbers of bacilli in urine for months or a year (Mandell, et al., 1985).

In schistosomiasis endemic areas there are high incidences of chronic S. typhi and S. paratyphi infections and carriers. Salmonella
colonises adult *Schistosoma* flukes and are protected from antibiotics (Cheesbrough, 2000).

Prolonged or recurrent typhoid fever is usually regarded as minor or even episodes of salmonelluria are not uncommon in patients infected with *Schistosoma* species. In such cases *Salmonella* infection is often apparently resistant to antibiotic therapy. *Schistosoma–Salmonella* interactions have been described with all species of *Schistosoma*, notably *S. haematobium*, *S. mansoni*, *S. intercalatum* and *S. japonicum*. The adult worms of *Schistosoma* live in the mesenteric venous plexuses; *Salmonellae* electively stick onto the outer wall of adult *Schistosomes*. As a result of this, *Salmonella* septicaemia is facilitated and sustained by schistosomal infestation, and several varieties of *Salmonella* may be involved. These *Salmonella* infections can not be cured without the treatment of the associated schistosomiasis (Gendrel, 1993).

A study of 150 files and records of patients suffering from typhoid or paratyphoid fever, in Libreville, revealed that the incidence rates of salmonellosis associated with schistosomiasis constituted 13% whilst with nematodiasis 2.3%, with sickle cell anaemia 7% and with HIV infection 8% (Okome, et al., 2000).

2.18.5 Diagnosis of carriers:
The Widal reaction is of no value in the detection of carriers in endemic countries. The identification of faecal carriers is by isolation of the bacillus from faeces or from bile (Mandell, et al., 1985). Culture of bile obtained by duodenal aspiration is usually positive and may be employed for the detection of carrier. The tracing of carriers in cities may be accomplished by screening food handlers and cooks. Testing for the presence of antibody to the Vi antigen has been suggested as a mechanism for screening for typhoid carriers (Rowe et al., 1997).

2.18.6 Treatment of chronic carriers:

While antimicrobial therapy has been so effective in the treatment of cases, it has been disappointing in the treatment of carriers. A combination of antimicrobial therapy along with a vaccine have been tried for the eradication of carrier state. Some chronic carriers have been cured by ampicillin alone (Brooks, et al., 1998). Elimination of the carrier state may require heroic measures such as cholecystectomy, pyeloithoctomy or nephrectomy (Johnson, et al., 1996).

2.19 Management of enteric fever:

2.19.1 Therapeutic approach to patients with enteric fever:

Specific antimicrobial therapy for enteric fever became available only in 1948 with the introduction of chloramphenicol (Rowe, et al.,
Many strains are sensitive to chloramphenicol, ampicillin, gentamicin, tetracycline, co-trimoxazole and some other antibiotics (Threlfall, Rowe and Ward, 1992). Chloramphenicol was considered to be the most effective agent for the treatment of typhoid fever; an alternative drug was co-trimoxazole (Mandell, et al., 1985; Collee, et al., 1996). Chloramphenicol was the first line of choice and in developed countries its use resulted in a reduction in mortality rates. Ampicillin not chloramphenicol may terminate the chronic carrier state and is preferred in intravascular infection. Trimethoprim-sulfamethoxazole (co-trimoxazole) is especially useful in Salmonella infections caused by organisms resistant to both chloramphenicol and ampicillin (Mandell, et al., 1985). Ciprofloxacin should be considered as the drug of choice for treatment of typhoid fever in patients likely to have acquired infection in areas reporting multi resistant strains (Collee, et al., 1996). All strains with decreased sensitivity to ciprofloxacin were also resistant to nalidixic acid (Threlfall and Ward, 2001). It is necessary to test antibiotic sensitivities of any strain isolated from a septicaemic illness (Collee, et al., 1996). Though S. typhi is susceptible, in vitro, to many antibiotics such as streptomycin and tetracycline, these drugs are ineffective in vivo, (Johnson, et al., 1996).

2.20 Complications of antimicrobial therapy:
Antimicrobial therapy may lead to complications in patients with typhoid fever. The rare idiosyncratic reaction to chloramphenicol, characterized by severe aplastic anaemia, occurs in about 1 of 25,000 persons treated with this antibiotic. Ampicillin and amoxicillin produce rashes and hypersensitivity reactions, and trimethoprim-sulphamexazole (co-trimoxazole), may cause bone marrow suppression and abnormalities in hepatic function. Toxic crisis in patients with typhoid are exacerbation of symptoms of toxaemia temporarily related to administration of antibiotics. These reactions have been attributed to sudden release of endotoxin and considered analogous to the Jarish-Herxheimer reaction seen in patients with syphilis treated with penicillin. The relationship of these “reactions” to antimicrobial therapy is by no means clearly established (Thong, et al., 1998).

2.21 Drug resistance:

In persons receiving antimicrobial drugs orally for several days, parts of the normal intestinal flora may be suppressed. Drug-resistant organisms may establish themselves in the bowel in great numbers and may precipitate serious enterocolitis (Brooks, et al., 1998). Resistance to individual drugs depends on serotype, phage type, and country of origin of Salmonella, (Threlfall, Rowe and Ward, 1991).
Though occasional strains had been identified in the laboratory, resistance to chloramphenicol did not pose any problem in typhoid fever till 1972, when resistant strains emerged in Kerala (India), where these strains became endemic. Subsequently such strains carrying drug resistant plasmids appeared in many other parts of India (Ananthanarayan and Paniker, 1997). Alternative drugs for typhoid fever are ampicillin and trimethoprim. However, following outbreaks in the Indian subcontinent, the Arabian Gulf, the Philippines, in the late 1980s and early 1990s, causative strains were found to be resistant to ampicillin and trimethoprim in addition chloramphenicol. The efficacy of these antimicrobials has also been impaired (Threlfall and Ward, 2001). Trimethoprim-sulfamethoxazole (co-trimoxazole), has been used in recent years in the treatment of enteric fever. Reports of efficacy have varied from claims of equivalence to chloramphenicol to unsatisfactory bacteriologic response. These variations in results have been attributed to the administration of insufficient doses of drugs and differences in strains of S. typhi, and variation in immunity in different geographical areas (Mandell, et al., 1985). During 1996-97, isolates uniformly susceptible to all antibiotics, including ciprofloxacin and ceftriaxone were commonly used in the treatment of enteric fever. However, in 1998 the incidence of enteric fever caused by drug resistant S. paratyphi A,
abruptly increased, and a number of drug resistant isolates susceptible to ciprofloxacin markedly decreased (Chandel, et al., 2000).

### 2.21.1 R plasmids:

Most wild antibiotic–resistant bacteria are not chromosomal mutants but carry the resistant genes in plasmids (R plasmids) and mechanism of resistance is usually quite distinct from that of chromosomal mutants resistant to the same antibiotic (Bull and Meadow, 1978). In all multidrug–resistant (MDR) strains of typhoid bacilli, so far examined, multiple resistance has been encoded by plasmids of the H₁ incompatibility group (Rowe, et al., 1997).

These plasmids cause considerable medical and veterinary problems because many of them are conjugative and can be transferred, particularly between different genera of enterobacteria. Beside many of them specify resistance to more than one drug, and in some cases to as many as six or seven unrelated drugs (Bull and Meadow, 1978).

### 2.21.2 Biochemical mechanism of plasmid-coded resistance:

R plasmids coding for resistance to, virtually, all the clinically used antimicrobial drugs have been described. The biochemical means
by which the resistance is achieved differs from one drug to another, but for any particular drug the mechanism appears almost universal, irrespective of the organism, from which the plasmids are isolated. Resistance to chloramphenicol is the result of plasmid-coded enzymes, which chemically modify the antibiotic to a non-toxic form (Bull and Meadow, 1978). The R plasmid coding for chloramphenicol resistance, also, codes for sulphonamide resistance; therefore only the trimethoprim portion of the drug is active against multiple resistant strains (Thong, et al., 1998).

CHAPTER THREE

3. Materials and Methods
3.1 Materials:

3.1.1 Culture Media:

Different types of culture media including solid, semi-solid, and liquid media were used to identify the aerobic bacteria. All culture media were prepared according to the methods described by the manufacturer.

3.1.1.1 Solid Media:

3.1.1.1.1 Brilliant green bismuth sulphite agar (modified Wilson & Blair’s medium) (Code CM 329):

Oxoid Ltd supplied this medium. It consisted of peptone (Oxoid L37), dextrose, disodium phosphate, ferrous sulphate, bismuth sulphite indicator, brilliant green and agar No3 (Oxoid L13). Four grams of the medium were dissolved completely by boiling in 100 ml of distilled water. The pH was adjusted to 7.7, then was sterilized by boiling only without autoclaving. The medium was cooled to 55°C and distributed into sterile petri dishes in 25 ml volumes (thick plates).

3.1.1.1.2 Salmonella Shigella Agar (SSA) (code CM108):

Oxoid Ltd., England supplied this medium. It consisted of peptone, lactose, bile salts, sodium citrate, sodium thiosulphate, ferric citrate, brilliant green, neutral red, and agar. Sixty-three grams of the medium were dissolved in one litre of distilled water by boiling. The pH was adjusted
to 7.0, cooled to 55°C and distributed aseptically without sterilization (according to the manufacturer) into sterile petri dishes, 20 ml in each dish.

3.1.1.1.3 MacConkey Agar (Code CM 47):

Oxoid Ltd., England supplied this medium. Fifty-two grams of the powder were dissolved in 1 litre of distilled water by boiling. The pH was adjusted to 7.4, then the medium was sterilized by autoclaving (121°C for 15 minutes), and cooled to 50°C, then distributed into sterile petri dishes 20 ml each.

3.1.1.1.4 Nutrient Agar:

Plasmatic Ltd., UK, supplied the medium. It was prepared according to the manufacturer by dissolving 28 g in 1 liter distilled water, the pH was adjusted to 7.4, then was sterilized by autoclaving (121°C for 15 minutes), cooled to 50-55°C and then distributed into sterile petri dishes, 20 ml each.

3.1.3.1.5 Christensen’s Urea Agar:

Plasmatic Ltd., UK supplied this medium. It was prepared by dissolving 2.4 g of the powder in 95 ml of distilled water by boiling. The pH was adjusted to 6.8, then the medium was autoclaved at 121°C for 15 minutes. The base medium was cooled to 50°C and aseptically 5 ml of sterile 40% urea solution were added. And the medium was distributed into screw-capped bottles 10 ml each, and then allowed to set in the slope position.
3.1.1.1.5 Kligler Iron Agar (KIA):

Plasmatic Ltd., UK supplied this medium. According to the manufacturer, 38.5 g of the powder was dissolved in 1 liter of distilled water by boiling. Then the medium was distributed into test tubes 5 ml each. The tubes were sterilized by autoclaving (121°C for 15 minutes), cooled and positioned to give a butt and slant in each tube.

3.1.1.1.6 Simmons’s Citrate Medium:

Plasmatic Ltd., UK supplied this medium. According to the manufacturer, 30 g of the powder were dissolved in 1 liter of distilled water by heating. The medium was distributed into clean test tubes 5 ml each. The tubes were sterilized by autoclaving (121°C for 15 minutes), then cooled and kept for the citrate utilization test.

3.1.1.1.7 Diagnostic Sensitivity Test (DST) Agar (Code CM 261):

Oxoid Ltd. supplied this medium. The medium was prepared by dissolving 40 g in 1 liter of distilled water by boiling. The pH was adjusted to 7.4. Sterilization was done by autoclaving (121°C for 15 minutes). Then, cooled to 50°C and enriched by bovine or sheep blood to enhance the growth of fastidious organisms. Then the medium was distributed into sterile petri dishes 20 ml each.
3.1.1.8 **Brain Heart Infusion Agar (Code CM 275):**

Oxoid Ltd. supplied this medium. It was prepared by dissolving 45 g in 1 liter distilled water by heating. The medium was distributed into clean bottles 10 ml each, the bottles were sterilized by autoclaving (121°C for 15 minutes), then cooled to 50°C and positioned to give slant media. The medium was used for the storage of the isolated bacteria.

3.1.1.2 **Semi-solid Media:**

It used for motility testing. Thirteen grams of the dehydrated nutrient broth were added to 4g of Oxoid agar No. 1 and dissolved in 1 litre of distilled water to give semi-solid medium. The pH was adjusted to 7.4, and then the medium was distributed into clean test tubes 5 ml each. The tubes were sterilized by autoclaving (121°C for 15 minutes), then cooled to ready for motility test.

3.1.1.3 **Liquid Media:**

3.1.1.3.1 **Selenite-F-broth (Code CM 395):**

Oxoid Ltd supplied this medium. It consisted of peptone, lactose, and sodium phosphate. Nineteen grams of the medium were dissolved in one litre of distilled water to which 4g of sodium biselenite (Oxoid L 121), was added. The medium was sterilized by boiling for 10 minutes, mixed well and filled into containers to depth 5 cm.
3.1.1.3.2 Nutrient Broth:

Plasmatic Ltd. supplied this medium. It was prepared by dissolving 13 g of the powder in 1 litre of distilled water. The medium was distributed into clean test tubes, and then tubes were autoclaved at 121°C for 15 minutes. The tubes were then cooled.

3.1.1.3.3 Peptone Water:

Plasmatic Ltd. supplied this medium. It was prepared by dissolving 15 g of the powder in 1 liter of distilled water by shaking or rotation. The pH was adjusted to 7.4, and then the medium was distributed into clean test tubes 5 ml each. The tubes were sterilized by autoclaving (121°C for 15 minutes) and cooled.

3.1.1.3.4 Peptone water sugar:

The medium was prepared according to Ochei and Kolhatkar (2000). Ninety ml of peptone water was prepared and the pH was adjusted to 7.1. Ten ml of Andrade’s indicator were added. The medium was distributed into test tubes with sterile Durham’s tubes inside each tube, in 5 ml volume for each tube. The tubes were autoclaved at 121°C for 15 minutes. Sugar solutions were prepared by dissolving 10 g of the appropriate sugar in 100 ml of distilled water. The sugar solutions in the volume of 0.25 ml were dispensed into each tube aseptically. The carbohydrates used in this test were glucose, lactose, sucrose, maltose and mannitol.
3.1.1.3.5 Glucose phosphate peptone broth:

The medium was prepared according to Barrow and Feltham (1993). Five grams of peptone, 5g of potassium phosphate were added to 1 litre of distilled water and steamed until the solids were dissolved. The pH was adjusted to 7.5, and then 5g of glucose were added and mixed. The medium was distributed into test tubes 5 ml each and sterilized by autoclaving at 115°C for 10 minutes.

3.1.1.3.6 Nitrate Broth:

This medium contained 1g of potassium nitrate; it was prepared according to Barrow and Feltham (1993), by dissolving the nitrate in 1 litre of nutrient broth, distributed into test tubes and then sterilized by autoclaving at 121°C for 15 minutes.

3.1.1.3.7 Brilliant green bile (2%) broth (Code CM 31):

Oxoid supplied this medium. It consisted of peptone, lactose, Ox-Bile (purified) and brilliant green. Forty grams of the medium were dissolved in one litre of distilled water. It was mixed well, and distributed into containers. Then it was sterilized by autoclaving at 121°C for 15 minutes.

3.1.1.4 Brilliant green bile diphasic medium (Code CM 31):

Brilliant green bile diphasic medium (Castañeda form) consisted of brilliant green bile broth and a slope of brilliant green bile agar.
3.1.1.4.1 Brilliant green bile agar (Code CM 263):

It was obtained by adding 1.5 g of agar to brilliant green bile broth and was dispensed in 25 ml amounts in medical bottles (200 ml), flat type fitted with screw-caps that had a central hole and rubber liner. It was sterilized by autoclaving at 121 C for 15 minutes. Then allowed to cool in a horizontal position to give an agar slope along one side.

3.1.1.4.2 Brilliant green bile broth (Code CM 31):

Twenty five ml of brilliant green bile broth were dispensed aseptically in each bottle containing an agar slope. Each bottle was covered with a foil cap. The bottles were stored in an upright position in a dark cool place.

3.1.2 Chemicals and Reagents:

All reagents were kept in well-closed glass stoppered bottles and protected from direct sun light.

3.1.2.1 Gram’s solutions (The British Drug Houses): The reagents were prepared according to Ochei and Kolhatkar (2000):

3.1.2.1.1 Crystal violet: Crystal violet powder was dissolved in distilled water to give 1% concentration.
3.1.2.1.2 Lugol’s Iodine: This was prepared by dissolving 20 grams potassium iodide in 50 ml distilled water and then dissolving 10 grams iodine by shaking and make up the volume to 1000 ml.

3.1.2.1.3 Decolourizing Reagent: This was prepared by mixture 1:1 of ethyl alcohol and acetone.

3.1.2.1.4 Counter Stain: This was prepared by dissolving 0.5 grams Safranin in 100 ml distilled water. Also was prepared by diluting strong Carbol fuchsin (1:10) distilled water.

3.1.2.2 Andrade’s Indicator: This was prepared according to Ochei and Kolhatkar (2000) by dissolving 5 grams of acid fuchsin in one litre of distilled water and 150 ml alkali solution (NaOH) was added. It was used in peptone water sugar medium.

3.1.2.3 Methyl red solution: This solution was prepared as described by Barrow and Feltham (1993) by dissolving 0.04 grams of methyl red powder in 40 ml of ethanol and the volume was diluted with distilled water to 100 ml. It was used in methyl red test.

3.1.2.4 Voges-Proskauer (V.P) test reagent: This reagent was composed of 40% potassium hydroxide (KOH) and 5% alpha-naphthol in absolute ethanol.

3.1.2.5 Nitrate test reagent: This reagent was composed of two solutions:
Solution A: Sulphanilic acid 0.33% in 5 N-acetic acid dissolved by gentle heat.

Solution B: Dimethyl-alphanaphthylamine 0.6% in 5 N-acetic acid. The complete reagent was used in nitrate test.

3.1.2.6 **Oxidase reagent:** The reagent is not stable. It was therefore prepared immediately before use. It was prepared by dissolving 0.1 grams tetramethyl-p-phenylene diamine dihydrochloride in 10 ml distilled water (Cheesbrough, 2000).

3.1.2.7 **Physiological saline:** It was prepared according to Ochei and Kolhatkar (2000) by dissolving 9 grams of sodium chloride in 1 litre distilled water.

3.1.2.8 **Kovac’s reagent:**

It was prepared according to Barrow and Feltham (1993) by dissolving 5 grams of para-dimethylaminobenzaldehyde in 75 ml amyl alcohol by gentle warming in a water bath, about 50-55 C. Then cooled and added to 25 ml concentrated HCL. Protected from direct sun light and stored at 4 C.

3.1.3 **Biological Materials:**

Bovine Blood or Sheep Blood (defibrinated blood), was used for the enrichment of media. They were obtained fresh and free from anticoagulants and antimicrobial agents.
3.1.4 Antibiotic Discs:

Plasmatic Ltd., UK supplied all antibiotic discs used in the test of antibiotic susceptibility test. The antibiotics used were ciprofloxacin (5 µg), chloramphenicol (30 µg), trimethoprim (10 µg), ampicillin (10 µg), nalidixic acid (10 µg) and co-trimoxazole (25 µg).

3.1.5 Test Control Organisms:

Organisms from American Type of Culture Collection (ATCC) (USA), and National Collection of Type Culture (NCTC) (UK), were used as test control organisms. They were *Salmonella* typhi (ATCC St. 19430), and *Salmonella paratyphi B* (NCTC).

3.1.6 API System:

API-20E Test kits for the identification of enteric bacteria were used to confirm the isolated bacteria. BioMerieux, Inc. Hazelwood, MO., France, supplied the kits.

3.2 Methods:

3.2.1 Sterilization and disinfectants:

The glassware (Petri dishes, Pipettes, test tubes, flasks, etc.), was sterilized in the hot air oven at 160 C for 1 hour

Media and solutions were sterilized by autoclaving at 15 pounds per square inch (psi) for 15 minutes at 121C. Carbohydrate media were sterilized by autoclaving at 110 C for 10 minutes.
Formalin of concentration 40% was used for sterilizing the room media.

Inoculating wire loops, needles and points of forceps were sterilized by direct flame.

Phenolic disinfectant was used for disinfecting floor, walls, and the roof of the laboratory. Alcohol (70%)(v/v), was used for disinfecting the benches.

3.2.2 The site of study:

El-rahad Irrigated Scheme, Gadarif State – Eastern Sudan, was chosen as the site for the present study. El-fao town comprises sporadically scattered villages and hamlets that rely largely on subsistence agriculture. The relatively close proximity of this town to Gadarif town provides practical advantages for epidemiologic and laboratory support.

3.2.3 Study Design:

A retrospective study was carried out in Gadarif State, Sudan (Gadarif town, El-hawata town, and El-fao town). The period of the study was from December 2004 to June 2005. The targeted population were the patients already diagnosed as suffering from typhoid fever.

3.2.3.1 Sample size: -

Seventy-one specimens were collected from patients, already diagnosed as suffering from typhoid fever with significant titres of 1/320
or more. Twenty of these had significant titre to *S*. *typhi*, 29 *S*. *paratyphi* B and 22 to both *S*. *typhi* and *S*. *paratyphi* B.

3.2.3.2 Specimen Collection:

The questionnaire included all 71 patients who had significant titres to the Widal test at the time of sample collection. Thirty eight of these patients already treated with one or more of the commonly used antibiotics for treatment of enteric fever, namely chloramphenicol, ciprofloxacin and ampicillin. Twenty three patients were treated with one antibiotic, 8 with two antibiotics while 7 had received three antibiotics. The remaining 33 patients did not start any treatment. Chloramphenicol was the most commonly used antibiotic followed by ciprofloxacin.

Blood specimens, clotted blood, and freshly passed stool were collected from patients clinically diagnosed as having enteric fever. Specimens were labelled, kept and transferred immediately to the laboratory for bacteriological examination.

3.2.4 Cultural Methods:

3.2.4.1 Blood culture: -

Five ml of venous blood was taken from every patient and put immediately in a culture bottle using Castañaeda method, where, a double
medium was used, containing bile broth with agar slant on the side. After the incubation of blood, the bottle was incubated in the upright position. For the subculture, the bottle was merely tilted so that the broth runs onto the surface of agar. It was re-incubated in the upright position. If *Salmonella* was present, colonies would appear in the slant side. A second subculture was performed on brilliant green bismuth sulphite agar, MacConkey agar and *Salmonella-Shigella* agar media. It was incubated at 37°C for another 24-48 hours. After that the plates were followed up for growth. If there have been growth non-lactose fermenters in (SSA), sometimes with clear edge and black colonies with metallic sheen in BBSA, then *Salmonella* has to be strongly suspected and further identification tests were performed.

3.2.4.2 Clot culture: -

Five ml of blood were withdrawn from the patient into a sterile test tube and left to clot. The serum was pipetted off and used for the Widal test. The clot was broken up with a sterile glass rod and added to a bottle of bile broth. Subculture was transferred onto SSA and BBSA.

3.2.4.3 Faeces culture: -

Freshly passed stool was collected in a stool container.

- **First day:** A suspension of sample in saline was used to inoculate plates of *Salmonella-Shigella* Agar (SSA), Brilliant green bismuth
sulphite agar (BBSA), and a bottle of selenite–F-broth, and then all were incubated at 37 C for 18-24 hours.

- **Second day:** The Selenite–F-broth was used to inoculate plates of *Salmonella Shigella* Agar (SSA) and Brilliant green bismuth sulphite (BBSA).

- **Third day and onwards:**
  Colonial morphology was observed for the presumptive colonies. Gram stain was done for these colonies followed by motility testing and biochemical tests which include indole production test, Simmons’s citrate, Voges Proskauer (VP), methyl red (MR), urease test and nitrate reduction test.
  
  Carbohydrate fermentation test were performed for further identification of the microorganism.

**3.2.5 Examination of Cultures:**

Cultures, which were semi-quantitatively determined as conforming to the concept of significant bacterial growth (i.e. presence of 100 or more colonies on the plate culture, because of the use of nichrome loop holding 0.001 ml), were subjected to further identification.

All cultures were examined with the naked eye for growth and colonial morphology as well as any changes in the media. Plates, which showed visible growth, were subjected to subsequent bacteriological tests.
Those which with no visible growth, were reincubated and examined daily for up to 7 days.

3.2.5.1 Purification of Isolates:

The primary isolates were subcultured on nutrient agar. This was achieved by several subcultures of a typical and a well-isolated colony from the corresponding medium. The process was repeated till purification was achieved. The resulting growth was checked for purity by Gram’s stain and examined microscopically.

3.2.5.2 Identification of the Isolated Bacteria:

3.2.5.2.1 Primary Identification:

Gram’s staining to see the shape, arrangement and Gram’s reaction were performed for primary identification.

Bacterial smears were prepared by emulsifying a small inoculum of the bacterial culture in a drop of normal saline and spreading it onto a clean glass slide (15-20 mm). The smears were allowed to dry on air and then fixed by gentle heating.

3.2.5.2.1.1 Gram’s Stain:

Gram’s stain was applied according to Ochei and Feltham (2000). The slides (smears), were placed on the staining rack and were flooded with crystal violet stain (base stain), for 1 minute, then the stain was washed off with distilled water. The smears were covered with Lugol’s
iodine (mordant) for 1 minute, and then washed. Acetone was used for the
decolourisation for 1-2 seconds, and then rinsed in distilled water. The
smears were counter stained with safranin for 1 minute, rinsed in water,
blotted with filter paper and allowed to air-dry. The prepared slides were
examined microscopically with oil immersion lens objective. Bacteria
coloured violet was labelled as Gram positive and red-coloured bacteria
were labelled Gram negative.

3.2.5.2 Secondary Identification of Isolated Bacteria:

This was done by using the biochemical tests, which were performed
according to Barrow and Feltham (1993).

3.2.5.2.1 Oxidase Tests:

A piece of filter paper approximately 2 cm in diameter, 5 cm in
length was placed in a petri dish and 2-3 drops of the solution of
tetramethyl-p-phenylene diamine dihydrochloride were added. A small
portion of the tested organism culture was picked with a sterile bent glass
rod and rubbed on the filter paper. A dark purple colour that developed
within 5-10 seconds was considered as a positive result.

3.2.5.2.2 Sugar Fermentation Tests:

The sugar media were inoculated with the test organisms and
incubated at 37 C overnight. They were examined daily for 7 days. Acid
production was indicated by the development of a pink colour in the
medium. Gas production was indicated by air trapped in the Durham’s tube. The sugars used in these tests were glucose, lactose, salacin, sucrose, maltose and mannitol.

**3.2.5.2.3 Indole Test:**

The tested organism was inoculated in peptone water and incubated at 37°C for 48 hours. Then 0.5 ml of Kovac’s reagent was added. To indicate indole the tube was shaken well and examined after one minute. The production of a red colour on the upper layer of the reagent indicated indole production.

**3.2.5.2.4 Voges-Proskauer (V.P) Test:**

The test was used to detect the production of acetyl-methyl carbinol. Glucose phosphate peptone medium was inoculated with the test organism and incubated at 37°C for 48 hours. Then 0.6 ml of 5% alpha-naphthol followed by the addition of 0.2 ml of 40% potassium hydroxide aqueous solution to one ml of the culture, shaken well and examined after 15 minutes and one hour. A bright pink colour indicated positive reaction.

**3.2.5.2.5 Methyl Red Test:**

The test was made to detect the production of prolonged acidity from glucose in buffered medium. Glucose phosphate peptone medium was inoculated with the tested organism. The inoculated tubes were incubated at 37°C for 48 hours. Five to six drops of methyl red solution were added and
the tube was then shaked. The development of red colour indicated a positive result.

3.2.5.2.6 Urease Test:

The tested organism was inoculated on a slope of urea agar medium and incubated at 37°C for 24 hours. A change in colour of the medium to red was considered positive.

3.2.5.2.7 Nitrate Test:

The test organism was grown in nitrate broth and incubated at 37°C for up to 5 days. One ml of nitrate reagent A was added followed by 1 ml of reagent B. A deep red colour indicated a positive test of nitrate reduction. To tubes that did not show red colour within 5 minutes, were treated with powdered zinc and allowed to stand. The formation of a red colour indicated that nitrate was present but the tested was not reduced by the organism.

3.2.5.2.8 Citrate Utilization Test:

Tubes of Simmons’s citrate medium were inoculated with the tested organism by stab inoculation. The inoculated tubes were incubated at 37°C for 24 hours. Positive results were indicated by change in the colour of the medium to blue. When there was no change in colour occurred, i.e. green), the result was considered negative.
3.2.5.2.9 Motility Test:

The motility test was done through inoculating motility media (semi-solid media) by stabbing with a bacteriological needle. Tubes were incubated at 37°C for 24 hours. The tested organism was considered motile when it formed a diffuse growth inside the medium. Non-motile organisms were those with growth along the stab area (line) only.

3.2.6 Susceptibility to Antibiotics:

All isolated bacteria were examined for their susceptibility to a number of antibiotics by disc diffusion technique (Kirby-Bauer method). The isolate was grown in peptone water and incubated at 37°C for 24 hours. A sterile cotton swab immersed into the growth was used to inoculate a plate of Diagnostic Sensitivity Test (DST) agar medium. The inoculation was evenly distributed all over the plate. Commercially prepared antibiotic discs of Plasmatic Ltd., UK were placed on the surface of the medium using sterile forceps. Discs were gently pressed to ensure full contact with the medium. Plates were incubated at 37°C for 24 hours. The zone of growth inhibition around each disc was measured in millimetres and the result was reported sensitive, intermediate or resistant. Antibiotics used in susceptibility test were ciprofloxacin (5µg), chloramphenicol (30 µg),
trimethoprim (10 µg), ampicillin (10 µg), co-trimoxazole (10 µg) and nalidixic acid (10 µg).

### 3.2.6.1 Interpretation of the susceptibility test

Inhibition zones of the test organisms were compared with those of the reference organisms they were *S. typhi* (ATCC St. 19430) from American Type of Culture Collection (ATCC) (USA), and *S. paratyphi* B from (National Collection of Type Culture (NCTC) (UK). The zones were measured by laying a millimetre ruler across the dish. Provided that the inoculum was optimal, results were interpreted and reported as follows:

- **Sensitive**: inhibition zone radius was equal to or wider than 3mm, yet smaller than the control by not more than 3mm.
- **Intermediate**: inhibition zone radius was wider than 2 mm yet smaller than the control by more than 3 mm.
- **Resistant**: inhibition zone radius was 2 mm or less.

### 3.2.7 API System:

All isolated bacteria were subjected to API system of identification to confirm primary and secondary identification; API-20E system was employed to identify members of the family enterobacteriaceae.

A plastic strip holding twenty mini-test tubes was inoculated with a saline suspension of a pure culture (according to manufacturer’s directions from BioMerieux, Inc. Hazelwood, MO, France). This process rehydrated
the already desiccated medium in each tube. Few tubes were completely filled and some tubes were overlaid with mineral oil so that anaerobic reactions could be carried out.

After incubation in a humidity chamber at 37°C for 18-24 hours, the colour reactions were read out, and the reactions were converted to a seven-digit code. The code was checked in the manufacturer’s manual to obtain the identification result, usually as genus and species with the corresponding percentage of identification.

3.2.8 Cases taken into consideration:

From all blood specimens, beside the bacteriological investigation, a blood film was tested for malarial infection and similarly from faeces specimens were tested for schistosomal infections. A wet mount technique was applied.
Out of 71 individual stool specimens that were collected from patients with significant titre to Widal test and cultured aerobically, 47 specimens gave growth to *Salmonella* species, of the remaining 24 the only non lactose fermenting bacteria isolated were *Proteus* species (16 isolates) and *Pseudomonas* species (five isolates). Of clotted blood specimens 33 gave growth to *Salmonella* species, 18 specimens gave growth to other types of bacteria and 6 specimens gave no growth. Of venous blood specimens 18 gave growth to *Salmonella* species, 15 specimens gave growth to other types of bacteria and 9 specimens gave no growth (table 1). *Salmonella* was not noted from any of the patients showing significant titres to *S. typhi* alone.

**Table (1): Bacteria isolated from different specimens obtained from 71 patients with significant titre to Widal test**

<table>
<thead>
<tr>
<th>Bacteria isolated</th>
<th>Type of specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stool</td>
</tr>
<tr>
<td></td>
<td>Clotted blood</td>
</tr>
<tr>
<td></td>
<td>Venous blood</td>
</tr>
</tbody>
</table>
All *Salmonella* isolates were completely identified biochemically as *Salmonella paratyphi* B. Results of biochemical tests used for the identification of suspected *Salmonella* isolates are shown in table (2). Some of these isolates were sent to the National Health Laboratory (Khartoum) for confirmation and the results were consistent with these obtained in this study.

**Table (2): Biochemical reactions of *Salmonella* isolated from patients & those of reference strains *Salmonella* typhi (ATCC 19430), and *Salmonella paratyphi B* (NCTC 15632).**

<table>
<thead>
<tr>
<th>Test</th>
<th>Control organisms</th>
<th><em>Salmonella</em> isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>S. typhi</em></td>
<td><em>S. paratyphi B</em></td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Indole</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Citrate</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Vogues Proskauer (VP)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methyl Red (MR)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>-</td>
<td>-</td>
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<tr>
<td>---------</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Urease</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H₂S (KIA)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glucose</td>
<td>+(A)</td>
<td>+(AG)</td>
</tr>
<tr>
<td>Lactose</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Maltose</td>
<td>+(A)</td>
<td>+(AG)</td>
</tr>
<tr>
<td>Mannitol</td>
<td>+(A)</td>
<td>+(AG)</td>
</tr>
<tr>
<td>Salacin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sucrose</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

ATCC = American Type of Culture Collection (USA).
NCTC = National Collection of Type Culture (UK).
A = acid, AG = acid and gas.
(+)= positive (-)= negative.

Of the 47 S. paratyphi B isolates, 34 (72.4%) isolates were from female patients and only 13 (27.6%) isolates were from male patients (table 3).

| Table (3): Isolation of S. paratyphi B According to Gender |
|------------------|------------------|------------------|
| Isolated Organism | Male | Female | Total |
|                  | No   | %     | No   | %     | No   | %     |
| S. paratyphi B    | 13   | 27.7% | 34   | 72.3% | 47   | 100%  |

Out of 38 specimens collected from patients previously treated with one or more of the commonly used antibiotics for treatment of enteric fever, 22 specimens gave growth to S. paratyphi B, 10 specimens gave growth to other types of bacteria and six specimens gave no growth. The remaining 33 specimens which were collected from patients who did not receive antibiotics, 24 specimens gave growth to S. paratyphi B, six gave
growth to other types of bacteria and three specimens gave no growth (table 4).

**Table (4): Isolation of bacteria from treated or untreated patients with antibiotics**

<table>
<thead>
<tr>
<th>Bacterial isolation</th>
<th>Specimens from Patients who received antibiotics</th>
<th>Specimens from Patients who did not receive antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. paratyphi B</em></td>
<td>22</td>
<td>24</td>
</tr>
<tr>
<td><em>Other type of bacteria</em></td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td><em>No growth</em></td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>38</strong></td>
<td><strong>33</strong></td>
</tr>
</tbody>
</table>

In addition to bacteriological culture, all blood specimens were tested for malaria. Twenty three specimens gave positive blood film for malaria parasites but blood specimens and stools from these patients were negative for *Salmonella*. Stools were also tested for *Schistosoma* parasites and 19 patients were found to have simultaneous infection with *Schistosoma* parasites and *S. paratyphi B*.

All 47 isolates of *Salmonella paratyphi B* were subjected to *in vitro* antimicrobial susceptibility tests using the disc diffusion method. The drugs used were chloramphenicol (30 µg), ciprofloxacin (5 µg), nalidixic acid, co-trimoxazole (10 µg), trimethoprim (10 µg), and ampicillin (10 µg). Table (5) shows the susceptibilities of isolates to different antimicrobial agents. Of the 47 strains examined 9 (19.2%) strains showed resistance to five antimicrobial agents namely ciprofloxacin, ampicillin, trimethoprim,
co-trimoxazole, and nalidixic acid. Fourteen isolates (29.8%) showed resistance to ciprofloxacin while 21 isolates (44.7%) showed resistance to co-trimoxazole. Most strains were resistant to ampicillin; 36 isolates (76.6%), whereas none of the isolates was resistant to chloramphenicol.
Fig (1) showing mucoid colonies of *S. paratyphi* B with the characteristic metallic sheen on brilliant green bismuth sulphate agar.
Human carriers are responsible for *S. typhi* infections in man (MacSween, 1992). According to Cheesbrough in (2000), *S. paratyphi* B can infect animals such as dogs or cows, which may act as sources for the human disease. So far, there are no reports of transmission of enteric fever from animals to the humans in Gadarif State. Infection takes place through ingestion of the organism from contaminated hands. *Salmonella typhi* is mainly water-borne, whilst *S. paratyphi* is mainly food-borne. In the present study it was found that *S. paratyphi* B, was the species commonly involved in enteric infections. Transmission was probably via contaminated food or carriers, as community, household and personal hygiene were poor. It was observed in this study that enteric fever was more prevalent in women than men. This observation is similar to that of Paniker and Vilma (1997), who reported that the development of the carrier state was common in women.

One of the most menacing problems that confront the epidemiological control of enteric fever is the phenomenon of carriers and chronic carriers. As reported by MacSween in (1992), despite recovery from the acute phase of the disease, some individuals continue to excrete the organism in the faeces and less often in urine. Mandell
(1985) observed that the excretion of organisms in stool of patients with enteric fever could persist from weeks to more than a year after recovery from the disease or after initial discovery of organisms in stool of chronic carriers. Johnson (1996) noted that the incidence of chronic enteric carrier state after typhoid fever was 1-3%. Chronic carriers are unidentified reservoirs and it appears that they may play a fundamental role in dissemination of the disease in Gadarif State. Patients with Schistosoma haematobium involvement in the urinary tract tend to become chronic urinary carriers for enteric fever organism and may continue to excrete large numbers of bacilli in urine for months or a year. Mandell (1985). Schistosomiasis is common in Gadarif State and the development of urinary carriers of enteric bacilli is likelihood. Such carriers may play a role in the epidemiology of the disease. The identification of carries in Gadarif State and similar areas in the Sudan remains a problem with no perspective of resolution because the Widal test is of no value in the detection of carriers in endemic countries. The identification of faecal carriers is by isolation of the bacilli from faeces or from bile. Rowe (1997), suggested that culture of bile obtained by duodenal aspiration is usually positive and may be employed for the detection of carrier. The tracing of carriers in cities may be accomplished by screening food handlers and cooks. Testing for the presence of
antibody to the Vi antigen has been suggested as a mechanism for screening for typhoid carriers. In this study, diagnostic identification of carriers was difficult, since detection by culturing for every suspected case was out of question, and so testing for antibodies against Vi antigens due to limited facilities in Gadarif State.

Widal test based on bacterial agglutination, is the most widely used test for diagnosis of enteric fever, however it is neither specific nor sensitive (Lim, 1998). It is popularly used due to the fact that it is simple and inexpensive. In Gadarif State Widal test proved excessively unspecific, for it frequently gave false-positive reactions in culturally negative patients. Enterobacteria having similar antigenic determinants to salmonellae causing enteric fever may stimulate production of antibodies that may cross react in the Widal test (Stratton, 2001). Cheesbrough (2000) asserted that the Widal test was entirely not specific, although it was highly sensitive, so its value in diagnosing enteric fever in endemic areas remains controversial.

Some of the blood specimens included in this work were found positive by the blood film test for malaria. Flagellar (H) antibodies of enteric fever bacilli persist long after recovery and their formation may again be stimulated, non-specifically, in subsequent febrile illnesses (Stockes, 1993). It was possible that malaria parasites elevated, non-
specifically, the H titres in some patients included in this study thus affecting results of the serological test.

Serological diagnosis of enteric fever was much used in the past when effective selective media were not available and it was more difficult to isolate the causal, *Salmonella* species Collee (1996). In this study, following serological diagnosis, confirmation was made by using highly selective media for the detection of *S. typhi* and *S. paratyphi* B. Out of the 71 specimens that gave significant titres to Widal test, only 47 showed growth of the causal pathogens of enteric fever in the culturing media, i.e. 66.1% of all the samples. This means that the Widal test cannot be regarded as a standalone method; it should be followed by culturing in highly selective media for the detection of the causal bacteria of enteric fever. In such cases, the Widal test will function only as a primary step for diagnosis for its simplicity and because it gives fast results. In developing countries where typhoid fever is endemic and there are few microbiology laboratories to provide diagnosis by culture, the search should continue forth for non-cultural techniques that provide rapid and reliable diagnosis (Okome *et al.*, 2000).

Emergence of multidrug-resistant enteric pathogens has remained a concern. Brown (2003) documented the emergence of multidrug-resistant *Salmonella paratyphi* B in Canada; similar observations were
made by Van Pelt in the Netherlands, and by Mitchell and co-workers in Scotland (2004). In the present work, it was found that *Salmonella paratyphi* B, which is the major causal of enteric fever in Gadarif State, developed drug resistance. Of 47 isolates from El-fao, Gadarif, and El-hawata regions 29.8% were not susceptible to ciprofloxacin, the drug of choice for treatment of enteric fever in the area. The isolates, also, displayed a resistance to co-trimoxazole and ampicillin. Mulvey (2004) was performed antimicrobial susceptibility testing on strains of *Salmonella paratyphi* B. Susceptibilities were determined for ampicillin, chloramphenicol, ciprofloxacin, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim. and found that some isolates displayed resistance to five of commonly used antibiotics, which were ampicillin, chloramphenicol, streptomycin, sulphamethoxazole, and tetracycline. In view of the findings of the current study, it is suggested that chloramphenicol should be considered the drug of choice for treating cases of enteric fever in Gadarif State, as the isolated *S. paratyphi* B exhibited no resistance to this drug.

One of the most serious complications of enteric fever is relapse. Antimicrobial therapy may increase the rate of relapse occurrence. The relapse rate was found to be doubled in patients receiving chloramphenicol therapy for 2 weeks yet to a less extent with co-
trimoxazole, ampicillin probably did not affect the rate of relapse (Pyatkin and Krivochein, 1987). Studies by Paniker (1997) proved that the relapse rate went as high as (15-20%), in patients treated with chloramphenicol. Convalescence was observed to be slow and in about 5-10% of the cases where the relapses occurred during convalescence.

The antibiotics commonly used in Gadarif State were chloramphenicol, ciprofloxacin and ampicillin. In this study it was observed that some patients complaining from recurring enteric fever. It was found through questionnaires that, 38 patients (53.5%) under study who had a previous infection and were treated with commonly used antibiotics complained from recurrence. It is noticeable that chloramphenicol was administered in most of these cases, followed by ciprofloxacin. Ampicillin was administered in rare cases.

Whether multidrug-resistant *Salmonella paratyphi* B is a more disseminating organism or otherwise, the underlying question remains; why did *salmonella paratyphi* B emerged as a main causative agent of enteric fever in Gadarif State. It may be attributed to poor sanitation in El-fao region. Other factors such as contaminated milk and its products obtained from infected animals could play a role in disseminating this organism. It has been suggested that schistosomiasis may simultaneously be associated with Salmonella infections. Nineteen of the patients
included in this study (40.4%) had schistosomiasis. The schistosomes may contribute to the dissemination of organism and development of carrier state. This has been pointed out by Cheesbrough (2000) who had explained that the *Salmonella* organisms colonised adult flukes, thus being protected from antibiotics and provide questions of a brooding danger on the epidemiological control, detection, diagnosis, and treatment of enteric fever. In other words, *Salmonella* is transmitted through a vector, which may be itself endemic in irrigated areas and areas of non-hygienic water supply sources. Gendrel (1993), reported that *Schistosoma–Salmonella* interactions had been described with all species of *Schistosoma*, The adult of which lives in the mesenteric venous plexuses, Salmonellae are stuck onto its outer wall. As a result of this, *Salmonella* septicaemia facilitated and supported by schistosomal infection, and several varieties of *Salmonella* may be involved. These *Salmonella* infections cannot be cured without the treatment of the associated schistosomiasis. It is suggestible that the shedding of *Salmonella* accompanies the excretions of the fluke. In such cases, *Salmonella* often appears to be resistant to antibiotic therapy and recurrent typhoid fever is not uncommon in patients infected with *Schistosoma*. A study carried out by Okome and co-workers on patients suffering typhoid or paratyphoid fever, in Libreville, the capital of
Gabon in (2000), documented that the incidence rates of salmonellosis
associated with schistosomiasis composed a rate of infection exceeding,
amongst others, the HIV infection.
CHAPTER SIX

Conclusions and Recommendations:

6.1 Conclusions:

- enteric fever appears to be endemic in the area in the vicinity of Gadarif State, Eastern Sudan.
- *Salmonella enterica* serotype *paratyphi* B was the main cause of the disease and *S. enterica* serotype *typhi* has not been isolated.
- Growing drug resistance of *S. paratyphi* B to commonly used antibiotics was noted especially to the drug of choice, ciprofloxacin.
- Some patients infected simultaneously with *Schistosoma* and *S. paratyphi* B did not respond to antibiotics, possibly because *Salmonella* invades the adult fluke of *Schistosoma* and become protected from antibiotics.
- Widal test proved to be unreliable for diagnosis of enteric fever as its titres elevated significantly in patients suffering from malaria.

6.2 Recommendations:

The following recommendations are relevant.
• Adoption Plans for Health education in Gadarif State particularly in community health, personal health, hygiene and careful disposal of waste (cow dung) and compost – through incineration or through any convenient methods.

• Establishment of laboratories competent in cultural techniques for the identification of the pathogens.

• Treatment of schistosomiasis prior to enteric fever to avoid complications created by possible Salmonella-Schistosoma association.

• Carrier-state is considered a major issue in the dissemination of enteric fever; better methods for early detection of carriers should be developed.

• Abuse of commonly used antimicrobials should be banned.

• Vaccination against enteric fever should be considered.
CHAPTER SEVEN

References:


Appendix (1)

Questionnaire about typhoid fever in Gadarif State

Patients name: ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... 
Age: ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... 
Residence: ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... 

Causative agent according to Widal test

1. *Salmonella typhi* 

2. *Salmonella paratyphi B* 

Antibiotics received in previous infection:

..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... 
..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... 
..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... 
..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... 

Type of specimen collected:

..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... 

Infection with other febrile illness:

..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... 
..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... 

Date of specimen collection: ..... ..... ..... ..... ..... ..... .....
Appendix (2)

SCHEME FOR IDENTIFICATION OF SALMONELLA SPECIES SUGGESTED BY F.J BAKER

On MacConkey medium

↓

Non lactose fermenting colonies

↓

Stage (1)
1- Oxidase test positive almost *Pseudomonas* species

↓

If negative

2- Urease test if positive almost *Proteus* species

↓

If negative

3- Indole test if positive almost *Shigella* species

↓

4- Motility test if positive suggestive of *Salmonella* species (except *S. galinarum- pullorum*)

Stage (2)

Glucose (fermentation with acid and gas)
Mannitol (fermentation with acid and gas)
Maltose (fermentation with acid and gas)
Sucrose (no fermentation)
Lactose (no fermentation)
Salacin (no fermentation)

Most probably *Salmonella* species