Clinical relevance of *Salmonella enterica* isolated from water and food in Eritrea

by

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Declaration

I the undersigned hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted by me in respect of a degree at any other University.

Signed: ……..

Date: ……..

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Summary

A total of 94 *Salmonella* isolates were collected from three catchments areas in Eritrea. These isolates were recovered from clinical and environmental sources. Biochemical tests using gelatin hydrolysis and tartrate utilization test were employed to differentiate between *Salmonella* subspecies. All *Salmonella* isolates were identified as *Salmonella* subspecies I and were then subjected to molecular characterization. Polymerase chain reaction (PCR) and amplified fragment length polymorphism (AFLP) were employed to identify and establish possible relationships between the clinical isolates and environmental sources. Two sets of oligonucleotide primers specific for genes from *S.* Typhimurium and *S.* Enteritidis were used for the PCR reaction. Of the 94 *Salmonella* isolates characterized only 6 were *S.* Typhimurium strains. To type the *Salmonella* isolates AFLP was used. Clustering the AFLP patterns using the un-weighed pair-group method using arithmetic means (UPGMA) revealed 15 clusters. Of the 94 *Salmonella* isolates collected, 48 (51%) strains were serologically identified. These serotypes include, 21 *Salmonella* Emek (43.7%), 19 *Salmonella* Heidelberg (39.5%), 7 of the 13, 22, 23; z undetermined serotype (14.5%), and 2 *Salmonella* Typhimurium strains (4.1%). The AFLP data in

the present study indicated a possible relationship between the clinical isolates and those obtained from environmental sources.

List of Abbreviations

AFLP	Amplified Fragment Length Polymorphism
AP-PCR	Arbitrary Primed PCR
BPW	Buffered peptone water
CDC	Centers of Disease Control
CFU	Colony-Forming Units
CHL	Central Health Laboratory
ELIFA	Enzyme-linked Immunofiltration Assays
ELISA	Enzyme Linked Immunosorbent Assay
ERIC	Enterobacterial Repetitive Intergenic Consensus
LPS	Lipopolysaccharides
OMP	Outer Membrane Proteins
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
PT	Phage Type
RAPD	Randomly Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RV	Rappaport-Vassiliadis
SS	*Salmonella-Shigela* agar
UPGMA	Unweighted-Pair Group Method using Arithmetic means
XLD	Xylose Lysine Deoxicolate agar

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Chapter 1
Introduction

Microbial diseases constitute a major cause of death in many parts of the world, particularly in developing countries. *Salmonella* has been identified as an important food and water-borne pathogen (Laconcha *et al*., 1998). Many strains of *Salmonella* can infect humans and animals and may cause pathological conditions resulting in morbidity and mortality. In industrialized countries, most of the outbreaks are associated with food-borne transmission by contaminated poultry, egg, meat, milk and other dairy products (Kariuki *et al*., 2002). In the United States alone, *Salmonella* accounts for 60% of all bacterial disease outbreaks (Chen *et al*., 2000).

In developing countries like Eritrea hygiene is often limited and therefore the probability of *Salmonella* contamination of water and food at various stages of processing and handling is always very high. There is also a tradition in Eritrea and the neighbouring countries like Ethiopia, that raw and undercooked meat is consumed at weddings, celebrations and other occasions, the so-called “zihla”. These conditions expose the people to a variety of food related and waterborne diseases. This is best seen in children who frequently suffer from intestinal infections including bacterial diarrhoea that carry a very high mortality.

Apart from the common problems associated with developing countries, Eritrea has for long been involved in a fight for independence and, more recently, a border dispute with Ethiopia. Due to the war, the water supply and sanitation infrastructure of the urban and rural areas is extremely fragile. In many areas of Eritrea untreated wastewater flow directly into rivers and streams. This contaminated water is often used to irrigate small plots of vegetables, which are then sold in nearby markets.

Despite the wide range of bacterial infections, limited data exist on the bacterial quality of water and food, the likely source of human infections. It is therefore difficult to evaluate the importance of salmonellosis in Eritrea due to the lack of co-

ordinated epidemiological surveillance systems. Epidemiological studies to investigate *Salmonella* infections could assist in identifying the major routes of transmission and, in the development of control measures to interrupt the transmission cycle. Without implementation of control measures, these continuous cycles of *Salmonella* infections and re-infections between humans and animals via the environment will not be broken.

Epidemiological studies cannot be performed accurately, based on phenotypic characteristics alone as they lack sensitivity, the interpretation of these phenotypic results may be highly subjective and they are not suitable for strain differentiation. Sensitive and reliable methods are therefore required to differentiate *Salmonella* beyond the phenotypic level. At present genotypic typing methods are widely used for epidemiological studies. These methods typically produce a strain-specific fingerprinting pattern that can be used for bacterial identification and strain differentiation. Using these methods, strains responsible for outbreaks can accurately be differentiated from other strains of the species or subspecies (Terletski *et al*., 2004).

In the present study the amplified fragment length polymorphism (AFLP) technique was employed to characterize clinical and environmental *Salmonella* isolates from water, food and stool samples obtained from the three defined areas in Eritrea. This was done in order to establish the possible genetic relationships amongst the isolates and in doing so to understand the epidemiological links between these isolates.

Chapter 2
Literature Review

2.1 Introduction

Despite global improvement in public health facilities bacterial infections still remain an important public health problem worldwide. *Salmonella* is one of the most important food-borne pathogens and is often linked to water-borne transmission as well (Weigel *et al*., 2004). The disease caused by members of *Salmonella* is called salmonellosis.

S. enterica subspecies *enterica* (subspecies I) is responsible for 99.5% of the infections in human and animals (Pignato *et al*., 1998). Most of the infections are zoonotic in origin but some serotypes like *S*. Typhi and *S*. Paratyphi infect only humans (Yan *et al*., 2003).

During the past ten years there has been a dramatic increase in non-typhoidal salmonellosis. In USA alone there are an estimated 1.5 million cases of non-tyhoidal salmonellosis each year, accounting for nearly one third of deaths associated with food-borne illness (Yan *et al*., 2003). The predominant serotypes responsible for non-typhoidal salmonellosis are *Salmonella enterica* sp. *enterica* serovar Enteritidis and Typhimurium (Schoeni *et al*, 1995; Soumet *et al*., 1999).

Molecular methods based on DNA, have become powerful alternatives in the identification of species and strains, which is essential for the effective investigation of both endemic and epidemic microbial diseases. Apart from the more traditional methods this review will also focus on a number of the molecular methods used for the identification and typing of *Salmonella* strains.

2.2 The Genus *Salmonella*

2.2.1 Definition

Salmonella is a gram-negative bacterium belonging to the family *Enterobacteriaceae*. All *Salmonella* species are usually motile, are facultative anaerobic, flagellated and rod shaped and can reduce nitrates to nitrites. With few exceptions, all *Salmonella* species also produce hydrogen sulfide abundantly in triple sugar iron (TSI) or Kligler's iron agar. The Salmonellae ferment glucose and yield positive methyl red and negative Voges-Proskauer and urease tests. With very few exceptions, indole is not produced, and malate is utilized. Beta-galactosidase is not produced, but inconsistent reactions may occasionally be observed (Mortemer *et al.*, 1981; Sneath *et al*., 1991).

2.2.2 Species

In the past, it was believed that there is only one species of *Salmonella* named as *Salmonella choleraesuis*, and this species has been subdivided in to five subgenera, namely I, II, III, IV and V (Ewing, 1986). At present the concept of having one species has changed and scientists use combinations of different nomenclatural systems to divide the genus into species, subspecies, subgroups and serotypes. Nowadays *Salmonella* is classified on the basis of genetic relatedness and biochemical tests. The genus *Salmonella* contains two species, *Salmonella enterica* and *Salmonella bongori* (formerly subspecies V). At present *S. enterica* is divided into six subspecies, namely: subsp. *enterica* (I); subsp. *salamae* (II), subsp. *arizonae* (IIIa), subsp. *diarizonae* (IIIb), subsp. *houtenae* (IV), and subsp. *indica* (VI) (Brenner *et al*., 2000; Rodriguez-Lazaro *et al*., 2003).

All *Salmonella* strains are serologically classified using the Kauffmann-White scheme, and at present the genus contains more than 2,400 serotypes (Yan *et al*., 2003). The majority of the serotypes belong to *S. enterica* subsp. *enterica* (about 60%), followed by subsp. *salamae* (20%), *diarizonae* (13%), *arizonae* (3.8%), *houtenae* (2.8%) and *indica* (0.45%), Only 0.8% of the serotypes belong to the second

species *S. bongori* (Pignato *et al*., 1998). Strains that belong to *S. enterica* subsp.*enterica* (I) are frequently pathogenic to humans and other mammals while those belonging to subspecies II, IIIa, IIIb, IV, IV and *S.bongori* are usually isolated from reptiles and other cold-blooded animals (Brenner *et al*., 2000).

2.3 Epidemiology of Non-Thyphoidal Strains

2.3.1 Salmonellosis

Salmonellosis is an infectious disease in both humans and animals. The infection is manifested in three forms: gastroenteritis, involving nausea, fever, vomiting and diarrhoea; enteric fever (typhoid and paratyphoid) and septicemia, which is usually characterized by fever, anorexia, anemia and local lesions on the visceral organs (Bennett, B. 1995; Cocolin *et al*., 1998; Yan *et al*., 2003). Human infections are usually associated with animal contact and the consumption of contaminated food products such as poultry, meat and other dairy products (Fratamico, 2003). Salmonellosis is usually considered as an asymptomatic or self-limiting diarrhoeal illness, but it can also become invasive and fatal, especially for patients who are young or immunocoprimized (Kariuki *et al*., 2002; Stephen *et al*., 2003).

Non-typhoidal *Salmonella* strains are important causes of infection in both humans and animals. This disease is caused by *Salmonella* serotypes other than *S*. Typhi and *S*. Paratyphi. It is a major food-borne infection with worldwide distribution. The majority cases are a self-limiting gastroenteritis (Kariuki *et al*., 2002). The clinical symptoms usually appear 8 to 72h after contact with the pathogen. The typical symptoms are usually nausea, vomiting, abdominal pain and diarrhoea with or without fever (Meng and Doyle, 1998; Ruan *et al*., 2002). Few (<5%) of the patients develop invasive *Salmonella* infections or bacteremia and about 10% of those with invasive disease develop localized infections (Yan *et al*., 2003).

During the past decade, there had been a significant worldwide increase of non-typhoidal salmonellosis especially in industrialized countries including, the United Kingdom, Germany, France, Austria, Denmark, and the United States of America

(Sakai and Chalermchaikit, 1996; Meng and Doyle, 1998). In the USA, 45,000 cases and 400 to 600 deaths are reported to the Centres for Disease Control and Prevention each year (Davis *et al*., 2002). The most common sterotypes responsible for the diseases are *Salmonella enterica* sp. *enterica* serotype Enteritidis and Typhimurium (Soumet *et al*., 1999).

In developing countries, including Eritrea there are no data available on *Salmonella* infections and the likely sources of salmonellosis due to limited epidemiological studies. Further, where incidence data are available, these are frequently out-dated. It is therefore difficult to evaluate the present situation of salmonellosis in Eritrea. In addition, under-reporting of cases and the presence of other infectious diseases considered to be of high priority may have also overshadowed the problem of salmonellosis.

2.3.2 Epidemiology of *S*. Enteritidis

Salmonella Enteritidis emerged as a pathogen of poultry in the mid 1970s, but later became an important human pathogen. Human *S*. Enteritidis infections showed a dramatic increase since the 1980s and has became the most commonly isolated serotype in many countries (Sakai and Chalermchaikit, 1996; Rabsch *et al*., 2001). Since 1989 it represents about 80% of all the *Salmonella* outbreaks in Slovakia (Sakai and Chalermchaikit, 1996). In 1993 it became the most frequently isolated serovar in England and Wales and similar trends were also reported from other countries such as Germany, North America, South America and Europe (Meng and Doyle, 1998).

Recently it has been identified that the main reservoir of *S*. Enteritidis is poultry, and the bacterium is usually transmitted through the consumption of eggs and poultry meat (Nayak *et al*., 2004). Moreover, the increased consumption of fast food of animal products, and the international food trade between countries have also played an important role in spreading *S*. Enteritidis (Landeras *et al*., 1998). *S.* Enteritidis can also spread via the environment through fecal contamination by humans and animals (Okafo *et al*., 2003).

Rodents are an important animal reservoir for *S*. Enteritidis. It is believed that *S*. Enteritidis may be introduced to the poultry farms through rodents. It has been suggested that, the recent *S*. Enteritidis pandemic may have been caused by the introduction of this pathogen into rodent populations because its use as rodenticids. In 1985, *S*. Enteritids has been used to control rodents during a *Yersinia pestis* outbreak in San Francisco.

There are two major evolutionary lineages of *S*. Enteritidis based on the phage types (PT). The one linage contains PT4 and PT1 and the other PT8 and PT13a (Rabsch *et al*., 2001). In the current *S*. Enteritidis epidemic in Western Europe the majority of human isolates belong to PT4 (Landeras *et al*., 1998). It has therefore been suggested that PT4 may have acquired the ability to enter and persist in poultry population. Moreover its introduction into breeding lines through international trade in the 1980s may have been responsible for the epidemic spread of PT4 in England and Wales (Rabsch *et al*., 2001).

In the United States of America, unlike Europe, phage type 8, 13a, and 13 are most commonly associated with human outbreaks, but phage type 14 has also been identified (Rychlik *et al*, 2000). In Canada phage type 8 is the most dominant phage type associated with outbreaks. In Germany phage type 4 is the most frequently phage type isolated during outbreaks but phage types 7, 25, 34, and 8 has also been isolated (Rabsch *et al*., 2001). It has been concluded that different geographical regions harbor their own *S*. Enteritidis subtypes. Since 1997 cases of salmonellosis caused by *S*. Enteritidis have started to decrease in UK and many countries (Liebana *et al*., 2004).

3.3.3 Epidemiology of *S*. Typhimurium

There has been a high incidence of multiresistant *S*. Typhimurium in the United Kingdom, Europe and North America in the 1990s (Stephen *et al*., 2003). This serotype was usually identified by two characters, resistance to multiple antimicrobial drugs (ampiciline, chloramphenicol, streptomycin, sulfonamides, and tetracycline [ACSSuT+] and the definitive phage type 104 (DT104a) (Duijkeren *et al*., 2002; Davis *et al*., 2002). *S*. Typhimurium DT104 first emerged in cattle in 1984 in England

and Wales and then spread very rapidly to Europe, Asia, and North America (Stephen *et al*., 2003). The incidence DT104 continues to increase in England and Wales, and has also been identified in Germany, the Netherlands, the United Arab Emirates, and the Philippines (Meng and Doyle, 1998; Rabsch *et al*., 2001).

Cattle served as the main reservoir for most multidrug resistant *S*. Typhimurium strains isolated in England and Wales including DT29, DT204, DT193 and DT204 (Rabsch *et al*., 2001). It is also suggested that the rapid spread of *S*. Typhimurium may be related to trading and movement of calfs as well as the wide use of antibiotics. Another possible factor contributing to the spread of the new phage types may be the horizontal transfer of virulence gene (Davis *et al*., 2002; Stephen *et al*., 2003).

In contrast to *S*. Enteritidis, which is usually associated with poultry and eggs, *S*. Typhimurium (DT104) is associated with a variety of different animals used for food production, including cattle, sheep, pigs, goats, chickens and turkeys as well as domestic pets (Meng and Doyle, 1998; Duijkeren *et al*., 2002). So far, the majority of bovine salmonellosis in England was caused by DT104, and 15 of the 16 new outbreaks of bovine salmonellosis, in Scotland during March 1996, were caused by DT104 (Rabsch *et al*., 2001).

Investigation into 46 outbreaks of DT104 infections in humans in the UK from 1992-1996 showed that 78% of these outbreaks were linked to food-borne transmission. Contact with sick animals is also a risk factor, since a number of families appear to have acquired DT104 infections while caring for sick farm animals. Moreover, household pets may also be a source of infection (Meng and Doyle, 1998). Human travel is also one of the routes of transmission of salmonellosis in developed countries and imported *Salmonella* infections are more likely to be multi-resistant than those domestically acquired. It has been suggested that the rapid dissemination of mr-DT104 is more consistent with human travel than with the movement of domesticated animals (Davis *et al*., 2002).

2.3.4 Epidemiology of *Salmonella* Heidelberg

Salmonella enterica serovar Heidelberg is also one of the most commonly occurring *Salmonella* serotypes. It is a group B *Salmonella* that causes non-typhoid salmonellosis in humans and septicemia and enteric disease in animals (Amavisit *et al*., 2001). In the USA about 50% of the food-borne salmonellosis outbreaks are caused by *S*. Enteritidis, *S*. Typhimurium and *S*. Heidelberg (Schoeni *et al*., 1995).

The isolation of *S*. Heidelberg from water, food and livestock has been reported in many studies. Most of the *S*. Heidelberg infections are due to eating contaminated food products. However, in some hospital outbreaks *S*. Heidelberg seemed to spread by cross-infection and most of the victims were infants, but some older children and adults were also affected (Edgar *et al*., 1963). Between 1963 and 1972, 28% of salmonellosis outbreaks reported to the Centers of Disease Control (CDC), occurred in hospitals, nursing homes and mental institutions were caused by *S*. Heidelberg (Rice *et al*., 1976).

S. Heidelberg outbreaks were also reported from equine veterinary hospitals and large horse farms. These outbreaks are usually associated with high population densities of horses on the farms and horses admitted to hospital were found to be more susceptible to *Salmonella* infections. Between 1992 and 1997, there were 31 cases of horses infected with *S*. Heidelberg in an equine veterinary hospital in Victoria, Australia. Infection of horses with *S*. Heidelberg can be asymptomatic or cause life-threatening septicemia. The asymptomatic carriers shed *Salmonella* in their faeces and are often the main source of outbreaks amongst the other animals (Amavisit *et al*., 2001).

2.3.5 Epidemiology of *Salmonella* Emek

Salmonella enterica serovar Emek is one of the rarely occurring *Salmonella* serotypes and causes non-thyphidal salmonellosis in humans. Like most *Salmonella* serotypes *S*. Emek show the same typical morphological, cultural and biochemical characteristics of the species. Hydrogen sulfide is produced, the production of indole or urease is negative but a positive reaction occurs with Simmons citrate and D-tatrate. The

Voges-Proskauer reaction is negative, and the bacterium is methyl red positive. Serologically, *S*. Emek contains O antigens and the H antigen is monophasic (Hirsch *et al*., 1950). It has first been isolated in Israel, in 1949 for the first time and has since been sporadically isolated from human cases of salmonellosis in Germany and some Middle East countries. However, no data have been obtained describing the epidemiology of *S*. Emek in any of the industrialized countries.

2.4 Detection Methods

2.4.1 Culture and Phenotypic Methods

Salmonella cells can be injured or stressed as the result of food processing conditions or environmental factors. The optimal isolation and cultivation of *Salmonella* therefore requires enrichment procedures, including non-selective and selective enrichment. For the resuscitation of injured *Salmonella* cells non-selective (pre-enrichment) medium such as buffered peptone water is usually used. The choice of pre-enrichment medium can, however, differ depending on the nature of the sample being examined.

The pre-enrichment stage is often followed by two enrichment steps. During the first step Selenite Cystine broth or Rappaport-Vassiliadis broth or both can be used to allow for the growth of *Salmonella* species but the inhibition of other organisms. Following this enrichment step, selective media is used for the confirmation of *Salmonella* species. The most commonly used media selective for *Salmonella* include xylose lysine deoxicolate agar (XLD), *Salmonella-Shigela* (SS) agar, Wilson-Blair bismuth sulfate agar, Hectoen enteric medium and brilliant green agar. After overnight incubation colonies can be examined for the required characterstic. *Salmonella* species grown on XLD plates, for example, usually produce H_2S, resulting in black centred colonies (Harrigan and Park, 1991).

Supplementation of the pre-enrichment, or enrichment media with ferrioxamine E could also improve the isolation of *Salmonella* quantitatively as well as qualitatively. It can significantly improve the isolation of low numbers of *Salmonella* from mixed cultures when used as a supplement in buffered peptone water (BPW) or Selenite-

Cystine broth (Reissbrodt *et al*., 1996). Ferrioxamine E supplies iron to *Salmonella* via the special uptake and utilization system of the cell. Other competitor organisms such *E.coli*, have no uptake and utilization system for feroioxamine E.

Presumptive *Salmonella* cultures are usually confirmed by means of a number of biochemical tests. A rapid screening test is the urease test, which should be negative for *Salmonella* isolates (Mortimer *et al*., 1981; Ewing, 1986). Following the urease test other biochemical tests can be performed. These tests could be performed individually or by using commercially available identification systems such as the API 20E system.

2.4.2 Immunological Methods

The traditional method for the detection of *Salmonella* is time consuming, and great efforts have therefore been made to develop rapid methods for the detection of *Salmonella.* Many of these rapid methods have been based on immunological techniques. In these tests antibodies produced by experimental animals are used to detect antigenic differences between microorganisms. Two classes of antibodies are used, polyclonal and monoclonal antibodies. In order to use antibodies to identify specific organisms, they must not cross react with other organisms (Towner and Cockayne, 1993). The speed and sensitivity of these immunological techniques vary, and are highly affected by the duration of the enrichment period required to get detectable amounts of target cells, as well as the complexity of the sample, and the specificity of the reporting mechanism.

2.4.2.1 Microscopy

Fluorescent antibodies can be used to detect bacteria under a fluorescent microscope that detects the light transmitted after excitation of the specific fluorochrome bound to the antibody (Guy *et al*., 2000). In this procedure organisms are fixed directly onto the slide, and are then incubated with the specific antibody. The antibodies can be labelled directly with a fluorochrome to allow visualization of the antibody (direct

immunofluorecence assay) or a fluorochrome-labelled antibody conjugate can be added to detect the unlabeled antibodies (indirect immunofluorecence assay) (Towner and Cockayne, 1993).

Immunofluorescence with polyclonal antiserum has been used for the detection of *Salmonella* species. O antisera are mostly applied but H antisera can also be used (Mortimer *et al*., 1981). A modification of the immunoflorescent technique has been developed to differentiate between dead and live *Salmonella* cells (Duffy *et al*., 2000). The method is based on combining anti-*Salmonella* antibodies labelled with Texas Red with a viability stain Sytox Green. The technique included pre-enrichment, enrichment and selective enrichment steps. The technique has been applied for the detection of *S*. Enteritidis. The main advantage of this technique is that it helps to distinguish between viable and non-viable cells, especially in processed and preserved food products in order to make an accurate assessment of the number of *Salmonella* in the product (Duffy *et al*., 2000).

2.4.2.2 Enzyme Linked Immunosorbent Assay

In a direct Enzyme Linked Immunosorbent Assay (ELISA), antibodies conjugated directly to an appropriate label are used to detect the specific antigen (Towner and Cockayne, 1993). In most applications, however, an indirect or sandwich ELISA technique is commonly used, where unlabelled primary antibody is detected with an appropriately labelled antibody conjugate. (Wang *et al*., 2001). In most of the ELISA methods developed for the detection of *Salmonella*, polyclonal antibodies were used, but examples of the use of monoclonal antibodies also exist (Kroll *et al*., 1993; Huang *et al*., 1999). The lipopolysaccharides (LPS) in the outer membrane of *Salmonella* are usually targeted as genus-specific antigens since the outer membrane proteins (OMP) are more conserved and less immunologically variable (Kroll *et al*., 1993).

ELISA methods were found not to be sensitive for the detection of low numbers of *Salmonella* in food or environmental samples. The detection limits of most ELISA methods vary between 10^4 and 10^7 colony-forming units (CFU) per ml *Salmonella* present in the enrichment broth (Huang *et al*., 1999). The inclusion of an enrichment

step in the ELISA procedure is therefore important to allow small numbers of *Salmonella* to increase above the detection level (Kroll *et al*., 1993). *Salmonella* can be enriched by using either nonselective or selective medium (Huang *et al*., 1999).

The use of ELISA based methods has a number of advantages over conventional culture methods. It is easy to perform and shortens the time required to obtain positive or negative results. The disadvantages of ELISA are amongst others the time required for the migration of antigen and enzyme label conjugate molecule from the bulk solution to the immobilized antibodies as well as the need for several labor-intensive manipulations (Huang *et al*., 1999).

2.4.2.3 Enzyme-linked Immunofiltration Assay

Enzyme-linked immunofiltration assays (ELIFA) have been developed as an alternative to the ELISA technique. In this technique a solution containing the antigen is filtered through an antibody-coated filter membrane, which results in high antigen-antibody binding and a reduction of the assay time. The enzyme-linked immunofiltration assay allows for the concentration of the antigen on the porous membrane but in most applications a pre-enrichment step is still required to improve the poor detection limit of the method. Enzyme immunofiltration assay has been used for the rapid detection of *E.coli* and *Salmonella*. (Abdel-Hamid *et al*., 1999).

2.4.3 Biosensors

Biosensors are analytical devices that incorporate a biologically active material such as an enzyme or antibody with a sensor that measures absorbance, scattering or fluorescence due to an interaction with the analyte of interest (Zhou *et al*., 1997). Many types of biosensors have been developed. These include amongst others conductometric biosensors, fiber optic fluorescent immunosensors as well as piezoelectric and quartz crystal biosensors (Dill, *et al*., 1996; Bacacan *et al*., 2000). Biosensors have been used for the detection of *Salmonella* serotypes, such as *S*. Typhimurium and *S*. Enteritidis from clinical and environmental samples. (Zhou *et al*,

1997; Zhou *et al*., 1998; Su *et al*., 2001; Kim *et al*., 2003; Muhammad-Tahir *et al*., 2003).

2.4.4 Hybridization

Hybridization has been widely used for the detection and identification of bacteria. (Olsen *et al*., 1995). In most cases short DNA fragments or probes are used to hybridize with the unique target nucleic acid (Olsen *et al*., 1995). Probes can be labelled radioactively or chemically with compounds such as biotin or digoxigenin (Liebana, 2002). Hybridization can be performed in a number of configurations such as in situ, in solution or on a solid support (Towner and Cockayne, 1993).

Since 1983, various probes have been developed for *Salmonella* detection. The probes consisted of either a specific cloned DNA fragment or a synthetic oligonucleotide (Towner and Cockayne, 1993).

Nowadays RNA based probes, particularly ribosomal RNA (16S and 23S rRNA) based, are used as part of *in situ* hybridisation (Olsen *et al*., 1995; Tolker-Nielsen *et al*, 1997). The detection of rRNA provides a good sensitivity for hybridization assays, since rRNA is highly conserved and the ribosomes are present in great numbers in actively growing bacterial cells (Pabbaraju and Sanderson, 2000). The rRNA based oligonucliotiede probes are considered as the best probe assay for *Salmonella* detection (Liebana, 2002).

2.4.5 Polymerase Chain Reaction

Polymerase chain reaction (PCR) uses a thermostable polymerase to produce multiple copies of specific nucleic acid regions (Towner and Cockayne, 1993). The DNA fragment is amplified in a three-step process. During the first step, the target DNA is denatured at a high temperature, this is followed by the annealing of the primers to the opposite strand of DNA at a temperature that only allows for hybridization with the

correct target sequence. During the third step, extension of the oligonucleotide primers occurs, using the target DNA as a template for the new strand that is formed. When this process is repeated for several cycles an exponential amplification of the targeted DNA fragment is obtained (Towner and Cockayne, 1993; Olsen *et al*., 1995).

The use of PCR is one of the most promising approaches for the detection of *Salmonella* species. PCR has advantages in terms of speed, sensitivity and specificity and has been widely used to detect *Salmonella* species in food, clinical and environmental samples. Combining the PCR with an enrichment step has improved the sensitivity of the assay and assisted in diluting out PCR-inhibitory substances (Olivera *et al*., 2002).

For the detection and identification of *Salmonella* species, several primers have been designed. Some of the primers are used for the identification of all isolates belonging to the genus. These primers sets target genes such as *invA*, *ST*, and *hin/H2*, which are specific for all *Salmonella spp.,* others primers are only specific for some individual *Salmonella* serotypes. These targets include *SefA*, that encodes the *SER* 14 fimbrial antigen, a virulence plasmid for *S*. Enteritidis, *S.* Pullorum and *S.* Gallinarum, and the *flic* gene, that encods flagelin *H1* present in *S*. Typhimurium (Trkov *et al*., 1999; Olivera *et al*., 2002; Agarwal *et al*., 2002).

2.4.5.1 Multiplex PCR

Multiplex PCR involves the amplification of more than one target gene per reaction by mixing multiple primer pairs with different specificities. (Zarlenga and Higgins, 2001). Multiplex PCR assay has been used for identification of *Salmonella* serotypes, such as Enteritidis and Typhimurium (Soumet *et al*., 1999). Three sets of primers are used, one specific for the genus *Salmonella* and two specific for *S*. Typhimurium or *S*. Enteritidis (Soumet *et al*., 1999; Carlson *et al*., 1999; Ebner and Mathew, 2001; Eceita, *et al*., 2002).

2.4.5.2 Real-time PCR

Real-time PCR has improved the rapid identification of several bacteria since amplification of the target sequence and analysis of the products can be done within a single instrument by adding fluorescent probes or dyes to the PCR reaction. This approach eliminates the need for separating the PCR products during subsequent gel electrophoresis. Various systems have been developed. Some of the methods use DNA-binding dyes (such as SYBR Green) or fluorescent oligonucleotides (such as the Scorpion primes); others use molecular Beacons or TaqMan probes (Rodriguez-Lazaro *et al*., 2003; Liming *et al*., 2004).

A real time-PCR approach has been used for the detection of *S.enterica* subspecies I, other subspecies such as II, III and IV, as well as *S.borgori* from food and environmental samples (Rodriguez-Lazaro *et al*., 2003; Bhagwat, 2003). In this case, class-I integrons and sequence variations in the *gyrA* gene were targeted. These markers have also been used previously for confirmation of epidemiological relationship between isolates (Liebana, 2002).

2.5 Typing Methods

Traditionally, epidemiological studies of *Salmonella* have been performed using serotyping and phage typing. The discriminatory power of these traditional methods is typically low and not always sufficient to study the population structure of the isolates (Torpdahl and Ahrens, 2004). The new typing methods are mainly based on the genotype of the organism. Molecular typing methods allow characterization of genotype of the organism and often produce a strain-specific fingerprinting pattern. This pattern is then used to investigate outbreaks and to differentiate between epidemiologically related and unrelated strains. Molecular typing methods have been widely used for tracing the spread of *Salmonella* strains from animals to humans as well as through contaminated food (Liebana, 2002; Terletski *et al*., 2004).

The new methods include amongst others ribotyping, IS200 typing, pulsed-field gel electrophoresis, random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP).

2.5.1 Serotyping

Serotyping is one of the oldest typing methods. It is the most commonly used method to differentiate members of the genus *Salmonella* following biochemical identification. Serotyping separates *Salmonella* isolates on the basis of their cell surface or somatic (O), capsular (Vi, if present) and flagellar (H) antigens in to different serotypes (Iankov *et al*., 2002). Serotyping is usually done in a suspension, which is incubated with antisera (polyclonal or monoclonal) that recognize specific O or H antigens. The agglutination profiles generated are used to determine the particular serotype of the isolate being tested (Yan *et al*., 2003). The disadvantages of serotyping are it is time consuming, it is difficult to standardize the method and it often has to be done in reference laboratories (Christensen *et al*., 2000). Even though it has many limitations, serotyping is widely used, as it is essential for the differentiation of *Salmonella* isolates.

2.5.1.1 The O antigen

The O antigens are heat-stable antigens, and are classified as major O or minor O antigens. The major O antigens are used to identify the O antigenic group whereas the minor O antigens have less discriminating value (Mortimer *et al*., 1981). The O antigens of *Salmonella* are numbered consecutively from 1 to 67 by means of Arabic numerals but this series is not completely continuous (Ewing, 1986).

2.5.1.2 The H antigen

Few *Salmonella* serotypes show only one flagellar antigen; in such a case the H antigen is called monophasic. Most *Salmonella* serotypes, however, can produce two

H antigens alternatively and the H antigen is then referred to as being diphasic (Mortimer *et al*., 1981). The two phases of the flagellar antigens are alternatively expressed by phase variation mechanism; this means in a single cell only one antigen is expressed at a time. In the case of phase I (the specific phase) the different antigens are designated using small letters and for phase II (the group phase) the first antigen discovered is numbered. (Yan *et al*., 2003). The strains must be actively motile to ensure development of H antigens.

2.5.2 Phage Typing

Phages are viruses that can infect bacteria and may lead to the lysis of the bacterial cell and the release of more phage particles. Phage typing is based on susceptibility of isolates to infection by specific phages (Towner and Cockayne, 1993). Bacterial isolates have different phage receptors on their surface and a phage will infect the bacterium when the appropriate receptor is present in the surface of the cell. Phage typing has been used for epidemiological studies of many bacteria and is the most commonly used typing method for primary differentiation within *Salmonella* serotypes.

The disadvantages of the method are that the set of phages is not generally available and the method can only be performed in reference laboratories. Certain phage types may also predominate in a particular area and the typing that may be useful in one area are therefore less effective in other areas (Towner and Cockayne, 1993; Landeras *et al*., 1998). Not all strains are susceptible to the recognized phage types (labelled UPT or UT) or some strains may show a reaction or lytic pattern that do not conform to any of the recognized phage types (labelled RDNC or NC) (Landeras *et al*., 1998).

Phage typing has been widely used for epidemiological studies of *S*. Enteritids isolates (Landeras *et al*., 1998). Strains that are susceptible to infection by the same phages are grouped as a specific phage type (PT). Based on the phage typing data a clonal dispersion of a limited number of stains has been reported for Europe and the USA (Laconcha *et al*., 1998). A few phage types, in particular PT4 in Europe and PT8 and PT13a in the USA, are most frequently isolated and are mostly responsible for food

poisoning associated with poultry products (Landeras *et al*., 1998; Rabsch *et al*, 2001).

2.5.3 Plasmid Profiling

Plasmids are extra chromosomal DNA elements that can replicate independently of the chromosome (Towner and Cockayne, 1993). Bacterial strains can be typed according to the plasmid content or plasmid profile of each isolate (Terletski *et al*., 2004). Plasmids are not considered as a stable strain characteristic as they can be lost or undergo re-arrangements by conjunctive transfer (Liebana, 2002; Domig *et al*., 2003) and therefore plasmid profiling is usually used as an additional method for typing. Some of the disadvantages of plasmid profiling it that it is not always true that strains with identical plasmid profiles are epidemiologically related. Some strains with the same chromosomal features may show different plasmid restriction patterns, and the same plasmid profile may be present in strains that are different at a chromosomal level (Chadfield *et al*., 2001; Liebana, 2002). The presence of different conformations of the same plasmid (linear or circular) may also result in altered profiles. Despite these limitations, plasmid profiling is a simple and rapid technique and can be a useful method for laboratories that are unable to perform more complex methods (Liebana, 2002).

Plasmid profiling is a wildly used method for the differentiation of *Salmonella* isolates. Many studies have shown that certain plasmid profiles are stable among *Salmonella* isolates even when isolated from cases of infection occurring in widely separated geographical areas over a long period of time (Chadfield *et al*., 2001). Plasmid analysis was found not to be suitable for the typing of *S*. Enteritidis strains, especially for the intraphage type differentiation of strains (Mohan *et al*., 1995; Domig *et al*., 2003). The method was, however, sensitive when it was applied for the typing of *S*. Typhimurium, *S*. Virchow, and *S*. Gallinarum strains (Mohan *et al*., 1995; Liebana, 2002).

2.5.4 Randomly Amplified Polymorphic DNA

Randomly amplified polymorphic DNA (RAPD) or arbitrary primed PCR (AP-PCR) is a method, which can discriminate between different isolates of the same species. RAPD analysis uses short PCR primers of a random sequence (Terletski *et al*., 2004). These primers detect polymorphisms in the absence of specific nucleotide sequence information and the polymorphisms then function as genetic markers (Millemann *et al*., 1996). The resulting PCR products are separated using agarose gel electrophoresis. The selection of appropriate primers and optimization of PCR condition is important to increase the discriminatory power of this method (Liebana, 2002). A large number or arbitrary primers can be tested to identify primers that might be used for a particular purpose. The main limitation of this technique is the lack of reproducibility for routine use, especially when performed in different laboratories using different equipment. This creates problems especially for inter-laboratory data comparison (Liebana, 2002). RAPD has provided useful information in several studies, when it is applied for the typing of *S*. Typhimurium, *S*. Eneritidis, *S*. Dublin and *S*. Pullorum isolates (Millemann *et al*., 1996; Landeras *et al*., 1998; Laconcha *et al*., 1998 and Liebana, 2002).

2.5.5 Eric-PCR and Box

Enterobacterial repetitive intergenic consensus (ERIC) and BOX elements are naturally occurring, highly conserved, repetitive DNA sequences, present in many copies on the genomes of most grams negative as well as many of the gram-positive bacteria (Towner and Cockayne, 1993; Tuang *et al*., 1999; Liebana, 2002). These repetitive sequences are located in distinct, intergenic positions situated throughout the genome (Liebana, 2002). The use of outward facing primers complementary to each end of the ERIC repeats in order to amplify the region between two of these repeats is called ERIC-PCR. Following PCR, fragments are separated using gel electrophoresis and differences in the banding patterns indicate polymorphisms in the distances between the repetitive elements on the different genomes (Towner and Cockayne, 1993). The ERIC sequence was originally found in *E.coli* and *S*.

Typhimurium (Millemann *et al*., 1996). The methods can be used to differentiate between *Salmonella* serotypes, but it has less discriminatory power than phage typing (Liebana, 2002).

2.5.6 Restriction Fragment Length Polymorphism and IS200

Restriction fragment length polymorphism (RFLP) is a technique, which is based on characterization of organisms by means of restriction enzyme digestion of the whole genome (Towner and Cockayne, 1993). In RLFP, the DNA of the organism is digested with endonucleases, which results in the generation of fragments of different sizes. The restriction enzymes used are usually 4 or 6 base pair cutters but 8 base pair cutters have also been used. The band patterns obtained after electrophoresis can be used to determine to which extent different organisms of one species differ from one another (Kroll *et al*., 1993; Liebana, 2002).

RFLP has been used for typing of *Salmonella* species such as *Salmonella enterica* serotype Gallinarum (Kwon *et al*., 2000). The advantage of the method is that it is fast, easy and a simple method for DNA typing. Its main disadvantage is the complexity of profiles generated, which makes the analysis of the patterns very difficult (Tenover *et al*., 1995). In order to avoid the problem of analyzing complex patterns generated by restriction enzyme, the electrophoresis is usually followed by blotting techniques using probes for repeated DNA elements (Kroll *et al*., 1993; Yan *et al*., 2003). Some of the common blotting targets are insertion elements present in the bacterial genome. IS200 is an insertion sequence that has been found in almost all *Salmonella* serotypes (Olsen *et al*., 1995). Because of its ability of transposing, it can be found on different DNA sites, and can be detected after hybridization with a specific probe (Liebana, 2002). IS200-RFLP has been found to be useful to study the phylogenetic relationships within serotypes. It has less discriminatory power within *S*. Enteritidis and *S*. Virchow, but is more sensitive when used for *S*. Typhimuruim or *S*. Infantis (Kwon *et al*., 2000; Chadfield *et al*., 2001).

2.5.7 Ribotyping

Ribotyping is another variation of the RFLP technique. In this method the typing of the bacteria is based upon differences in the positions of the rRNA operons on the genome. The method was first described by Grimont in 1986 (Towner and Cockayne, 1993). The method involves digestion of the whole DNA with restriction endonuceleases, southern blotting and then hybridization with labelled rRNA probes (Nair *et al*., 1999). It generates highly reproducible fingerprints that can be used to classify bacteria from the genus through species level and even below the species level (Towner and Cockayne, 1993). The technique, however, requires high levels of laboratory expertise and it is rather time consuming. The effectiveness of ribotyping also depends on the restriction enzyme used for digestion, the probe and the origin of the isolates (Chadfield *et al*., 2001).

Ribotyping has been applied for the typing of different *Salmonella* serotypes. *Salmonella* contains seven *rrn* loci distributed throughout the chromosome (Liebana, 2002). If genomic DNA is digested with a restriction enzyme that does not cut within the operons, seven or fewer DNA hybridizing fragments will be observed. However, if the enzyme cuts into some of the *rrn* loci, the number of fragments will be more than seven (Liebana, 2002; del Cerro *et al*., 2002). Ribotyping has found to be a suitable fingerprinting method for *S*. Enteritidis and *S*. Typhimurium isolates (Nastasi and Mammina, 1995; Landeras *et al*., 1998, Terletski *et al*., 2004). The appropriate enzyme must be determined for each serotype, and the use of two or more enzymes in combination can increase the discrimination power of the method. The combination of *Pst*I-*Sph*I gave the most sensitive result for differentiation of *S*. Enteritidis strains of animal origin (Landeras *et al*., 1998; Liebana, 2002).

2.5.8 Pulsed Field Gel Electrophoresis

Pulsed Filed Gel Electrophoresis (PFGE) was first described in 1984 by Swartz and Cantor (Liebana, 2002). In PFGE, DNA is digested with infrequent cutting endonucleases, which results in the generation of fewer fragments with high

molecular mass (Terletski *et al*., 2004). These fragments can be separated on the basis of their size using pulsed field gel electrophoresis (Laconcha *et al*., 1998). PFGE usually separates DNA fragments of less than 50kb, since DNA fragments above 50kb produce large and diffused bands (Kroll *et al*., 1993; Towner and Cockayne, 1993). PFGE uses electric fields of alternating direction, which cause the DNA fragments to continuously change direction. This results in the resolution of high molecular weight DNA into separate bands (Liebana, 2002). PFGE has been used for epidemiological studies of a wide range of microorganisms. The pattern of restriction fragments gives information about the genetic relatedness of strains (Towner and Cockayne, 1993). The discriminatory power of the method can be increased by the use of more than one rare cutting enzyme (Laconcha *et al*., 1998). The main advantage of PFGE is that it is highly reproducible and produces fewer bands that are clearly visible and can be compared to one another. Disadvantages of this method include that it is a tedious and time-consuming technique and requires equipment of relatively high cost (Terletski *et al*., 2004).

PFGE has been applied for many epidemiological studies of *S. enterica*. The enzymes *XbaI* and *BlnI* have been identified as the most discriminative enzymes for the analysis of *S*. Enteritidis strains (Laconcha *et al*., 1998; Seno *et al*., 2003). Several studies have, however, reported the limitation of PFGE for intraphage-type differentiation of *S*. Enteritidis strains (Seno *et al*., 2003). It was reported that PFGE has high discriminating power for typing of *S*. Typhimurium, *S*. Dublin, *S*. Infantis, *S*. Virchow and some of the other serotypes mainly associated with animals (Wegener *et al*., 1996; Liebana *et al*., 2002; Moore *et al*., 2003).

2.5.9 Amplified Fragment Length Polymorphism

AFLP is a recently developed typing method that was originally described by Vos in 1995 (Lindstedt *et al*., 2000). The method is based on the detection of DNA polymorphisms, which is a result of point mutations, insertions or deletions in the genome (Masiga and Turner, 2000). In AFLP, genomic DNA is digested with two restriction endonucleases (one frequent cutting and another rare cutting enzymes), followed by ligation of specific adaptors to the created fragments. Thereafter one or

more selective amplification of the restriction fragments can be performed (Blears *et al.*, 1998). The sequences of the adaptors and the adjacent restriction site serve as primer binding sites for the selective primers used in AFLP.

Pre-amplification is usually performed using primers with a single or non-selective nucleotide at their 3'end (Nair *et al*., 2000; Lan *et al*., 2003). Pre-amplification helps to reduce the complexity and background smear in the fingerprint pattern resulting form mismatched amplification (Liebana, 2002). The pre-amplification products are used as templates for the selective amplification reaction. The selective amplification reaction uses primers with one to three selective nucleotides at their 3' ends. Only restriction fragments in which the nucleotides flanking the restriction site are complementary to the selective nucleotides on the primers will be amplified (Blears *et al*., 1998; Okano *et al*., 1998; Liebana, 2002). For small genomes one or two selective nucleotides at the 3' end of each primer is used but for a complex genome, three selective nucleotides are required to produce the desired number of amplified fragments. Following selective amplification, the PCR products are resolved on a denaturing polyacrylamide gel (Masiga and Turner, 2000; Liebana, 2002).

The advantages of AFLP are that it is highly reproducible and shows variation around the whole genome by selectively amplifying a subset of restriction fragments for comparison. It allows for the identification of highly related bacterial strains. AFLP has been found to possess higher discrimination potential than RAPD and PFGE fingerprinting techniques (Terletski *et al*., 2004; Wang *et al*., 2004). The major disadvantage of the method is that it requires high cost equipment and consumables (Velappan *et al*., 2001; Terletski *et al*., 2004).

Recently, AFLP has been successfully used for the typing of *Salmonella* serotypes (Terletski *et al.*, 2004; Torpdahl and Ahrens, 2004). The method has been used for the identification of clones of *S*. Enteritidis (PT4) and *S*. Typhimurium associated with human infections. (Lindstedt *et al.*, 2000). It has been reported that the combination of *EcoR1-MseI* provides sufficient sensitivity to separate *Salmonella* serotypes (Lindstedt *et al*., 2000; Nair *et al*., 2000; Liebana, 2002).

2.6 Conclusions

Salmonella is widespread and many of the serotypes can infect animals and humans. Infection may cause pathological conditions resulting in morbidity and mortality. The detection of *Salmonella* in food and water is therefore important in the prevention of such infections. The combination of conventional culturing and molecular typing methods described above is useful to track specific strains in the environment and to identify their possible link to outbreaks of salmonellosis. It also provides important insights into understanding the epidemiology of this pathogen.

Chapter 3

Isolation of non-typhoidal *Salmonella* from three areas in Eritrea

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3.1 Introduction

Diarrhoeal disease remains a major public health problem worldwide. In developing countries, an estimated 12 or more diarrhoea episodes per child per year occur within the first 5 years of life (Maltezou *et al*., 2001). In Eritrea, diarrhoeal disease is one of the most frequent causes of childhood illnesses and a major cause of child mortality. The major contributors to the enteric infections are *Salmonella*, *Shigella*, and pathogenic *E.coli*.

Asmara, Massawa and Keren are among the most densely populated areas in Eritrea. Due to limited sanitary facilities the people are exposed to a variety of water and food related diseases. The community in these areas are supplied with water through either individual house pipe systems; public standpipe systems or many people use water directly from rivers and wells. The result of previous studies on the microbiological quality of the water supply in the three areas indicated that most of the water sources were contaminated with coliforms (Ministry of Land and water sources, Eritrea, Personal communications, 2003)

Apart from the data concerning microbial contamination, no data exist on the occurrence of enteric bacteria such as *Salmonella* in Eritrea. Studies to isolate *Salmonella* from environmental and clinical sources would help to determine the prevalence of *Salmonella* infections in Eritrea. Therefore, in this study water, food and clinical (stool) samples were collected from the above-mentioned three areas in Eritrea.

3.2 Materials and Methods

3.2.1 Study Areas

The study areas consisted of three catchments areas in Eritrea (Figure 1). Eritrea is an eastern African country of 124,320 km^2 and with 3.56 million inhabitants. Eritrea is bordered to the West by the Red sea, to the North by Sudan and to the South by Ethiopia and Djibouti. The three areas selected were Asmara, Massawa and Keren. These three areas were selected because they are the most densely populated areas in Eritrea.

Asmara is the capital city of Eritrea, located on the highland region. Asmara is the most densely populated city in Eritrea, and is the residential and as well as commercial centre of the country. The city uses five dams, Maynefhi, Adinifas, Adisheka, Mayserwa, and Myanmbessa as a source of water. Mynefhi is the largest dam and is used as the main water supply for the inhabitants of Asmara. The dam is located at Gala Nefhi, a few kilometres away form Asmara. Many hand-dug wells exist and are also used for domestic purposes.

Massawa is a port city and located 110 km away from Asmara. Its main water source is a subsurface (aquifer) dam in the Dogoli River 22 km of Massawa. Keren is about 100 km away from Asmara. Water is mainly obtained from hand-dug wells in the riverbed of the Anseba River 3 km north west of Keren and the Shifshifit dam 3 km west of Keren.

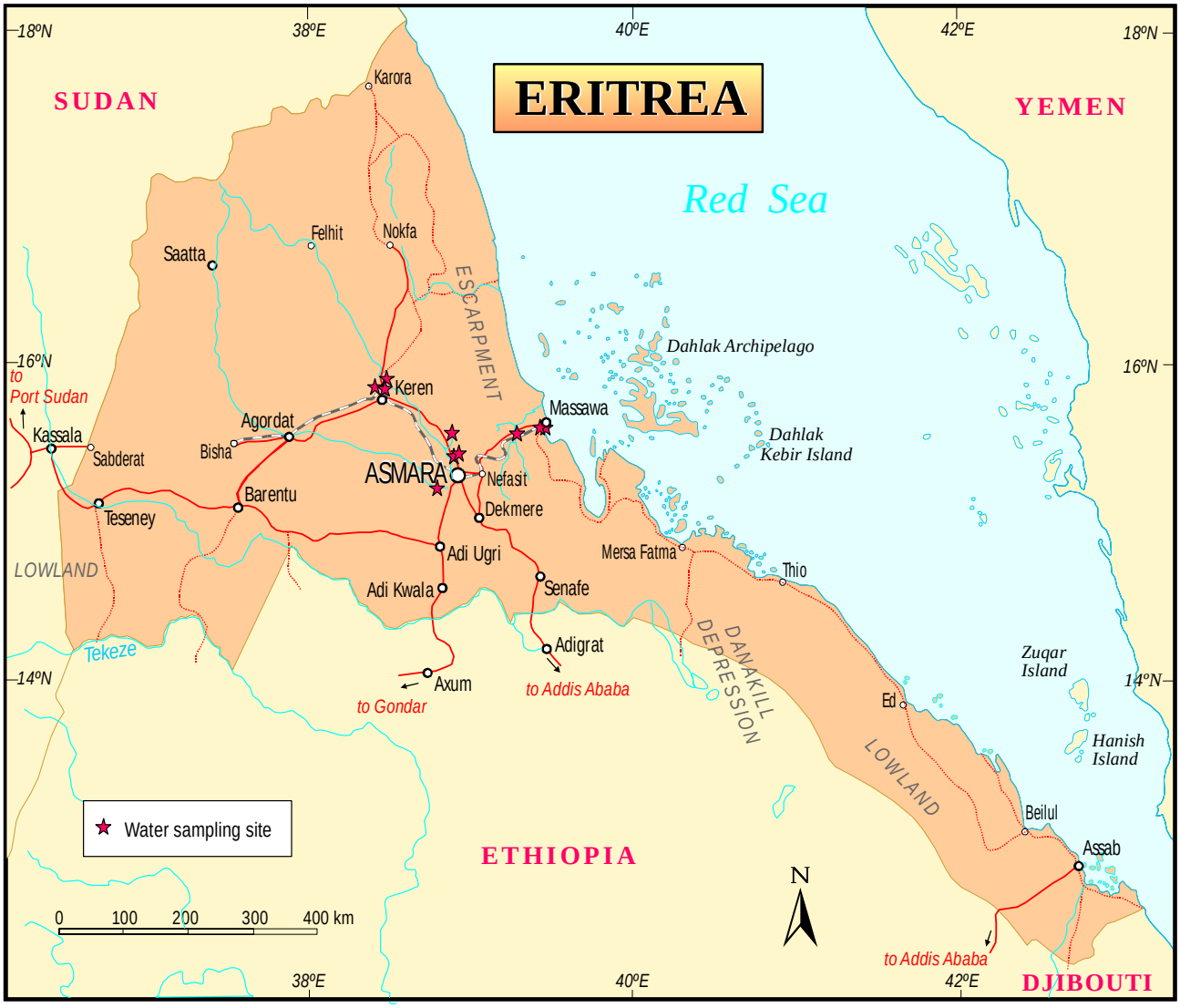


Figure 3.1 Locations of water sampling sites in Eritrea

3.2.2 Sample Collection

Asmara

Polluted water sources used for domestic purposes were identified with the help of the Ministry of Land and Water resources. Their evaluation of the microbiological quality of the water is based on total and faecal coliform indicators as predictors of the presence of pathogenic microorganisms. All the water sources and hand dug wells previously found to be contaminated with faecal coliform bacteria were selected for sampling.

Water samples were collected from four dams (Mynefhi, Adinifas, Mayserwa, and Myanmbessa); two hand-dug wells (Sembel and Mychihot) and from taps on four occasions. Samples were collected in sterile bottles and were transported on ice to the Microbiology laboratory, Asmara Central Health Laboratory (CHL). Analyses were performed within six hours of collection.

Food samples collected included meat and vegetables. Beef and intestine samples were purchased from retail outlets, and were placed in sterile plastic bags. The samples were taken to the laboratory and were analyzed within 6 hours of collection. The vegetables sampled were salad, spinach, cucumber and cabbage. Vegetables are grown in Villajo (Adisegdo) using untreated wastewater that flows through channels, the so-called My Bella. These produce are sold in the nearby urban markets and are even transported to the lowlands regions such as Keren and Massawa. Samples were purchased directly from the farmers at Villajo (Adisegdo) as well as from a market located in the centre of the city. Samples were aseptically cut, placed in separate plastic bags and were transported on ice to the laboratory where they were analyzed within 6 hour of collection.

Stool samples were collected from the Paediatric, Halibet and Hazhaz hospitals in Asmara for the isolation of *Salmonella* strains. The samples were taken as swabs or in small containers and were analyzed within 6 hours of collection in the laboratory.

Massawa

Water samples were collected from the main water source used by inhabitants of Massawa. The subsurface dam at Dogali River, 22 km west of Massawa was sampled at two collection points. The first sample was from the main dam (untreated) and the second (treated with chlorine) a few kilometres away from the main dam. The samples were collected in sterile bottles at these two points on four occasions. Samples were transported on ice to the laboratory and were analyzed within 6 hours of collection.

Meat, intestine and a range of fresh vegetables, such as spinach, salad and cabbage were purchased from Edaga market located in the centre of the city. The vegetables were separately placed in sterile plastic bags and transported on ice to the laboratory where they were analyzed within 6 hours of collection.

Fresh stool samples were collected from Massawa hospital and were placed in transport media. The samples were transported to the Microbiology laboratory, Central Health Laboratory where they were analyzed within 6 hours of collection.

Keren

Water samples were collected from the two main water supply areas of the city. The samples were from a hand dug well in the riverbed of the Anseba River 3 km north west of Keren and from the Shifshifit hand-dug well at the base of Shifshshifit dam 3 km west of Keren. The samples were transported on ice to the laboratory and were analyzed within 6 hours of collection.

A range of fresh vegetables were purchased from the market. The vegetables selected were spinach, salad, cabbage and girgir (local salad). The vegetables were separately placed in a sterile plastic bag and transported on ice to the laboratory where they were analyzed within 6 hours of collection.

Fresh stool samples were collected from Keren hospital. The swab samples were taken, placed on transport media and were transported to the Microbiology laboratory, Central Health Laboratory for analysis.

3.2.3 Isolation and identification of *Salmonella* strains

3.2.3.1 Culture method

Isolation of *Salmonella* strains from stool, water and food samples collected from the three catchments areas were performed using standard culturing techniques. During cultivation pre-enrichment for the resuscitation and multiplication of damaged *Salmonella* isolates were followed by selective enrichment and plating.

The isolation of *Salmonella* bacteria from water samples collected from the three areas were performed as follows: 1 ml of water samples were added to 9 ml of buffered peptone water (Oxoid CM509) and appropriate dilutions were performed. The samples were incubated at 37 ^{o}C for 24 hours. For the selective enrichment Rappaport-Vassiliadis (RV) (Oxoid, CM 669) broth was used. For the isolation of *Salmonella*, 0.1 ml of the pre-enrichment buffered peptone water culture was transferred into 10 ml of RV broth and then incubated at 42 ^{o}C for up to 48 hours. Following incubation, a loopful of inoculum from the enrichment broth was streaked onto Xylose Lysine Deoxicolate (XLD) agar (Oxoid, CM0469) plates and then incubated at 37 ^{o}C for 24 hours.

Isolation of *Salmonella* from food samples collected from the three catchments areas were performed as follows: 10g of each food samples were homogenized in a sterile plastic bag with 90 ml of diluent (Zwittergent 3-12) solution using a stomacher. The diluent solution was prepared adding, 0,3355 mg Zwittegent 3-12, 3.804 mg EDTA, 0,1211g Tris (0,01 M; pH 7), and 0,1 g peptone to 90 ml distilled water (LeChevallier, *et al*, 1984) before sterilizing the diluent at 121 ^{o}C for 20 minutes. Appropriate tenfold dilutions were made using ¼ strength Ringers solution. One millilitre of the dilution was then transferred to 3 tubes containing 9 ml buffered peptone water. The tubes were then incubated at 37 ^{o}C for 24 hours. After incubation 0.1 ml buffered

peptone water was then transferred to 10 ml Rappaport-Vassiliadis (RV) broth and incubated at 42 ^{o}C for up to 48 hours. Following selective enrichment, the presence of *Salmonella* was determined by streaking a loopful of broth onto Xylose Lysine Deoxicolate (XLD) plates. The plates were incubated at 37 ^{o}C for 24 hours. Clinical stool samples collected from hospitals in the three areas were analysed according to the same procedure described above.

Suspected *Salmonella* colonies on XLD plates were subcultured on nutrient agar plates (Oxoid, CM3469). Presumptive *Salmonella* isolates were streaked on agar slants and were transported to South Africa. After transportation, the purity of cultures was evaluated using Bismuth Sulphite Agar, Hektoen Enteric Agar, Brilliant Green Agar and Blood Agar.

3.2.3.2 API 20E

The identity of all 94 presumptive *Salmonella* isolates collected from the three areas in Eritrea was confirmed using API 20E strips. Tests were performed according the manufacturers instruction.

3.2.3.3 Biochemical tests

Tartrate utilization and gelatine hydrolysis tests were employed to differentiate between the different *Salmonella* subspecies. The tartrate utilization test was carried out according to the method described by Ewing (1986). Organic acid media consisted of 10g peptone; 12 ml of a 0.2% bromothymol blue solution, and 10 g sodium potassium tartrate, which were added to 1 L of distilled water. The pH was thereafter adjusted to 7.4 with 10 N sodium hydroxide. The tubes, containing 3 ml of the medium, were inoculated with a loop of overnight culture and were incubated at 37 ^{o}C for 24 hours. A volume of 0.5 ml of a 50% neutral lead acetate solution was added to each of the tubes (experiment and control). Tartrate utilization was indicated by a decrease in the volume of precipitate formed when compared with the control tube.

Tartrate utilization was also confirmed using Phenol Red Tartrate agar, as described by Ewing (1986). The agar deeps were inoculated by stabbing into the column of media with a straight wire, using young overnight cultures of the *Salmonella* isolates. The tubes were incubated at 37 ^{o}C for 48 hours. Positive tests for tartrate utilization were indicated by the development of an acid reaction (yellow colour). Cultures that tested positive for tartrate utilization were considered to belong to *Salmonella enterica* subsp. *enterica* or Sub-species 1 and negative isolates were considered to belong to the environmental isolates or *Salmonella* subspecies II-VI.

The gelatin hydrolysis test was performed using Nutrient Gelatin and the medium was prepared according to the method described by Ewing (1986). The agar deeps were inoculated by stabbing into the column of media with a straight wire, using young overnight cultures of the *Salmonella* isolates. The tubes were incubated at 37 ^{o}C for 48 hours. Positive tests for gelatin hydrolysis were indicated by the liquefaction of the gelatin. Isolates testing negative for gelatin liquefaction are considered to be of possible the human origin (*Salmonella enterica* subspecies I).

3.2.4 Determination of specific *Salmonella* serovars using PCR

The 94 *Salmonella* isolates were subjected to PCR in order to determine whether the stains belong to the Typhimurium serovar or specific members of the D_1 O antigen group (*S.* Enteritidis, *S.* Pullorum and *S.* Gallinarum).

DNA was extracted for the various cultures by resuspending a loopful of an overnight culture into 100 µl of distilled water and then boiling it for 10 minutes in order to release the bacterial DNA from the cells. The suspension was centrifuged for 30 seconds to separate bacterial cell debry (pellet) from the bacterial DNA present in the supernatant. A 5 µl volume of the lysate was used as DNA template for the PCR reaction.

Two PCR assays were carried out. The first primer set Fli15 - Tym (Table 3.1) was specific for the *fliC* gene of *S.* Typhimurium encoding flagellin H1 and the second set

A058 - A01 (Table 3.1) was specific for the *sefA* gene found in *S.* Enteritidis, *S.* Pullorum and *S.* Gallinarum (Olivera *et al*., 2002).

Amplification reactions were carried out in a total volume of 50 µl, containing 1.5 U Taq polymerase (Southern Cross), 6 µM of each primer, 0.2mM of each dNTPs, 1 X PCR buffer, 1.5 mM $MgCl_2$ for the *S.* Typhimurium assay and 2.5 mM for *S.* Enteritidis assay. Five µl of the bacterial DNA solution was added. Nuclease free water (Promega) was added up to a final volume of 50µl. The PCR reaction was carried out in a Gene Amp 2700 Thermocycler (Applied Biosystem). The cycling profile consisted of initial denaturation at 94 ^{o}C for 1 minute, followed by 35 cycles of denaturation at 94 ^{o}C for 30 seconds, annealing at 56 ^{o}C for the *S.* Typhimurim assay and 55 ^{o}C for *S.* Enteritidis assay and extension at 72 ^{o}C for 30 seconds. The cycle was completed with a final terminal extension at 72 ^{o}C for 7 minutes.

Table 3.1 Primers used for the detection of *S.* Tyhimurium or *S.* Enteritidis, *S.* Gallinarum and *S.* Pullorum serovars

Target Primer	Length	5'-3'	Target sequence	Amplification region
*fli*C gene	Fli15	22	CGGTGTTGCCCAGGTTGGTAAT	488
	Tym	22	ACTCTTGCTGGCGGTGCGACTT	
*sef*A gene	A058	21	GATACTGCTGAACGTAGAAGG	559
	A01	24	GCGTAAATCAGCATCTGCAGTAGC	

The amplified DNA products were resolved by agarose gel electrophoresis and visualized by UV transillumiation after ethidium bromide staining. Amplified products (10 µl PCR product with 2 µl loading buffer) were separated by electrophoresis on a 1.2 % agarose gel (Agarose, LE analytical Grade). The gel was run in 1X TBE buffer and DNA Molecular Weight Marker VI (Roche) was used for sizing of the fragments.

3. 3 Results

3.3.1 Sampling results

In this study a total of 225 samples were tested. The water (50), food (51), and clinical (124) samples were collected from the three catchments areas (Asmara, Massawa and Keren) between July and September 2003. Samples were tested using non-selective enrichment in BPW at 37 ^{o}C, selective enrichment in RV broth at 42 ^{o}C and XLD to culture presumptive *Salmonella* colonies. The study showed higher isolation rates of *Salmonella* from water (64%) and food (41%) samples compared to clinical (33%) stool samples as indicated in Table 2.

In the study, 32 of the 50 water samples testes positive for *Salmonella*. The majority of the samples were from Asmara. Of the six water sampling points in the Asmara region, *Salmonella* were detected in the two main water sources (Mynefhi and Adinifas tahtay), in the two hand-dug drinking wells (Sembel and Mychihot) and from the tap water sampled. As each spot has been sampled four times, a total of 16 *Salmonella* positive samples were obtained from the two main water sources and two hand-dug wells, but only two *Salmonella* positive samples were obtained from the tap water samples tested. *Salmonella* were detected in 41 of the 67 clinical samples obtained from Asmara and 9 of the food samples tested positive.

Water samples from Massawa were collected from the untreated main source (Dogoli River) and the chlorinated drinking water supply. *Salmonella* were detected at both sampling sites. Each spot were sampled four times and a total of six *Salmonella* positive samples were collected. Four of the samples were from the untreated main water source (Dogoli) while two were from the chlorinated drinking water. From the variety of vegetables and meat tested, *Salmonella* were detected in meat, spinach and salad. *Salmonella* were detected in 8 of the 33 clinical samples obtained from the Massawa region.

A total of 21 *Salmonella* isolates were collected form Keren. Eight of the *Salmonella* were isolated from water collected from the two sampling sites (Ruba anseba and

Shifshifit), 6 of the isolates were collected from food (spinach, salad, and girgir) and 7 isolates were obtained from the 24 clinical samples collected at Keren hospital.

3.3.2 Biochemical test

Salmonella isolates collected from the three catchments were tested for tartrate utilization and gelatine hydrolysis in order to determine to which sub-species they could possibly belong. Tartrate utilization was confirmed by the addition of lead acetate to the Organic acids medium. The water isolates collected from the three areas (18 samples for Asmara, 6 samples from Massawa, 8 samples from Keren) and the isolates from food samples like spinach, meat, intestine, and salad all gave a positive reaction compared to the control tube. Except for few samples, however, most of the clinical samples didn't show a positive reaction.

Tartrate utilization was also confirmed using Phenol Red Tatrate agar. All the isolated obtained from the water samples showed a positive reaction. The food as well as clinical isolates also showed a positive reaction. A positive test for gelatin hydrolysis was indicated by the liquefaction of the gelatine. All the water, food and clinical samples were negative for the gelatin hydrolysis test. The results of the biochemical tests are summarized in Table 3.2.

Based on the results of the biochemical tests (tartrate utilization and gelatine hydrolysis), all *Salmonella* isolates collected from the three catchments areas were identified as *Salmonella* subspecies I, commonly associated with humans and warm-blooded animals.

3.3.3 Determination of specific *Salmonella* serovars using PCR

Two PCR based assays were used to identify possible *Salmonella* Enteritidis and *Salmonella* Typhimurium strains. The PCR tests targeted either the *fliC* or *sefA* genes specific for *S*. Typhimurium and *S*. Enteritidis respectively. The expected fragment was 559 bp for *S*. Typhimurium and 488 bp for *S*. Enteritidis. Out of the 94

Salmonella isolates tested only 6 were positive for the PCR reactions. These six isolates gave a 559 amplification product from the *fliC* gene. The results are summarized in Table 3.2.

Table 3.2 Summary of the results of the biochemical and PCR tests performed on the *Salmonella* isolates

Sample	Strain source	API 20E	Tartrate utilization		Gelatin hydrolysis	PCR	
			Lead acetate	Phenol red		*Salmonella* Enteritidis	*Salmonella* Typhimurim
Clinical 1	Asmara	*Salmonella*	–	+	–	–	–
Clinical 2	Asmara	*Salmonella*	+	+	–	–	–
Clinical 3	Asmara	*Salmonella*	+	+	–	–	–
Clinical 4	Asmara	*Salmonella*	–	+	–	–	–
Clinical 5	Asmara	*Salmonella*	+	+	–	–	–
Clinical 6	Asmara	*Salmonella*	–	+	–	–	–
Clinical 7	Asmara	*Salmonella*	–	+	–	–	–
Clinical 8	Asmara	*Salmonella*	–	+	–	–	–
Clinical 9	Asmara	*Salmonella*	–	+	–	–	–
Clinical 10	Asmara	*Salmonella*	–	+	–	–	–
Clinical 11	Asmara	*Salmonella*	–	+	–	–	–
Clinical 12	Asmara	*Salmonella*	–	+	–	–	–
Clinical 13	Asmara	*Salmonella*	–	+	–	–	–
Clinical 14	Asmara	*Salmonella*	–	+	–	–	–
Clinical 15	Asmara	*Salmonella*	–	+	–	–	–
Clinical 16	Asmara	*Salmonella*	–	+	–	–	–
Clinical 17	Asmara	*Salmonella*	–	+	–	–	–
Clinical 18	Asmara	*Salmonella*	–	+	–	–	–
Clinical 19	Asmara	*Salmonella*	+	+	–	–	+
Clinical 20	Asmara	*Salmonella*	–	+	–	–	–
Clinical 21	Asmara	*Salmonella*	+	+	–	–	–
Clinical 22	Asmara	*Salmonella*	+	+	–	–	–
Clinical 23	Asmara	*Salmonella*	+	+	–	–	–
Clinical 24	Asmara	*Salmonella*	+	+	–	–	+
Clinical 25	Asmara	*Salmonella*	+	+	–	–	+
Clinical 26	Asmara	*Salmonella*	+	+	–	–	–

Table 3.2 (continued)

Sample	Strain source	API 20E	Tartrate utilization		Gelatin hydrolysis	PCR	
			Lead acetate	Phenol red		*Salmonella* Enteritidis	*Salmonella* Typhimurim
Mynefhi 1	Asmara	*Salmonella*	+	+	–	–	–
Mynefhi 2	Asmara	*Salmonella*	+	+	–	–	–
Mynefhi 3	Asmara	*Salmonella*	+	+	–	–	+
Mynefhi 4	Asmara	*Salmonella*	+	+	–	–	+
Mychihot 1	Asmara	*Salmonella*	+	+	–	–	–
Mychihot 2	Asmara	*Salmonella*	+	+	–	–	–
Mychihot 3	Asmara	*Salmonella*	+	+	–	–	–
Mychihot 4	Asmara	*Salmonella*	+	+	–	–	–
Sembel 1	Asmara	*Salmonella*	+	+	–	–	–
Sembel 2	Asmara	*Salmonella*	+	+	–	–	–
Sembel 3	Asmara	*Salmonella*	+	+	–	–	–
Sembel 4	Asmara	*Salmonella*	+	+	–	–	–
Adinifas 1	Asmara	*Salmonella*	+	+	–	–	–
Adinifas 2	Asmara	*Salmonella*	+	+	–	–	–
Adinifas 3	Asmara	*Salmonella*	+	+	–	–	–
Adinifas 4	Asmara	*Salmonella*	+	+	–	–	–
Meat 1	Asmara	*Salmonella*	+	+	–	–	+
Meat 2	Asmara	*Salmonella*	+	+	–	–	–
Intestine	Asmara	*Salmonella*	+	+	–	–	–
Spinach 1	Asmara	*Salmonella*	+	+	–	–	–
Spinach 2	Asmara	*Salmonella*	+	+	–	–	–
Salad 1	Asmara	*Salmonella*	+	+	–	–	–
Salad 2	Asmara	*Salmonella*	+	+	–	–	–
Salad 3	Asmara	*Salmonella*	+	+	–	–	–
cabbage	Asmara	*Salmonella*	+	+	–	–	–

Table 3.2 (continued)

Sample	Strain source	API 20E	Tartrate utilization		Gelatin hydrolysis	PCR	
			Lead acetate	Phenol red		*Salmonella* Enteritidis	*Salmonella* Typhimurim
Clinical 1	Massawa	*Salmonella*	–	+	–	–	–
Clinical 3	Massawa	*Salmonella*	–	+	–	–	–
Clinical 4	Massawa	*Salmonella*	–	+	–	–	–
Clinical 5	Massawa	*Salmonella*	–	+	–	–	–
Clinical 7	Massawa	*Salmonella*	–	+	–	–	–
Clinical 8	Massawa	*Salmonella*	–	+	–	–	–
Clinical 9	Massawa	*Salmonella*	–	+	–	–	–
Clinical 10	Massawa	*Salmonella*	+	+	–	–	–
Water 1 (2)	Massawa	*Salmonella*	–	+	–	–	–
Water 1 (4)	Massawa	*Salmonella*	+	+	–	–	–
Water 2 (1)	Massawa	*Salmonella*	+	+	–	–	–
Water 2 (4)	Massawa	*Salmonella*	+	+	–	–	–
Water 2 (3)	Massawa	*Salmonella*	+	+	–	–	–
Water 2 (4)	Massawa	*Salmonella*	+	+	–	–	–
Meat 1	Massawa	*Salmonella*	+	+	–	–	–
Meat 2	Massawa	*Salmonella*	+	+	–	–	–
Spinach 1	Massawa	*Salmonella*	+	+	–	–	–
Spinach 2	Massawa	*Salmonella*	+	+	–	–	–
Salad 1	Massawa	*Salmonella*	+	+	–	–	–
Salad 2	Massawa	*Salmonella*	+	+	–	–	–

Table 3.2 (continued)

Sample	Strain source	API 20E	Tartrate utilization		Gelatin hydrolysis	PCR	
			Lead acetate	Phenol red		*Salmonella* Enteritidis	*Salmonella* Typhimurim
Clinical 1	Keren	*Salmonella*	–	+	–	–	–
Clinical 2	Keren	*Salmonella*	–	+	–	–	–
Clinical 3	Keren	*Salmonella*	–	+	–	–	–
Clinical 4	Keren	*Salmonella*	–	+	–	–	–
Clinical 5	Keren	*Salmonella*	–	+	–	–	–
Clinical 6	Keren	*Salmonella*	–	+	–	–	–
Clinical 7	Keren	*Salmonella*	–	+	–	–	–
Ruba anseba 1	Keren	*Salmonella*	+	+	–	–	–
Ruba anseba 2	Keren	*Salmonella*	+	+	–	–	–
Ruba anseba 3	Keren	*Salmonella*	+	+	–	–	–
Ruba anseba 4	Keren	*Salmonella*	+	+	–	–	–
Shifshifit 1	Keren	*Salmonella*	+	+	–	–	–
Shifshifit 2	Keren	*Salmonella*	+	+	–	–	–
Shifshifit 3	Keren	*Salmonella*	+	+	–	–	–
Shifshifit 4	Keren	*Salmonella*	+	+	–	–	–
Spinach 1	Keren	*Salmonella*	+	+	–	–	–
Spinach 2	Keren	*Salmonella*	+	+	–	–	–
Salad 1	Keren	*Salmonella*	+	+	–	–	–
Salad 2	Keren	*Salmonella*	+	+	–	–	–
Girgir 1	Keren	*Salmonella*	+	+	–	–	–
Girgir 2	Keren	*Salmonella*	+	+	–	–	–

3.4 Discussion

Although it is known that *Salmonella* infections are widespread in Eritrea no data exist on the bacterial quality of water and food and their likely role in human infections. This study has, therefore, been conducted to assess the role of water and food in *Salmonella* transmission. In this study a total of 225 water, food and clinical samples were collected from three selected areas in Eritrea. The collection has been done over a period of three months between July and September 2003. The 225 samples included 124 from clinical cases, 50 from water, and 51 from food. Of the 94 *Salmonella* positive isolates, 41 were isolated from the clinical samples, 32 from water and 21 from food.

The incidence of *Salmonella* in water was relatively high, as 32 of the 50 water samples were found positive for the organism. This indicates high contamination of water with *Salmonella*. In the three areas selected many of the communities are supplied with treated piped water, but others in those areas do not have access to treated water. These communities use untreated surface or ground water or water from nearby hand-dug wells for drinking. These water sources are usually contaminated by faecal material from humans and animals, especially during the wet season when wastes and faecal material are washed into the water. In all three these areas water poses a health risk to consumers if the water is consumed without treatment. *Salmonella* was also isolated from treated water samples (chlorine treated) collected from Asmara and Massawa. This indicates that the water was not treated efficiently to kill all the microbes and additional treatment is therefore required to prevent transmission of *Salmonella* and other pathogenic microorganisms.

The isolation of 21 *Salmonella* isolates from 51 food samples also indicates a high incidence of *Salmonella* in certain food products. It has been reported in many studies that *Salmonella* is usually isolated from food of animal origin especially poultry meat, beef and raw eggs (Swanenburg *et al*., 2001, Fratamico, 2003; Nayak *et al*., 2004). In this study only meat and intestine samples were tested since poultry is not as common in Eritrea as in other countries. *Salmonella* was isolated from meat and intestine samples from Asmara and meat samples from Massawa, but none were recovered from samples collected from Keren.

In this study, most of the vegetable samples examined were found to be contaminated with *Salmonella*. The vegetables may be contaminated while growing, during harvest or during post-harvest handling and distribution. In many areas of Eritrea untreated wastewater flow into rivers and streams. This contaminated water is used to irrigate some plots of vegetables and salad that are sold in the nearby urban markets. The use of contaminated containers or animal wastes as fertilizer could also be an important source of contamination. Some of the vegetables sampled in Asmara were purchased from Villajo, a place where vegetables are irrigated with the untreated wastewater (Mybella). Not only are these vegetables sold in markets in Asmara but they are also sold in markets in Keren, Massawa and other lowland areas. This reinforces the need for a proper system to manage the risk of infection due to fresh produce of poor microbial quality.

Following the isolation, *Salmonella* species and subspecies were differentiated phenotypically using biochemical test. The biochemical tests included tartrate utilization and gelatin hydrolysis. Based on the result of the biochemical tests, all the 94 *Salmonella* isolates were identified as *Salmonella enterica* subspecies I, which is commonly associated with humans and warm-blooded animals. Many serotypes of subspecies I are pathogenic and are able to cause infections such as gastroenteritis, septicaemia and other serious conditions in humans and animals.

S. Typhimurium and *S*. Enteritidis are the most common serotypes recovered worldwide (Soumet *et al*., 1999). To establish whether this was also the case with the isolates obtained from Eritrea all isolates were screened using two PCR reactions. The first primer set, Fli15 - Tym was specific for the *fliC* gene from *S*. Typhimurium which encodes for flagellin H1. The second set, A058 - A01, was specific for the *sefA* gene found in *S*. Enteritidis, *S*. Pullorum and *S*. Gallinarum.

In this study only six out of 94 isolates gave positive results. This indicates that the common serotypes *S*. Typhimurium and *S*. Enteritidis are not as widespread in Eritrea as is often found in other parts of the world. Other serotypes may therefore be of epidemiological importance in the areas investigated.

3.5 Conclusions

In this study, the isolation of *Salmonella* from water, food and clinical samples indicated the wide spread distribution of *Salmonella* in Eritrea. The results of this study also showed that the occurrence of *Salmonella* was not confined to a particular location as *Salmonella* was recovered from all three areas sampled. Due to logistical reasons the sample collection was only done over a three-month period, which corresponded with the wet season in Eritrea. Collection of samples over an extended period of time, which include both the wet and the dry season, would provide a better idea of the possible seasonal variation in the level of *Salmonella* present in the environment.

Chapter 4

AFLP Typing of *Salmonella* Strains Isolated From Three Areas in Eritrea

4.1 Introduction

Amplified fragment length polymorphism (AFLP) is a genotyping method that combines restriction endonuclease digestion and polymerase chain reaction (PCR) amplification. The method involves digestion with two suitable restriction endonucleases, ligation of suitable adapters and then followed by PCR amplification (Terletski *et al*., 2004). The resulting PCR products are resolved on polyacrlamide sequencing gels. AFLP detects DNA variation at specific restriction enzyme sites and it has been found to be of higher sensitivity and resolution than RAPD and PFGE fingerprinting methods (Lindstedt *et al*., 2000; Wang *et al*., 2004).

In this study AFLP was performed to establish possible relationships between the clinical and environmental *Salmonella* isolates collected from three areas in Eritrea. AFLP banding patterns from the isolates associated with clinical cases (hospital isolates) were compared with those obtained from environmental sample isolates (water and food). *Salmonella* types were also identified by serotyping of representative strains from the AFLP clusters obtained.

4.2 Materials and Methods

4.2.1 AFLP

DNA extraction

Nutrient broth medium was inoculated with the *Salmonella* isolates and incubated overnight at 37°C. Genomic DNA was extracted and purified using the DNeasy tissue extraction kit (Qiagen) according to the manufacturer's instructions. An aliquot of 1 ml broth was centrifuged for 10 minutes at 7500 rpm to harvest the bacterial cells. The supernatant was discarded and the bacterial pellet was resuspended in 180µl of ATL buffer. For the cells to be lysed, 20µl of Proteinase K solution was added, mixed and the solution was incubated at 55 °C for one hour. After spinning for 15 s, 200µl AL buffer was added and thoroughly mixed by vortexing to give a homogenous solution. The solution was then incubated at 70 °C for 10 minutes. Thereafter 200µl ethanol (96%) was added, mixed thoroughly and then transferred onto the DNeasy spin column. The DNeasy column was centrifuged for 1 minute to remove the liquid phase; the column was washed using 500 µl of AW2 buffer and thereafter centrifuged for 3 minutes at full speed to dry the DNeasy membrane. The elution of the DNA was performed by adding 100µl of AE buffer directly onto the DNeasy membrane and incubating it for one minute at room temperature. After spinning the column for one minute the buffer containing the DNA was collected. The elution process was repeated to collect the DNA that still remained on the column.

Restriction enzyme digestion

Genomic DNA was digested with *Mse*I and *EcoR*I. *Mse*I is known to cut DNA frequently whereas *EcoR*I cuts DNA infrequently. The restriction enzyme digestion was carried out in a total volume of 15 µl, which contained 5 µM of *EcoR*I and 4 µM of *Mse*I, 1X R/L Buffer, 3 µl bacterial DNA and 8.1 µl nuclease free water. The digestion reaction was incubated at 37 °C for two hours.

Ligation of adaptors

In order to generate DNA template for the subsequent PCR amplification, the digested DNA fragments were ligated with double stranded adaptors with sticky ends, which corresponded to the single stranded DNA ends generated by the restriction enzyme digestion. The ligation reaction was performed in a total volume of 20 µl, which contained 1.0 µl of 1X R/L Buffer, 2.0µl of ATP, 0.1 µl of *EcoR*I adaptor, 0.1 *Mse*I of adaptor, 0.5 µl of T4 DNA ligase, 15µl of the digested DNA reaction and 1.3µl of nuclease free water. The ligation reaction was incubated at 20 ^{o}C for two hours. The solution, which contained the ligated DNA fragments, was diluted 1:10 with TE buffer. These fragments served as unique primer binding sites for the subsequent PCR amplification.

Pre-amplification

A PCR reaction was performed on the template DNA using *EcoR*I-0 and *Mse*I-0 (Table 4.1) primers. Only genomic fragments, which have an adaptor at each end, would be amplified exponentially. Pre-amplifications reactions were carried out in a total volume of 25 µl, containing 3 U Taq Polymerase (JMR, Southern Cross), 2 µM of each primer, 0.2 mM of each dNTPs, 1 X PCR buffer, 3mM $MgCl_2$, nuclease free water and 2 µl of the ligation reactions. The PCR reaction was carried out in a Gene Amp 2700 Thermocycler (Applied Biosystem). The cycling profile was performed with an initial denaturation step at 94 ^{o}C for 3 min, followed by 20 cycles of denaturation at 94 ^{o}C for 30 s, annealing at 56 ^{o}C for 1 min and extension at 72 ^{o}C for 1 minute.

Selective amplification

The pre-amplification product was diluted 1:50 in TE buffer and used for the selective PCR amplification reaction. The selective amplification reaction was carried out in a total volume of 20 µl, containing 2.5 U Taq Polimerase (JMR, Southern Cross), 6 µM *EcoR*I-G and 2.5 µM *Mse*I- T primer (Table 4.1), 0.25 mM of each dNTPs, 1 X PCR buffer, 2.5 mM $MgCl_2$, nuclease free water and 5 µl of the diluted pre-amplification

product. The PCR reaction was carried out in a Gene Amp 2700 Thermocycler (Applied Biosystem). The cycling parameters included an initial denaturation at 94ºC for 5min, followed by denaturation at 94 ^{o}C for 30 seconds, annealing at 56 ^{o}C and extension at 72 ^{o}C for 1min. The first cycles were touch-down from 65 ^{o}C to 56 ^{o}C decreasing by 1 ^{o}C per cycle, and then 23 cycles of denaturation at 94 ^{o}C for 30 seconds, annealing at 56 ^{o}C for 30 seconds and extension at 72 ^{o}C for 1minute.

Table 4.1 Primers used for pre and selective PCR amplification reaction

Primer	Sequence (5'→3')
*Eco*RI-0	GACTGCGTACCAATTC
*Mse*I-0	GATGAGTCCTGAGTAA
*Eco*RI-G	GACTGCGTACCAATTCG
*Mse*I- T	GATGAGTCCTGAGTAAT

Gel analysis

Amplification products were separated on an 8 % polyacrylamide gel using the LI-COR DNA analyzer system (LI-COR). The gel was allowed to polymerise at room temperature for at least 1 hour. Each sample (2µl) was added to 2µl of loading dye solution (composed of 98 ml of 98 % formamide, 0.294 g EDTA, 50 mg Bromophenol blue and 50 mg xylene cyanide). The sample mix was denatured at 90^{o}C for 3 minutes, cooled on ice immediately and loaded onto the gel. The gel was electrophoresed for 4 hours using 1X TBE as the buffer. The IRD 700 AFLP marker was run in a number of separate lanes to allow rapid identification of bands and normalization of the gels.

4.2.2 Data interpretation

The AFLP image was captured and imported into the GelCompar II programme (Applied Maths) for further analysis. The AFLP marker 700 was used as reference for the necessary normalization of the gels. The standard is composed of 18 IRD-labelled DNA fragments with equal banding intensities. These fragments cover a size range of 50 to 700 bp. Profiles were compared by means of the Dice coefficient and the unweighted-pair group method using arithmetic means (UPGMA) was used to cluster the AFLP patterns of all the *Salmonella* isolates.

4.2.3 Serotyping of selected strains

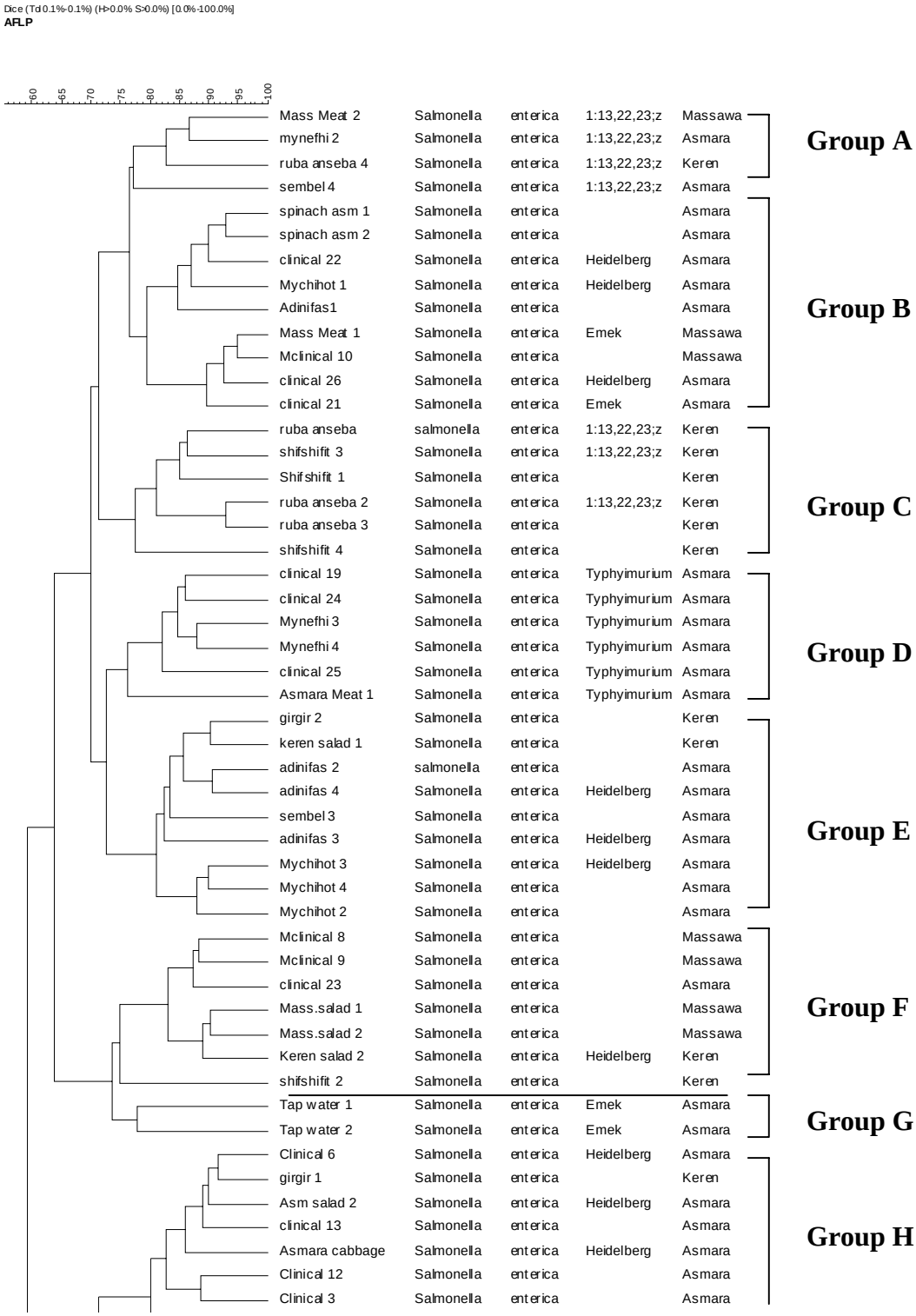
Serotyping of *Salmonella* is the most commonly used typing method for this genus (Yan *et al*., 2003). It represents an important tool for identification and grouping of strains especially when it is used for epidemiological studies. Using the dendrogram based on the AFLP patterns of the 94 Eritrean isolates of *Salmonella*; representative isolates from each cluster were selected and sent for serotyping at the National Health Laboratory Services, Johannesburg.

4.3 Result

The digestion of chromosomal DNA of 94 *Salmonella* isolates was performed using the restriction enzymes *Eco*RI and *Mse*I. The digestion products were amplified with two primer combinations, *EcoRI*-0/ *MseI*-0 as non-selective primers and a further selective primer set *Eco*RI-G/ *Mse*I-T. The AFLP fingerprints of the 94 *Salmonella* isolates generated a total of between 70 - 85 separate bands.

The AFLP data were used to construct a dendrogram using the unweighed pair-group method using arithmic means (UPGMA) with Gel Compare II software. The AFLP dendrogram revealed 15 clusters designated as A, B, C, D, E, F, G, H, I, J, K, L, M, N, O and four *Salmonella* isolates remained ungrouped. Clusters were designated at a

similarity level of 76%. The linkage level of the various clusters ranged from 90% to 76%. The dendrogram is shown in Fig 4.1.



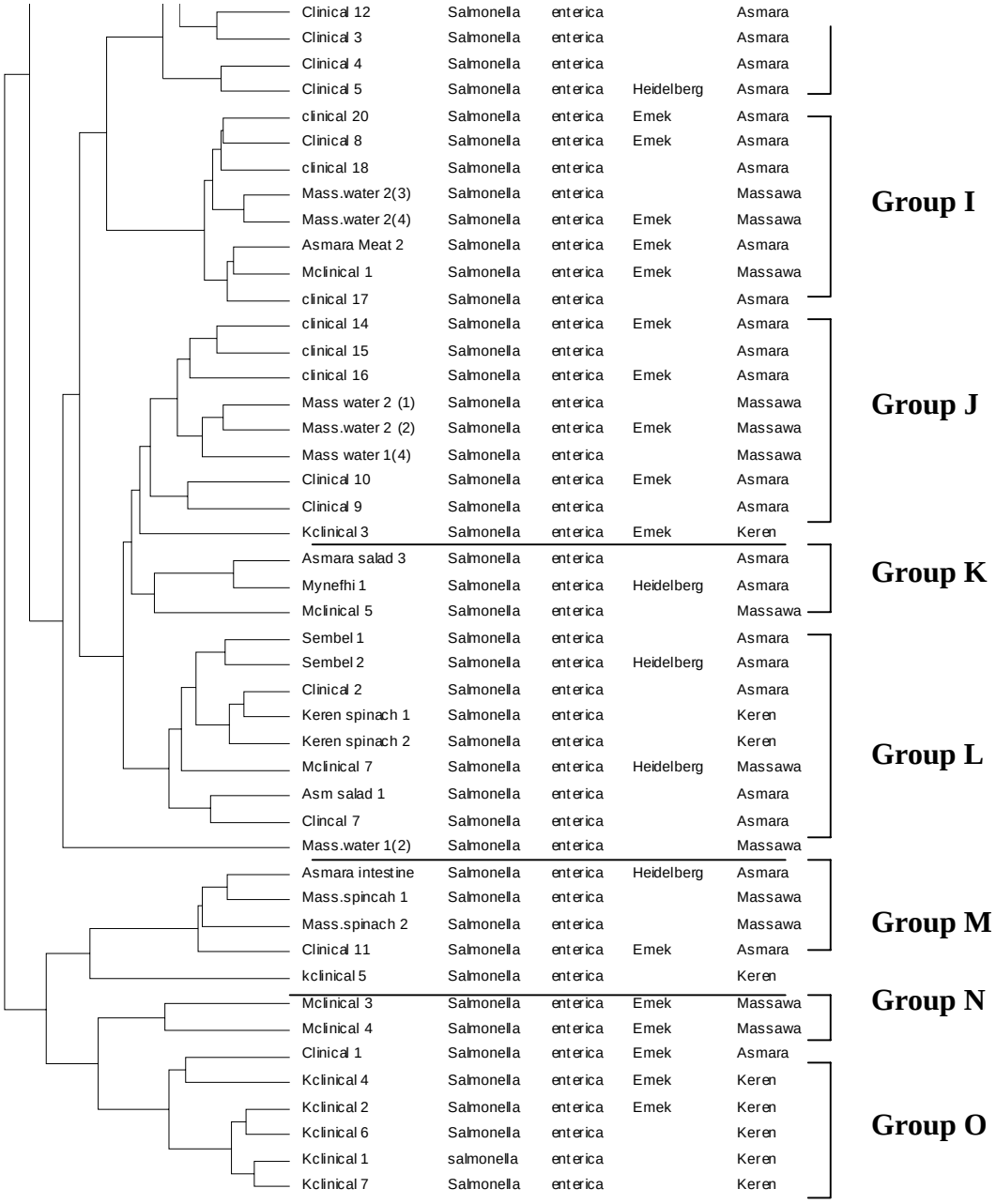


Fig. 4.1 Dendrogram based on the AFLP profiles of 94 *Salmonella* strains isolated from environmental and clinical samples in Eritrea. AFLP groups are indicated in parenthesis and the isolates which are ungrouped are underlined.

The resulting dendrogram shows the genetic relationship among the *Salmonella* isolates. At least two representative isolates from each cluster were selected for serotyping. Six (6) of the *Salmonella* isolates were already identified using the PCR method. According to the result obtained, the majority of *Salmonella* isolates within a cluster belonged to one particular serotype, but in two clusters (group B and M) different serotypes were identified within one cluster.

Of the 94 *Salmonella* isolates collected, 48 (51%) strains were serologically identified. These serotypes include, 21 *Salmonella* Emek (43.7%), 19 *Salmonella* Heidelberg (39.5%), 7 of the 13, 22, 23; z undetermined serotype (14.5%), and 2 *Salmonella* Typhimurium strains (4.1%). The two *S*. Typhimurium isolates were previously identified using PCR and this was confirmed by the seroptyping. The AFLP grouping and the results of the serotyping are summarized in Table 4.2.

AFLP cluster A consisted of four strains obtained from two water samples from Asmara, a water sample from Keren and a food sample from Massawa and were linked at 76% similarity. Even though these four strains belong to *Salmonella enterica* subspecies *enterica* their serotype cannot be determined (1:13, 22, 23; z) because there was over expression of the Z in the H phase.

Group B consisted of two subclusters joined together at 80%. Subcluster 1 consisted of five isolates obtained from two food samples; two water samples and one clinical sample from Asmara and the isolates linked together at 85% similarity. Of the five isolates two strains were serologically identified as *S*. Heidelberg. Sub cluster 2 consisted of four isolates obtained from two clinical samples from Asmara as well as a clinical and food sample from Massawa and were linked at 90% similarity. Of the four isolates three strains were serologically identified as *S*. Emek and one as *S*. Heidelberg.

Group C consisted of six isolates obtained from water samples form Keren and were linked at 76% similarity. Of the six isolates three were serologically identified as 1:13, 22, 23; z (undetermined serotype).

Group D consisted of six isolates obtained form three clinical, two water and one food samples from Asmara and were linked at 76% similarity. Of the six isolates two strains were serologically identifies as *S*. Typhimurium. Those six isolates were also previously identified as *S*. Typhimurium using the PCR method.

Group E consisted of nine isolates obtained from seven water samples from Asmara and two food samples from Keren. All were linked at 82% similarity. Of the nine isolates three strains were serologically identified as *S*. Heidelberg.

Group F consisted of seven isolates obtained form two clinical and two food samples from Massawa, one clinical sample form Asmara and one water and one food sample from Keren. These strains were joined at 76% similarity. Of the seven isolates one was serologically identified as *S*. Heidelberg.

Group G consisted of two isolates obtained from tap water from Asmara and were linked at 78% similarity. Both isolates were serologically identified as *S*. Emek.

Group H consisted of nine isolates obtained from six clinical and two food samples form Asmara and one food sample from Keren. The isolates were joined at a similarity of 82%. Of the nine isolates four were serologically identified as *S*. Heidelberg.

Group I consisted of eight isolates obtained from four clinical and one food sample from Asmara, two water and one clinical sample form Massawa. These strains linked at a similarity level of 85%. Of the eight isolates three were serologically identified as *S*. Emek.

Group J consisted of eight isolates obtained from five clinical samples collected from Asmara and three water samples form Massawa. The cluster formed at a 76% similarity level. Of the eight isolates, two were serologically identified as *S*. Emek.

Group K consisted of three isolates obtained from food and water samples from Asmara and a clinical sample from Massawa. These isolates joined at a similarity level of 76%. Of the three isolates one was serologically identified as *S*. Heidelberg.

Group L consisted of eight strains isolated from two clinical, two water and one food sample obtained from Asmara, two food samples obtained from Keren and one clinical sample from Massawa. The strains in cluster L grouped at a similarity level of 82%. Of the eight isolates two isolates were serologically identified as *S*. Heidelberg.

Group M consisted of four isolates obtained from two food samples from Massawa, one clinical and one food sample from Asmara and were linked together at 90% similarity. Of the four strains, two were serologically identified as *S*. Emek (clinical 11) and *S*. Heidelberg (Asmara intestine).

Group N consisted of two isolates obtained from clinical samples from Massawa. Both were serologically identified as *S*. Emek.

Group O consisted of six isolates obtained form five clinical isolates form Keren and one clinical sample form Asmara They joined at a similarity level of 90%. Of the six isolates three were serologically identified as *S*. Emek.

Table 4.2 List of *Salmonella* strains used in the AFLP analysis

Group	Sample	Species	Serotype	Stain source
A	Massawa meat 2	Massawa	*S. enterica*	1:13,22,23;z
	Mynefhi 2	Asmara	*S .enterica*	1:13,22,23;z
	Rubaanseba	Keren	*S .enterica*	1:13,22,23;z
	Sembel 4	Asmara	*S. enterica*	1:13,22,23;z
B	Spinach Asmara 1	Asmara	*S. enterica*	
	Spinach Asmara 2	Asmara	*S. enterica*	
	Clinical 22	Asmara	*S. enterica*	*S.* Heidelberg
	Mychihot 1	Asmara	*S. enterica*	*S.* Heidelberg
	Adnifas 1	Asmara	*S. enterica*	
	Meat 1	Massawa	*S. enterica*	*S.* Emek
	Clinical 10	Massawa	*S. enterica*	
	Clinical 26	Asmara	*S. enterica*	*S.* Heidelberg
	Clinical 21	Asmara	*S. enterica*	*S.* Emek
C	Rubaanseba1	Keren	*S. enerica*	1:13,22,23;z
	Rubaanseba2	Keren	*S. enerica*	1:13,22,23;z
	Rubaanseba 3	Keren	*S. enterica*	
	Shifshifit 1	Keren	*S. enterica*	
	Shifshifit 3	Keren	*S. enterica*	1:13,22,23;z
	Shifshifit 4	Keren	*S. enterica*	
D		Asmara	*S. enterica*	*S.* Typhimurium
	Clinical 19	Asmara	*S. enterica*	*S.* Typhimurium
	Clinical 24	Asmara	*S. enterica*	*S.* Typhimurium
	Clinical 25	Asmara	*S. enterica*	*S.* Typhimurium
	Mynefhi 3	Asmara	*S. enterica*	*S.* Typhimurium
	Mynefhi 4			

Table 4.2 (continued)

Group	Sample	Species	Serotype	Stain source
E	Girgir 2	Keren	*S. enterica*	
	Salad 1	Keren	*S .enterica*	
	Adinifas 2	Asmara	*S. enterica*	
	Adinifas 4	Asmara	*S. enterica*	*S*. Heidelberg
	Sembel 3	Asmara	*S. enterica*	
	Adinifas 3	Asmara	*S. enterica*	*S*. Heidelberg
	Mychihot 3	Asmara	*S. enterica*	*S*. Heidelberg
	Mychihot 4	Asmara	*S. enterica*	
	Mychihot 2	Asmara	*S. enterica*	
F	Clinical 8	Massawa	*S. enterica*	
	Clinical 9	Massawa	*S. enterica*	
	Clinical 23	Asmara	*S. enterica*	
	Salad 1	Massawa	*S. enterica*	
	Salad 2	Massawa	*S. enterica*	
	Salad 2	Keren	*S. enterica*	*S*. Heidelberg
G	Tap water 1	Asmara	*S. enteica*	*S*. Emek
	Tap water 2	Asmara	*S .enterica*	*S*. Emek
H	Clinical 6	Asmara	*S. enterica*	*S*. Heidelberg
	Girgir 1	Keren	*S. enterica*	
	Salad 2	Asmara	*S. enterica*	*S*. Heidelberg
	Clinical 13	Asmara	*S. enterica*	
	Asmara cabbage	Asmara	*S. enterica*	*S*. Heidelberg
	Clinical 12	Asmara	*S. enterica*	
	Clinical 3	Asmara	*S. enterica*	
	Clinical 4	Asmara	*S. enterica*	
	Clinical 5	Asmara	*S. enterica*	*S*. Heidelberg

Table 4.2 (continued)

Group	Sample	Species	Serotype	Stain source
I	Clinical 20	Asmara	*S. enterica*	*S*. Emek
	Clinical 8	Asmara	*S. enterica*	*S*. Emek
	Clinical 18	Asmara	*S. enterica*	
	Water 2 (3)	Massawa	*S. enterica*	
	Water 2 (4)	Massawa	*S. enterica*	*S*. Emek
	Meat 2	Asmara	*S. enterica*	*S*. Emek
	Clinical 1	Masswa	*S. enterica*	*S*. Emek
	Clinical 17	Asmara	*S. enterica*	
J	Clinical 14	Asmara	*S. enterica*	*S*. Emek
	Clinical 15	Asmara	*S. enerica*	
	Clinical 16	Asmara	*S. enterica*	*S*. Emek
	Water 2 (1)	Massawa	*S. enterica*	
	Water 2 (2)	Massawa	*S. enterica*	*S*. Emek
	Water 1 (4)	Massawa	*S. enterica*	
	Clinical 10	Asmara	*S. enerica*	*S*. Emek
	Clinical 9	Asmara	*S. enerica*	
K	Salad 1	Asmara	*S .enterica*	
	Mynefhi 1	Asmara	*S. enterica*	*S*. Heidelberg
	Clinical 5	Massawa	*S. enterica*	
L	Sembel 1	Asmara	*S. enterica*	
	Sembel 2	Asmara	*S. enterica*	*S*. Heidelberg
	Clinical 2	Asmara	*S. enterica*	
	Spinach 1	Keren	*S .enterica*	
	Spinach 2	Keren	*S. enterica*	
	Clinical 7	Asmara	*S. enterica*	*S*. Heidelberg
	Salad 3	Asmara	*S. enterica*	
	Clinical 7	Massawa	*S. enterica*	

Table 4.2 (continued)

Group	Sample	Species	Serotype	Stain source
M	Intestine	Asmara	*S. enterica*	*S*. Heidelberg
	Spinach 1	Massawa	*S. enterica*	
	Spinach 2	Massawa	*S .enterica*	
	Clinical 11	Asmara	*S .enterica*	*S*. Emek
N	Clinical 3	Massawa	*S. enterica*	*S*. Emek
	Clinical 4	Massawa	*S. enterica*	*S*. Emek
O	Clinical 1	Asmara	*S. enterica*	*S*. Emek
	Clinical 4	Keren	*S .enerica*	*S*. Emek
	Clinical 2	Keren	*S .enterica*	*S*. Emek
	Clinical 6	Keren	*S .enterica*	
	Clinical 1	Keren	*S .enterica*	
	Clinical 7	Keren	*S .enterica*	

4.4 Discussion

AFLP analysis allows for the grouping of related bacterial strains by comparison of their individual banding pattern profiles. In this study, *Salmonella* genomic DNA was digested using restriction enzymes *EcoR*I and *Mse*I. This enzyme combination has been previously applied to *Salmonella* and gave highly reproducible bands (Lindstedt *et al*., 2000; Torpdahl and Ahrens, 2004).

The banding patterns of the isolates associated with clinical cases (hospital isolates) were compared with those obtained from environmental isolates (water and food) to determine if isolates from the environmental samples were genetically similar to the clinical isolates. This was done in order to determine any possible indirect link between common sources of infection and clinical cases.

The dendrogram (Figure 4.1) shows the genetic relationship among the *Salmonella* isolates examined. In some instances the same *Salmonella* serotypes clustered in different groups while in other cases, different *Salmonella* serotypes clustered together. In spite of these problems the cluster analysis of the AFLP fingerprints provided a consistent presumptive identification, as most of the strains clustered according to their serotype. In this study, an empirically determined cut off value of 76% was used to define the clusters. The choice of this similarity value of 76% was based on the six strains, which were previously identified using the PCR method.

As it is mentioned above, 15 groups were identified while four strains remained ungrouped. Serotyping of the representative strains from each group showed that the *Salmonella* isolates belong to a number of serotypes including, *S*. Emek, *S*. Heidelberg, *S*. Typhimurium, and 1:13,22,23;z (undermined serotype). Despite few exceptions, the serotyping of selected strains confirmed that *Salmonella* isolates within each cluster were possibly of the same serotype. Based on the result of the serotyping, it can be said that *Salmonella* Heidelberg was the most prevalent *Salmonella* serotype, followed by *Salmonella* Emek, *Salmonella* 1:13, 22, 23; z

(undetermined serotype), and *Salmonella* Typhimurium. No other studies to confirm the *Salmonella* serotypes prevalent in Eritrea have been found.

In the dendrogram, strains from the same serotypes did not necessarily group in one cluster; they were often present in a number of clusters. *Salmonella* isolates belonging to *S*. Heidelberg were distributed into seven clusters in the dendrogram. The result of the serotyping showed that all the isolates in group E, F, H, K and L possibly belong to *S*. Heidelberg which seems to be serotype-specific clusters. However, in group B and M some *S*. Heidelberg strains were clustered along with some *S*. Emek strains. This data and the fact that there was no single clustering of the serotypes shows that both the *S*. Heidelberg and *S*. Emek serotypes are genetically heterogeneous. It also shows that the current AFLP typing system used is not optimal for the determination of serotypes.

In group B, E, F, H, K, L and M, the majority of *S*. Heidelberg isolates were collected from Asmara. In group B (sub cluster 1) and group L, the *S*. Heidelberg isolates recovered from clinical, water and food (vegetables) samples from Asmara clustered together. This data indicates a high degree of genetic similarity between clinical and environmental isolates. In group H clinical and food *S*. Heidelberg isolates collected from Asmara also clustered together indicating a high degree of genetic similarity between clinical and food isolates. In group K both food and water *S*. Heidelberg isolates from Asmara clustered together indicating genetic similarity between food and water *S*. Heidelberg isolates. The above data indicates a possible link between clinical and environmental isolates and therefore, water and food (vegetables) could be the main vehicles for *S*. Heidelberg transmission in Asmara.

In group E, *S*. Heidelberg isolates recovered from water samples from Asmara showed close similarity to isolates recovered from food (vegetables) samples from Keren. In group L, isolates recovered from food (vegetables) samples from Keren also showed close similarity to water, food and clinical isolates from Asmara. This data indicates genetic relatedness between food *S*. Heidelberg isolates from Keren and water and food *S*. Heidelberg isolates from Asmara. In this study, *S*. Heidelberg was not isolated from any of the clinical or water samples colleted from Keren. No association could therefore be made between clinical, water and food isolates from

this area. It could however be possible that *S*. Heidelberg contaminated vegetables were brought in from Asmara since most of the vegetables in Eritrea are grown in the highland region, including Asmara. These produce are afterwards sold in lowland areas such as Massawa and Keren.

In group F, *S*. Heidelberg isolates recovered form clinical and food (vegetables) isolates collected from Massawa clustered together indicating that food could be the main vehicle for *S*. Heidelberg transmission in Massawa. *S*. Heidelberg was, however, not detected in any of the water isolates, so no association could be made between clinical and water isolates. In group K and L clinical *S*. Heidelberg isolates collected from Massawa clustered along with clinical, water and food isolates from Asmara, and food isolates from Keren indicating a genetic relationship between these strains.

The serotyping results showed that all *Salmonella* isolates recovered from water samples from Asmara possibly belong to *S*. Heidelberg. These water sources might be contaminated due to the defecation of people and livestock around the water and during the wet season when the wastes might be washed into the water. Moreover, the majority of *S*. Heidelberg isolates were isolated from vegetables, which might be irrigated with contaminated water. As was mentioned before, many vegetables in Asmara are irrigated with the untreated wastewater (e.g. Mybella) and are sold in nearby markets including Keren and Massawa. Based on the above findings, water could be the main direct and indirect source of *S*. Heidelberg transmission in Eritrea. This could be either through the consumption of untreated water or through the use of contaminated water to irrigate vegetables. In many studies the importance of water in the transmission of *Salmonella* and other microorganism has been well documented (Momba and Kaleni, 2002).

In this study *S*. Emek was the second most prevalent serotype. *S*. Emek was present in seven clusters in the dendrogram. The result of the serotyping showed that all the isolates in group G, I, J, N and O possibly belong to *S*. Emek as these clusters seem to be serotype-specific clusters. Some of the *S*. Emek strains in group B and M, however, grouped with *S*. Heidelberg strains.

In this study, *S*. Emek was recovered from clinical, water and food samples. The majority of the isolates came from clinical sources. This was unexpected as *S*. Emek is not frequently involved in disease outbreaks in other countries. According to the Robert Koch Institute, Wernigerode Branch, in Germany (Wolfgang Rabsch, Personal communications, 2004), a few *S*. Emek strains has only sporadically been isolated from human cases of salmonellosis.

In group G, I and J the majority of *S*. Emek strains were collected from Asmara. Most of the strains were recovered from clinical sources. A few *S*. Emek strains were, however also recovered from water and food samples. In group I, one of *S*. Emek strains recovered from a food (meat) sample showed close similarity with the clinical strains. Food of animal origin could therefore be one of the routes of *S*. Emek transmission in Asmara. The two *S*. Emek isolates recovered from tap water clustered separately in group G, and no association could be made between the water and clinical isolates.

In group I and J most of the *S*. Emek strains recovered from clinical samples from Asmara and water samples from Massawa clustered together. Although these strains were obtained from two different areas, they exhibited similar AFLP patterns. This indicates a high genetic similarity between the strains and can be due to the wide spread distribution of this serotype in Eritrea.

In group N, two *S*. Emek strains recovered from clinical samples from Massawa clustered together. In group I, one *S*. Emek strain recovered from a clinical sample clustered with *S*. Emek strains recovered from the water samples taken at Massawa. This indicates that water could be one of the routes of *Salmonella* transmission in Massawa. In group B (sub cluster 2) one of clinical isolate (possibly *S*. Emek) from Massawa clustered with a *S*. Emek isolate recovered from a food (meat) sample. It is therefore possible that food of animal origin could also be the source of *S*. Emek transmission in Massawa but more data are required to confirm this result.

In group O *S*. Emek strains recovered from clinical samples from Keren clustered along with one clinical strain from Asmara. *S*. Emek was however not detected in any of the water or food samples collected from Keren. The route for *S*. Emek

transmission can therefore not be linked to either water or food sources. Further studies would need to be performed to clarify the possible transmission route.

In this study, *S*. Emek isolates were recovered from food of animal origin but none were isolated from vegetables. Food of animal origin could therefore be an important source of *S*. Emek transmission in Eritrea. The study also showed that water could be an important route of transmission but further studies are required to confirm these results.

The result of the serotyping showed that all the isolates in group A and C possibly belong to the same serotype. The serotype of these isolates could, however, not be determined (1:13, 22, 23; z) since there was an over expression of the Z in the H phase. It has been confirmed that these isolates belong to *Salmonella enterica* subspecies *enterica.*

S. Typhimurium is one of the most common serotypes recovered worldwide, but in this study it was the least isolated serotype. All six *S*. Typthimurium isolates were recovered from samples collected from Asmara. The results of the serotyping of two isolates were in agreement with the previous PCR identification. *S*. Typhimurium isolates recovered from water, food and clinical samples clustered together, indicating a possible link between the clinical and environmental sources. Either water or food or both could therefore be the source of *S*. Typhimurium transmission in Eritrea. *S*. Tyhimurium was not detected in either the clinical nor environmental samples collected from Massawa and Keren. Based on the finding of this study it can be said that this serotypes is not as widespread in Eritrea as in other countries.

4.5 Conclusions

In conclusion, the present study has demonstrated the usefulness of AFLP typing to differentiate between and group *Salmonella* strains recovered from clinical and environmental sources. The use of LICOR DNA analyzer for the analysis of the PCR products was able to accelerate the AFLP method and make the process less labour intensive.

In this study, most of the isolates were grouped according to their serotype, but few isolates belonging to different serotypes also grouped together indicating genetic heterogeneity of these serotypes. The current AFLP typing system currently used is, therefore, not optimal for determining serotypes. The result of the AFLP analysis, however, showed that water and food have important roles in *Salmonella* transmission in Eritrea. AFLP can therefore play an important role in epidemiological studies to determine the source of transmission.

Chapter 5
Conclusions

The isolation of *Salmonella* from water, food and clinical samples indicated the wide spread distribution of *Salmonella* in Eritrea. The results of this study also showed that the occurrence of *Salmonella* was not confined to a particular location as *Salmonella* was recovered from all three areas sampled.

Based on the results of the present study it can be concluded that water and food have an important role in *Salmonella* transmission in Eritrea. The prevalence and contamination level of *S*. Heidelberg is high in water and food (vegetables). This should be of concern because *S*. Heidelberg is commonly associated with human infections. On the other hand the prevalence of *S*. Emek is relatively high in clinical sources. The present study also showed that the prevalence *S*. Typhimurium was very low in Eritrea compared to other countries. Due to a lack of available information concerning the prevalence of *Salmonella* serotypes in Eritrea, verification of the results using similar studies could not be performed.

There is a need to improve hygiene practices and control measures to prevent the discharge of wastes into water sources. It is important to use clean and treated water for domestic purposes; drinking water should be continuously disinfected. Chlorination is widely used to disinfect water; however, *Salmonella* has been isolated from chlorinated water collected from Asmara and Massawa. Therefore, the disinfection process at these treatment works should be optimised to provide a safe supply of water.

The potential problem of spreading of *Salmonella* through raw vegetables should also not be underestimated. In many studies vegetables were implicated in outbreaks of salmonellosis (Awang, *et al*., 2003). In Eritrea several vegetables are consumed raw including salad and cabbage, which were among the vegetables contaminated with *S*. Heidelberg. It is therefore important to manage the contamination levels of the water

used for irrigation of these vegetables and to wash the vegetables carefully before consumption.

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