IMPREGNATED DRY FILTER PAPER AS AN ALTERNATIVE MEDIUM FOR TRANSPORTING DIARRHOEAL STOOL SAMPLE

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A RESEARCH THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE (MEDICAL BIOCHEMISTRY) IN THE SCHOOL OF PURE AND APPLIED SCIENCES OF KENYATTA UNIVERSITY

JANUARY 2009
DECLARATION

This thesis is my original work and has not been presented for a degree in any other university or any other award.

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DEDICATION

This thesis is dedicated to the memory of my father, the late Aggrey Akhanyinya Luchera, my mother, Elizabeth Ongoro Akhanyinya and my brothers, Justus Abuko Akhanyinya and Wellington Muleshe Akhanyinya.
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>EPEC</td>
<td>Enteropathogenic <em>Escherichia coli</em></td>
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<td>ETEC</td>
<td>Enterotoxin <em>Escherichia coli</em></td>
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<td>FP</td>
<td>Unimpregnated Filter Paper</td>
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<tr>
<td>GEL</td>
<td>Gelatine</td>
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<td>Gn</td>
<td>Gentamicin</td>
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<td>GoK</td>
<td>Government of Kenya</td>
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<td>GTPase</td>
<td>Guanidine Triphosphatase</td>
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<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<td>H₂S</td>
<td>Hydrogen Sulfide</td>
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<tr>
<td>IgA</td>
<td>Immunoglobulin A</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<td>INF-γ</td>
<td>Interferon-gamma</td>
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<tr>
<td>IND</td>
<td>Indole</td>
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</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
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<tr>
<td>KEMRI</td>
<td>Kenya Medical Research Institute</td>
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<tr>
<td>LB</td>
<td>Lauria Bertani broth</td>
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<tr>
<td>LDC</td>
<td>Lysine Decarboxylase</td>
<td></td>
</tr>
<tr>
<td>LT</td>
<td>Heat-labile enterotoxin</td>
<td></td>
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<tr>
<td>MH</td>
<td>Mueller Hinton agar/broth</td>
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<tr>
<td>MIC</td>
<td>Minimum Inhibitory Concentration</td>
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<td>MRVP</td>
<td>Methyl Red Voges Proskauer</td>
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<tr>
<td>Abbreviation</td>
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<tr>
<td>NCCLS</td>
<td>National Committee on Clinical Laboratories Standards</td>
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<td>NLF</td>
<td>Non-Lactose Fermenting</td>
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<td>NTS</td>
<td>Non-typhoid Salmonella</td>
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<tr>
<td>N-WASP</td>
<td>Nural-Wiskott-Aldrich Syndrome protein</td>
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<tr>
<td>ODC</td>
<td>Ornithine Decarboxylase</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>SEL</td>
<td>Selenite-F-broth</td>
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<td>SIM</td>
<td>Sulfide-Indole-Motility</td>
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<td>SPI 1</td>
<td>Salmonella Pathogenicity Island 1</td>
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<tr>
<td>SS</td>
<td>Salmonella Shigella agar/broth</td>
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<tr>
<td>Sxt</td>
<td>Trimethoprim-Sulfamethoxazole (Co-trimoxazole)</td>
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<tr>
<td>TBE</td>
<td>Tris Borate ethylene diamine-tetra-acetic acid</td>
<td></td>
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<tr>
<td>TCBS</td>
<td>Thiosulfate Citrate Bile Salt</td>
<td></td>
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<tr>
<td>TDH</td>
<td>Thermostable direct hemolysin</td>
<td></td>
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<tr>
<td>Te</td>
<td>Tetracycline</td>
<td></td>
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<tr>
<td>TE</td>
<td>Tris ethylene diamine-tetra-acetic acid</td>
<td></td>
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<tr>
<td>TSI</td>
<td>Triple Sugar Iron agar</td>
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<td>URE</td>
<td>Urea agar</td>
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<tr>
<td>VASP</td>
<td>Vasodilator-stimulated phosphoprotein</td>
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<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>XLD</td>
<td>Xylose Lysine Desoxycholate</td>
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ABSTRACT

Diarrhoea due to bacterial infections is a major cause of morbidity and mortality both in children and adults and hence lowers economic activity especially in developing countries. This is especially important in areas with poor hygiene such as slums and refugee camps. Bacterial diarrhoea is caused by several enteric pathogens among them *Shigella*, *Salmonella*, *Vibrio cholerae* and *Escherichia coli*. Some, especially *Shigella dysenteriae* Type 1 are very fragile organisms whose viability is difficult to maintain in transport medium or placing such stool specimens in Cary-Blair for a limited time while on transit to diagnostic laboratories. Failure to isolate and identify enteric bacterial pathogens because of poor transport conditions or inadequate laboratory facilities has often resulted in delayed and/or misdiagnosis of enteric bacterial pathogens as the cause of epidemics. The increasing multidrug resistant strains do not make matter any better. This study was carried out to determine the viability of impregnated dry filter paper as an effective transport medium for diarrhoea stool specimens, identify and characterize multidrug resistant enteric bacteria in the stool specimens. Whatman filter paper was impregnated with 0.1% of glucose and used to transport stool samples from Budalang’i, a remote area in Western Kenya to Centre for Microbiology Research laboratories, KEMRI, Nairobi. During the five months period, stools from 336 diarrhoeal patients aged between birth to 85 years were cultured and (14.9%) samples presented bacterial growth and were included in this study. Impregnated dry filter paper was as good as Cary-Blair in the recovery of the isolates. More than 75% of all isolates were resistant to locally available antibiotics while all isolates remained susceptible to ciprofloxacin. Six (6) resistotypes were determined with ampicillin, chloramphenicol, cotrimoxazole and tetracycline most frequently isolated. Small molecular weight plasmids of approximately 7MDa, 10MDa, 30MDa and 47MDa were transferred to *E. coli* K-12 (F’ Na’) in the conjugation experiments. Increasing multidrug resistance remains a matter of great concern. Impregnated dry filter paper may be used to transport stool samples suspected for enteric bacterial pathogens in impoverished areas in absence of conventional media and/or with conventional media to increase the recovery rates of enteric bacterial pathogens.
CHAPTER ONE
INTRODUCTION

1.1 Background

Diarrhoea causes substantial illness among rural sub-Saharan African (WHO, 1999; Brooks et al., 2005; Kariuki et al., 2006) and visibly bloody diarrhoea causes proportionally greater morbidity and mortality (Farshad et al., 2006; Ronsmans et al., 1998). This is especially common in areas with compromised hygiene such as slum areas and refugee camps (Chiou et al., 2001; Lee et al., 2003; Totaro et al., 2004). Enteric bacterial pathogens of public health importance in the developing world are *Shigella*, *Salmonella* and *Vibrio cholerae* (WHO, 2003). There have, however, been problems associated with isolation and subsequent identification of the causative agent especially in outbreak areas (Oundo et al., 1996). This has been due to the absence of properly equipped laboratories, qualified staff and adequate transport medium (Mutwewingabo et al., 1984; Kariuki et al., 2006). Major epidemics of diarrhoeal diseases have often originated in remote rural areas without immediate access to laboratories for bacteriologic analysis (Ries et al., 1994). This has led to major problems in identification on site, of the causative agents.

*Shigella* organisms, the causative agents of bacillary diarrhoea, are fastidious and therefore, die easily during transport (Niyogi, 2005). They are usually found in low numbers in diarrhoeal stool specimen and the absence of a satisfactory enrichment
medium for them further complicates their identification (Diarrhoeal Diseases Control Programme, 1987; Moyer, 2002). *Shigella dysenteriae* Type 1 in particular is known to cause more severe, prolonged and frequently fatal illness (Pazhani *et al.*, 2004). These and the fact that it develops antimicrobial resistance more quickly makes it a formidable enteric bacterial pathogen even in this era of antibiotics hence generates a lot of concern (WHO, 2003; Niyogi, 2005).

One major contributor to the persistence of *Salmonella* as a major cause of diarrhoeal and invasive disease is the variety and abundance of animal reservoirs they are able to infect (Boyle *et al.*, 2007). Of concern are the increasing incidences of non typhoidal salmonella (NTS) infections caused by multidrug resistant strains (Wolfgang *et al.*, 2001; Boyle *et al.*, 2007). In developing countries, salmonellosis and typhoid fever are frequently diagnosed solely on clinical grounds; however isolation of the causative organism is necessary for the performance of antimicrobial susceptibility testing which is essential in recommending treatment.

Since antimicrobial resistance has been a growing problem in most parts of the world, the susceptibility of *Vibrio cholerae* 01 strains to antimicrobial agents should be determined at the beginning of an epidemic and be monitored periodically (WHO, 2003). This necessitates for an alternative media for transportation of stool specimens in the absence of the gold standard medium...
rarely found in the settings in which this infection occur.

Various transport media are used for transporting diarrhoeal stool specimen suspected for enteric bacterial infections and include Cary-Blair. Other transport media that are similar to Cary-Blair are Amie’s and Stuart’s transport media. Both of these are acceptable for *Shigella* and *Salmonella* but they are inferior to Cary-Blair for transporting *V. cholerae*. Alkaline peptone water (APW) may be used to transport *V. cholerae* specimens but this medium is also inferior to Cary-Blair (WHO, 2003). In addition, other organisms overgrow *Vibrios* in APW when sub-cultured for more than six hours.

It is also very important to note that these media are rarely available in the rural set-ups where most of the outbreaks frequently occur. Where transport medium has been available, there have been poor recovery rates of etiologic agent due to non-viability of the bacteria in these media. This has often led to poor diagnosis with no bacteria being detected at all, thus resulting in the overall unchecked spread of morbidity and mortality. More so, the cost of conventional transport medium and its related low isolation rate of 30% (Saidi et al., 1997) for enteric bacterial pathogens contribute to the delayed confirmation of outbreaks. The provision of an inexpensive, easily available and cost-effective means of transporting these diarrhoeal samples to well equipped laboratory is a requirement in order to increase the recovery rates. The use of dried filter paper as a means of transporting
diarrhoeal specimens to the laboratory was first described by Joe (1950). This method was modified to increase the recovery rates of enteric bacterial pathogens that cause diarrhoea.

1.2 Justification

Due to problems associated with the transportation of diarrhoeal stool specimen and the eventual isolation and identification of enteric bacterial pathogens, there is a need to develop an inexpensive, easily available and cost-effective transport medium for use in impoverished areas during epidemics. This will aid in the improvement of recovery rates of enteric bacterial pathogens from the suspected diarrhoeal stool specimens thus increasing the level of accurate diagnosis and subsequent treatment.

1.3 Problem statement

Poor recovery rates of etiologic agents due to non-viability of enteric bacterial pathogens in the conventional media have led to poor diagnosis of diarrhoeal diseases resulting in unchecked spread of morbidity and mortality.
1.4 Hypotheses

i. Impregnated dry filter paper is a better transport medium for diarrhoeal stool samples suspected for enteric bacterial pathogens.

ii. Impregnated dry filter paper increases the recovery rates of enteric bacterial pathogens in diarrhoeal stool samples.

1.5 Objectives

1.5.1 General objectives

To determine the viability of impregnated dry filter paper as an effective transport medium for diarrhoeal stool specimens.

1.5.2 Specific objectives

i. To develop an impregnated dry filter paper transport medium for enteric bacterial pathogens.

ii. To isolate and identify enteric bacterial pathogens from both the developed and conventional transport media.

iii. To assess the recovery rates of the enteric bacterial pathogens in both the impregnated dry filter paper and the conventional transport media.

iv. To identify and characterise the multidrug resistance enteric bacterial pathogens.
CHAPTER TWO
LITERATURE REVIEW

2.1 Enteric bacterial pathogens

2.1.1 Shigella

*Shigella* belong to the family Enterobacteriaceae and are small, gram-negative, non-motile, non-sporulating, non-capsulate and facultative anaerobic bacilli, which form pale colonies when cultured on differential media (Niyogi, 2005). Currently, *Shigella* genus is biochemically and serologically divided into: *Shigella dysenteriae* (subgroup A, consisting of 13 serotypes), *Shigella flexneri* (subgroup B, consisting of 15 serotypes including subtypes), *Shigella boydii* (subgroup C, consisting of 18 serotypes) and *Shigella sonnei* (subgroup D, consisting of a single serotype) (Farshad *et al.*, 2006; Moyer, 2002). This is based on the structure of the ‘O’ antigen component of lipopolysaccharide present on the outer membrane of the cell wall (Niyogi, 2005; Stoll *et al.*, 1982). Thus, at least 47 serotypes of *Shigella* have been recognized (WHO, 1999). Serogroups A, B and C are very similar physiologically while *S. sonnei* can be differentiated from the other serogroups by positive beta-D-galactosidase and ornithine decarboxylase biochemical reactions (Niyogi, 2005).

Both *S. sonnei* and *S. boydii* are usually associated with mild illness of short duration in which the stool may be watery or bloody (Keusch and Bennish, 1989). *S. flexneri* is generally more severe, lasts longer and causes blood in stools
(Subekti et al., 2001). *S. dysenteriae* causes the most severe diarrhoeal illness, reflected in high death rates (WHO, 1994). *S. flexneri* is a principal cause of endemic shigellosis in many developing countries (Navia et al., 1999), while shigellosis in both endemic and epidemic form has been attributed to *S. dysenteriae* Type 1 (Pazhani et al., 2004).

### 2.1.2 Salmonella

*Salmonella* are gram negative rod bacteria, motile, facultative anaerobic, non-sporing members of Enterobactericeae (Center for Infectious Diseases, Research and Policy, 2008). They form colourless, opaque, transparent colonies when cultured on differential media (WHO, 2003). *Salmonella* bacteria produce acid on glucose fermentation, reduce nitrates and do not produce cytochrome oxidase. Differential metabolism of sugars and other biochemical properties can be used to distinguish serotypes. *Salmonella* species possess two sets of antigens: O antigens which is heat stable polysaccharides that form part of the cell wall lipopolysaccharide and H antigens formed from the structural proteins that make up the flagella (Center for Infectious Diseases, Research and Policy, 2008). Additional surface (Vi) antigens and habitat also can be used to classify serotypes (Bopp et al., 2003). These antigens are useful in serological identification of *Salmonella* serotypes.
More than two thousand (2000) serotypes of *Salmonella* exist and are known to cause a wide variety of human and animal infections (Center for Infectious Diseases, Research and Policy, 2008). *Salmonella* are commonly grouped for convenience into typhoidal salmonella (*S. typhi*) and non-typhoidal salmonella (NTS) species. Four species most important for human diseases are: *Salmonella typhi*, *Salmonella paratyphi*, *Salmonella enteritidis* and *Salmonella typhimurium*.

### 2.1.3 Vibrio cholerae

*Vibrio cholerae* belong to the family Vibrionaceae and are short, gram-negative rods usually curved and actively motile by a single polar flagellum. They are fermentative but anaerogenic and reduce nitrate to nitrite. Nearly all are oxidase positive and produce indole (Albert, 1994). Of the more than 150 serogroups of *V. cholerae* known only two serogroups are toxigenic: serogroups 01 and 0139 (WHO, 2003). These are the serogroups associated with epidemic cholera outbreaks. Two biotypes of *V. cholerae* 01 are: El Tor and classical classified on the basis of phenotypic characteristics. El Tor is responsible for virtually all of the cholera cases throughout the world but classical isolates are not encountered outside India or Bangladesh. Endemic *V. cholerae* 0139 appears to be confined to the Asian continent (Albert, 1994).
(Cimons, 2000). In Japan, a total of 1628 cases of shigellosis were reported between 2003 and 2005 (as of 6 February 2006) (National Institute of Infectious Diseases, 2006). In Taiwan, 200 to 500 cases of shigellosis were identified annually from 1998 to 2002 with the majority of infections occurring in children younger than 9 years of age (Department of Health, Taiwan, 2003).

*Shigella* infections remain a global public health concern, causing diarrhoea in both the developing and developed regions (Guerrant *et al.*, 1990). Shigellosis is also common among children less than five years of age in developing countries and in persons who travel from industrialized to less developed countries (Jucket, 1999; Shlim *et al.*, 1999). In the developing world, it is estimated that 113 million episodes of shigellosis occur annually, resulting in more than 400,000 deaths (Kotloff *et al.*, 1999).

Of the four serogroups known, *S. dysenteriae* Type 1 has been responsible for epidemic dysentery with high death rate worldwide (WHO, 2003). In recent years, *S. dysenteriae* Type 1 has caused epidemic dysentery in central America, south Asia and central and southern Africa with African countries suffering most from the disease (WHO, 1999). In 1968, *S. dysenteriae* Type 1 caused a four-year epidemic that resulted in more than 500,000 cases and at least 20,000 deaths (WHO, 1995). In 1979, another epidemic was reported in Zaire, which subsequently spread to Rwanda and Burundi. In early 1990s, epidemic dysentery
moved southward affecting first Zambia, then Malawi, Mozambique, Zimbabwe and South Africa. In 1995, a severe outbreak of bloody diarrhoea with 600 cases and 104 deaths was reported in the central African country of Equatorial Guinea. This was reported concurrently with another outbreak in the west African country of Liberia (O undo et al., 1996).

In Kenya, the outbreak of \textit{S. dysenteriae} Type 1 was first detected in a Somali refugee camp in Mombasa in 1994. However, the number of cases and fatalities has not been documented due to initial confusion in the correct identification and diagnosis of the dysentery by health workers (O undo et al., 1996). Diagnostic difficulties occur for several reasons. Enteric bacterial pathogen infections tend to occur in remote areas, impending specimen transport to laboratories for bacteriological diagnosis. \textit{Shigella} species and in particular \textit{S. dysenteriae} Type 1 is fastidious and the organism dies during transport if not handled properly (Niyogi, 2005). \textit{Shigella} species can be difficult to culture even under good conditions, because often low numbers of organism are present in the stool and no satisfactory enrichment medium is available for routine use (Moyer, 2002; Ries et al., 1994). Some standard enteric media such as \textit{Salmonella-Shigella} agar actually inhibit growth of \textit{S. dysenteriae} Type 1 markedly (Rahaman et al., 1975). Once the organism is identified, antisera for typing may not be available (Ries et al., 1994).
Prevention of *Shigella* infections has proven difficult, mainly due to the low inoculum needed to produce disease and inadequate empirical therapy options secondary to antimicrobial resistance (Edwards, 1999; Replogle *et al.*, 2000). Problems associated with the development and spread of antibiotic resistance have been increasing since the early 1960s and are a major threat to global public health (WHO, 2001).

The ability of bacteria to acquire and disseminate exogenous genes via mobile genetic elements such as plasmids, transposons, insertion sequences and genomic islands has been the major factor in the development of multidrug resistant strains (Rowe-Magnus *et al.*, 2002). The matter became more serious after the discovery of another mechanism for the dissemination of resistance, involving integrons (Stokes and Hall, 1989). Integrons are genetic elements that acquire and exchange exogenous DNA, known as gene cassettes, by a site-specific recombination mechanism. The most notable gene cassettes identified within integrons are those conferring resistance to antibiotics.

The increasing incidence of multidrug resistant microorganisms has led to tremendous interest in the genetics and mechanisms of resistance evolved by bacteria to counteract the effects of antimicrobial agents. Recent reports have determined the molecular basis of multidrug resistance phenotypes of *Shigella* species in Australia (Mclver *et al.*, 2002), Ireland (Delappe *et al.*, 2003), Korea (Oh
et al., 2003), Italy (Mammina et al., 2005) and Brazil (Peirano et al., 2005).

A rapid, simple and inexpensive laboratory test to detect *S. dysenteriae* Type 1 in stool sample in the absence of a complete microbiology laboratory would be useful. Fluorescent antibody staining and polymerase chain reaction (PCR) procedures with or without immunomagnetic isolation have been developed for the rapid diagnosis of *Shigella* infections but require specialized equipment that is rarely available where *S. dysenteriae* Type 1 epidemics occur (Niyogi, 2005; Islam and Lindberg, 1992). Biotinylated DNA probes have been effective for characterising colonies of diarrhoea causing *Escherichia coli* but do not offer obvious advantage in cost or time for *S. dysenteriae* Type 1 (Gicquelais et al., 1990).

2.2.2 Salmonella

Few established surveillance systems for typhoid exist in the developing world, especially in community settings; hence the true burden is difficult to estimate (Bhutta and Dawraji, 2006). This is shown by recent revisions in the global estimates of the true burden of typhoid. In contrast to previous estimates, which were 60% higher, Centre for Disease Control and Prevention (CDC) estimate that there are 21.6 million typhoid cases annually, with the annual incidence varying from 100 to 1000 cases per 100 000 population (Crump et al., 2004; WHO, 2008). The global mortality estimates from typhoid have also been revised downwards
from 600 000 to 200 000, largely on the basis of regional extrapolations (Crump et al., 2004). Recent population based studies from South Asia suggest that the incidence is highest in children aged less than 5 years, with higher rates of complications and hospitalisation, and may indicate risk of early exposure to relatively large infecting doses of the organisms in these populations (Brooks et al., 2005; Siddique et al., 2006). These findings contrast with previous studies from Latin America and Africa which suggested that S. typhi infection causes a mild disease in infancy and childhood (Brooks et al., 2005).

Although the overall ratio of disease caused by S. typhi to that caused by S. paratyphi is about 10 to 1, the proportion of S. paratyphi infections is increasing in some parts of the world (Ochiai et al., 2005). In contrast to the Asian situation, the HIV and AIDS epidemic in Africa has been associated with a concomitant increase in community acquired bacteraemia due to non-typhoidal salmonella such as S. typhimurium (Berkley et al., 2005; Graham, 2002), an illness that may be clinically indistinguishable from typhoid. The exact reasons for these differences in the epidemiology and spectrum of salmonella infections between Asia and Africa remain unclear.

In most cases, outbreaks of NTS infection are caused by S. typhimurium and S. enteritidis. NTS infections are acquired as food poisoning and are usually self-
limiting (Fierer and Swancutt, 2000). A variety of foods have been implicated as vehicles transmitting salmonellosis to humans. These include; poultry, beef, pork, eggs, milk, cheese, fish, shellfish, fresh fruit and juice, and vegetables (Espié et al., 2005; Mazurek et al., 2004; Varma et al., 2005). Contamination can occur at multiple steps along the food chain. Contact with farm animals, pets, reptiles and natural pet treats have also been associated with infection (CDC, 1999; Wall et al., 1996).

In all sub-Saharan African countries where they have been studied, NTS are the commonest or second-commonest cause of bacteraemia in children under 5 years of age (Bahwere et al., 2001; Berkley et al., 2005; Graham, 2002). NTS are also the second-commonest cause of neonatal meningitis, the third-most-common cause of bacterial meningitis in children over 2 months of age in Malawi (Molyneux et al., 2003), an important cause of septic arthritis (Lepage et al., 1990) and neonatal sepsis (Milledge et al., 2005). It is estimated that the minimum incidence of community-acquired NTS in rural and urban populations of children may be as high as 166 per 100,000 per year for children under 5 years of age (Berkley et al., 2005; Mwangi et al., 2002). Of all admissions with febrile illness, NTS constitute 18% of cases and result in 28% mortality, compared to 5.7% mortality in children that do not have bacteraemia. In particular, multidrug resistant S. typhimurium causes serious outbreaks. In Zaire (Cheesbrough et al., 1997) and Rwanda (Lepage et al., 1990), multidrug resistant S. typhimurium is the predominant cause of
bacteraemic illness in children, while in Kenya this serotype is the predominant isolate in children with Salmonella bacteraemia (Kariuki et al., 2005; Mwangi et al., 2002). The source and mode of transmission of NTS in the African context have remained unknown; although it is thought that human-to-human transmission may play an important role (Kariuki et al., 2002).

In many sub-Saharan African countries community-acquired bacteraemia is a major cause of high morbidity and death among children especially from resource poor settings (Kariuki et al., 2006). NTS account for a steadily increasing proportion of these infections and represent from 20-50% of cases (Blomberg et al., 2005; Adejuyigbe et al., 2004). In Kenya invasive NTS infections in children less than 5 years of age are an important cause of morbidity and high mortality; they are ranked second only to pneumococcal pneumonia in importance as the leading bacterial cause of child mortality (Mwangi et al., 2002; Berkley et al., 2005). In contrast, in industrialized countries the most common manifestation of NTS infection is gastroenteritis, which is usually a self-limiting and benign disease, invasion beyond the gastrointestinal tract occurs in only approximately 5% of patients with salmonellosis, even among children (Fierer and Swancutt, 2000). In this small proportion of children from industrialized countries with NTS bacteraemia, the infection usually occurs in previously healthy young children; it appears in the context of gastroenteritis and has a favourable outcome (Shimoni et al., 1999). However, lack of adequate diagnostic capabilities in poor resource
settings that is prevalent in most public health facilities in Africa may hinder prompt diagnosis of infection and may often lead to under-diagnosis, delay appropriate treatment and often result in poor prognostic outcomes compared to similar cases in industrialized countries (Kariuki et al., 2006).

Emergence of drug resistant typhoid continues to cause a lot of concern. After sporadic outbreaks of chloramphenicol resistant typhoid between 1970 and 1985, many strains of S. typhi developed plasmid mediated multidrug resistance to the three primary antimicrobials used (ampicillin, chloramphenicol, and co-trimoxazole) (Rowe et al., 1997). This was countered by the advent of oral quinolones, but chromosomally acquired quinolone resistance in S. typhi and S. paratyphi has been recently described in various parts of Asia, possibly related to the widespread and indiscriminate use of quinolones (Renuka et al., 2005; Shirakawa et al., 2006). Multidrug resistant typhoid and paratyphoid infections are more severe with higher rates of toxicity, complications, and mortality than infections with sensitive strains (Bhatta, 1996). This may be related to the increased virulence of multidrug resistant S. typhi as well as a higher number of circulating bacteria (Wain et al., 1998).
2.2.3 *Vibrio cholerae*

Cholera is a widespread, severe diarrhoeic disease, which continues to be a global threat (Salim *et al.*, 2005). Cholera is thought to have its ancestral home in the Ganges Delta of the Indian subcontinent. There have been seven pandemics of cholera of which the last three were due to *V. cholerae* 01. The seventh pandemic of cholera, caused by *V. cholerae* 01 El Tor, originated in Celebes, Indonesia in 1961 and spread far and wide reaching South America continent in 1991 (Barue, 1992). The pandemic entered Africa in 1970, where it remains an ongoing source of morbidity and mortality (Gaffga *et al.*, 2007). The pandemic reached the Americas in 1991, causing nearly 400,000 cases that year for a total of nearly 1 million cases over a 10 year period. However, the number of Latin American countries reporting cholera and the number of cases reported has since diminished sharply, and in 2005, only one Latin American country (Brazil) reported cholera (5 cases).

The number of Asian countries reporting cholera cases decreased from a mean of 18 during the period 1970-1979 to a mean of 11 during the period 2000-2005. In 2005, 9 Asian countries reported a total of 6824 cholera cases to WHO. However, each year since 1991, more than 100,000 cases and 1800 cholera deaths have been reported by between 45 and 92 member nations (WHO, 2006). Although cholera incidence and the number of countries reporting cholera has decreased in Asia and
Latin America over the past decade, the number of countries in sub-Saharan Africa reporting cholera has increased, and the incidence has remained largely unchanged.

In recent years, the pandemic has been sustained in several African regions (Gaffga et al., 2007). Among the 39 African countries that reported cases of cholera in any year from 2000 through 2005, 18 (46%) reported cases in all 6 years: Benin, Burundi, Cameroon, Democratic Republic of Congo, Ghana, Guinea, Liberia, Malawi, Mozambique, Niger, Nigeria, South Africa, Swaziland, Togo, Uganda, United Republic of Tanzania, Zambia and Zimbabwe. Countries with such high endemicity are found in East, Southern, Central and West Africa. During that 6 year period, no cholera cases were reported by 11 (28%) countries in sub-Saharan Africa: Angola, Botswana, Cape Verde, Eritrea, Lesotho, Mauritius, Namibia, Reunion, St. Helena, Seychelles and Sudan.

The absence of reported cases may reflect a real lack of detectable cholera or the national Health Ministries’ inability to recognize or unwillingness to report cholera cases. It is noteworthy that three countries that did not report cholera in 2000-2004 experienced cholera outbreaks in 2005: Gambia, 214 cases and 13 deaths; Mauritania, 4132 cases and 70 deaths; Sao Tome, 1966 cases and 33 deaths. Similarly, in 2006, two countries that did not report cholera in the preceding 5 years had dramatic cholera epidemics: Angola, 43 076 cases and 1642 deaths as of
June 6, 2006 and Sudan, 8923 cases and 238 deaths as of March 20, 2006 (WHO, 2006).

In 2006, 98.9% of the cholera cases reported worldwide, and all but 8 of the 6311 deaths, were reported from Africa (Anon, 2007). Angola, the Democratic Republic of Congo, Ethiopia and Sudan were the worst hit countries, but most coastal West African countries reported large numbers of cases and case fatality rates of 1% to 6.2%. Between 2 January and 25 June 2006, 1869 cases and 79 deaths (4.2% case fatality rate) were reported in Ghana (Opintan et al., 2008). By the end of the year, the count was 3357 cases and 107 deaths, with an overall case fatality rate of 3.19% (Anon, 2007).

Cholera remains a disease of the world’s poorest people (Mugoya et al., 2008). Since 1970 when the seventh pandemic of El Tor biotype of V. cholerae O1 reached sub-Saharan Africa, epidemic cholera has persisted in many African countries (WHO, 2005). While in some parts of Africa, measurable improvements in sanitation, hygiene and overall infrastructure have occurred during recent decades, the majority of Africans still live in environments without safe drinking water or modern sanitation, putting them at ongoing risk for cholera (WHO, 2005). Cholera outbreaks in Africa have been linked to multiple sources, most of these related to consumption of unsafe water and food, such as drinking river and lake water, eating at funeral feasts and consuming food or beverages from street vendors.
(Acosta et al., 2001; Hutin et al., 2003; Dubois et al., 2006). Despite the multiple potential sources of cholera contamination in Africa, simultaneous countrywide or multinational epidemics, such as the explosive Latin American epidemic of the 1990s, have been rarely reported in Africa (CDC, 1991; Tauxe et al., 1995).

Since 1971, Kenya has suffered several waves of cholera recrudescence. Its largest epidemic started in 1997 with 17 200 cases notified to the WHO (Shapiro et al., 1999). In 1998 and 1999, the epidemic progressed with more than 33 400 notified cases (Scrascia et al., 2001). The final official figures of the Kenyan epidemic were 10% of all cholera cases reported from the African continent in the same 3 years (WHO, 1999; WHO, 2000). In the first half of 2005, Kenya experienced 5 cholera outbreaks with nearly 1000 reported suspect cases in geographically discrete locations (Mugoya et al., 2008). Cholera outbreaks are associated with crowded living conditions, inadequate or unprotected water supply and poor sanitation, which exist in much of Kenya, making most of the country susceptible to outbreaks if V. cholerae are introduced (WHO, 2003). In addition, Kenya has 2 large refugee camps, which are known to be fertile grounds for explosive cholera out-breaks (Swerdlow et al., 1997).

Good sanitation practices, if instituted in time, are usually sufficient to stop an epidemic. There are several points along the transmission path at which the spread may be halted:
Sterilization: Proper disposal and treatment of the germ infected fecal waste produced by cholera victims (and all clothing and bedding that come in contact with it) is of primary importance. All materials that come in contact with cholera patients should be sterilized in hot water using chlorine bleach if possible. Hands that touch cholera patients or their clothing and bedding should be thoroughly cleaned and sterilized.

Sewage: Treatment of general sewage before it enters the waterways or underground water supplies prevents undiagnosed patients from spreading the disease.

Sources: Warnings about cholera contamination posted around contaminated water sources with directions on how to decontaminate the water.

Water purification: All water used for drinking, washing or cooking should be sterilized by boiling or chlorination in any area where cholera may be present. Boiling, filtering and chlorination of water kill the bacteria produced by cholera patients and prevent infections from spreading. Water filtration, chlorination and boiling are by far the most effective means of halting transmission. Cloth filter, though very basic, have significantly reduced the occurrence of cholera when used in poor villages in Bangladesh that rely on untreated surface water. Public health education and appropriate sanitation practices can help prevent transmission.
2.3 Pathogenesis of enteric bacterial pathogens

2.3.1 *Shigella*

All the enteric pathogens, which enter the gastrointestinal tract through the mouth with food and water, colonise a certain part of the intestine in a process termed colonization (Levine *et al.*, 1983). *Shigella* colonisation of ileocaecum and colon is mediated by the production of specific surface antigens called colonisation factors and specific receptors for the factors.

*Shigella* is an enteroinvasive pathogen, which invades epithelial cells of the intestine (Miwatani *et al.*, 1988). *Shigella* directs its uptake into the colonic mucosa through membrane ruffling and macropinocytosis in a manner similar to *Salmonella* uptake (Adam *et al.*, 1995). After engulfment, the pathogen is surrounded by a membrane bound vacuole within the host. Unlike *Salmonella*, however, *Shigella* rapidly lysis the surrounding vacuole and is released into the cytosol, where it grows and divides (Sansonetti *et al.*, 1986). Once the microbe has escaped from the vacuole, it quickly becomes coated with filamentous actin and ultimately forms an actin tail at one pole of the bacterium (Bernardini *et al.*, 1989). This actin polymerisation propels the bacterium through the cytoplasm at speeds reaching 0.4 μM/sec. On reaching the plasma membrane, the pathogen forms a long protrusion into the neighbouring cell, which subsequently internalises the microbe (Kadorugamuwa *et al.*, 1991). The bacterium again breaks out of the vacuole to start another new cycle of infection in a new host cell. This process
allows *Shigella* to move from cell to cell without ever contacting the extracellular milieu.

### 2.3.1.1 Bacterial factors involved in *Shigella* motility

IcsA (also called VirG) is a 120-kDa outer membrane protein that hydrolyses Adenosine Triphosphate (ATP) and is localised to one pole of the bacterium at the junction between the microle and the actin tail (Goldberg *et al.*, 1993). IcsA expression on the surface of *Shigella* is sufficient to direct actin-based motility. SopA (IcsP) is a bacterial protease that proteolytically cleaves IcsA. This cleavage is required for polarized distribution of IcsA on the bacterial surface and for proper actin-based motility of *Shigella* in infected cells (Shere *et al.*, 1997).

### 2.3.1.2 Host factors involved in *Shigella* motility

IcsA expression on the *Shigella* surface promotes rapid accumulation of actin around the bacterium. Following bacterial division and IcsA polarisation, actin tails begin to form on one end of the bacterium. Host cytoskeletal proteins involved in tail formation include: α actinin (Zeile *et al.*, 1996), filamin, fimbrin (Prevost *et al.*, 1992), vasodilator-stimulated phosphoprotein (VASP) (Chakraborty *et al.*, 1995), vinculin (Kadurugamuwa *et al.*, 1991) and Neural-Wiskott-Aldrich Syndrome protein (N-WASP) (Laine *et al.*, 1997).
*Shigella* infection results in the cleavage of intact vinculin (120 kDa) to produce a 90-kDa fragment (Laine *et al.*, 1997). This proteolysis unmasks an actin-based motility site on vinculin, which contains a polyproline region capable of binding VASP. VASP recruitment to the bacterial surface in turn allows the recruitment of other cytoskeletal proteins such as actin and profilin and forms the basis of an actin-based motor for *Shigella* movement.

Like vinculin, N-WASP can bind IcsA directly and in addition to VASP, can recruit profilin and actin to the surface of *Shigella* thereby mediating actin polymerisation.

### 2.3.1.3 Invasion of the underlying tissue

Intercellular dissemination of *Shigella* is facilitated by the IpaB protein, which are encoded on the virulence plasmid and lyses the plasma membrane (Dorman and Porter, 1998). In the lymphoid follicle, *Shigella* is phagocytosed by macrophages. The infected macrophages undergo apoptosis, resulting in the release of the bacteria, which then infect adjacent enterocytes at the basolateral surface. The apoptotic process is also mediated by the IpaB protein and occurs following activation of the cysteine protease caspase-1. The infected host cells produce an inflammatory response, which is mediated most importantly through interleukin-8 (IL-8) and interleukin-10 (IL-10) production. IL-8 and IL-10 are responsible for chemotaxis of polymorphonuclear leukocytes, which cause disruption of the
epithelial barriers integrity. Degeneration of the epithelium and inflammation of 
the lamina propria are pathognomonic of *Shigella* infection. These changes induce 
disruption of epithelial absorption producing the characteristic diarrhoea and 
abdominal cramps. *Shigella* may cause mucosal destruction in the form of 
ulceration of the colonic mucosa, which explains the presence of blood in the stool 
(Niyogi, 2005).

2.3.1.4 Toxin production

*Shigella* strains produce 3 distinct enterotoxins: (a) chromosome encoded *Shigella* 
enterotoxin 1 (SHET 1) which is present in all *S. flexneri* 2a (Yavzoru *et al.*, 2002; 
Niyogi *et al.*, 2004) but rarely found in other *Shigella* serotypes (Noriega *et al.*, 
1995), (b) *Shigella* enterotoxin 2 (SHET 2) which is located on a large plasmid 
associated with virulence of *Shigella* (Nataro *et al.*, 1995). SHET 2 was found in 
many, but not all, *Shigella* of different serotypes and also in enteroinvasive 
*Escherichia coli* (EIEC) (Vargas *et al.*, 1999). The soluble toxins, SHET 1 and 
SHET 2, show significant enterotoxic activity *in vitro* when tested in rabbit ileal 
loops and Ussing chambers. (c) Phage-borne Shiga toxin produced by *S. dysenteriae*. Shiga toxin is neurotoxic, cytotoxic and enterotoxic, encoded by 
chromosomal genes (O'Brien and Holmes, 1987; Niyogi, 2005).

Shiga toxin is active in Vero cells. Shiga toxin is a protein, which has a 1-A and 5-
B type structure similar to the Shiga-like toxins of enterohaemorrhagic *E. coli*
infection (Acheson et al., 1991). Enterotoxic effect Shiga toxin adheres to small intestine receptors and blocks absorption (uptake) of electrolytes, glucose and amino acids from the intestinal lumen. Cytotoxic effect B subunit of Shiga toxin binds host cell glycolipid in large intestine, A1 domain internalized via receptor-mediated endocytosis and cause irreversible inactivation of the 60S ribosomal subunit, thereby inhibiting protein synthesis, causing cell death, microvasculature damage to the intestine and haemorrhage (blood and faecal leukocytes in stool). Shiga toxin increases the release of Tumor Necrosis Factor alpha (TNF-α) and IL-1, and appears to be responsible for Haemolytic Uremic Syndrome (HUS).

2.3.2 Salmonella

Salmonella ingested in food may survive the gastric acid barrier, travel through the mucus layer overlying the epithelium of the small intestine and evade intestinal defences such as specific IgA to result in infection (Ohl and Miller, 2001; Pegues et al., 2005). Invasion into the host intestinal cell results in dramatic morphologic changes to the cells that are due to exploitation of the host cytoskeleton. Once in close contact with the epithelium, Salmonella induces degeneration of enterocyte microvilli (Takeuchi et al., 1967). Loss of microvilli structure is followed by profound membrane ruffling localized to the area of bacterial-host cell contact (Ben-Ami et al., 1998). Membrane ruffling is accompanied by profuse macropinocytosis, which leads to the internalisation of bacteria into the host cells (Garcia del Portillo and Finlay, 1994).
Finally, *Salmonella* resides within membrane bound vesicles and the cytoskeleton returns to its normal distribution. Once *Salmonella* invades the intestinal epithelial barrier, the organism interacts with macrophages and lymphocytes in Peyer’s patches, which results in marked enlargement and necrosis of the lymphoid tissue (Hackett *et al*., 1986). Invasion of epithelial cells also stimulates the release of pro-inflammatory cytokines, which induce an inflammatory reaction. The acute inflammatory response causes diarrhoea and leads to ulceration and destruction of the mucosa. From the sub-mucosal lymphoid tissue or Peyer’s patches, the organism enters the systemic circulation, causing fever and other systemic diseases.

2.3.2.1 Bacterial factors involved in *Salmonella* invasion

*Salmonella* entry into non-phagocytic epithelial cells requires several chromosomal genes (inv/spa) clustered in a pathogenecity island termed *Salmonella* Pathogenecity Island 1 (SPI 1) (Galan, 1996). Like Enteropathogenic *Escherichia coli* (EPEC), SPI 1 encodes a type III secretion system and several potential virulence factors secreted by this machinery. The type III secretion system is activated upon host-cell contact and allows export of virulence determinants directly into the host cell, where they effect bacterial uptake (Ginocchio *et al*., 1994). SptP, a bacterial protein encoded within SPI 1 is translocated into the host epithelial cell, where it modulates the host actin cytoskeleton through its tyrosine phosphatase activity (Fu and Galan, 1998).
Disruption of a critical Cys residue in the catalytic domain of SptP results in loss of phosphatases activity (Kaniga et al., 1996). It is hypothesised that SptP may function in disrupting host actin stress fibres, thereby facilitating membrane ruffling and subsequent bacterial uptake into host cells.

A virulence factor encoded on the genome of a cryptic bacteriophage found in the Salmonella chromosome; SopE is required for efficient bacterial entry into host cells. SopE requires the type III secretion system to be translocated into the host cell where it can directly stimulate actin cytoskeletal rearrangements. It acts as a guanidine exchange factor for members of the Rho subfamily of small Guanidine Triphosphatases (GTPases). SopE mutants exhibit less extensive actin cytoskeletal rearrangement upon entry into epithelial cells than do wild type Salmonella (Hardt et al., 1998). This illustrates how pathogens can craftily subvert the host’s own signalling machinery within the cell by mimicking host proteins.

2.3.2.2 Host factors involved in Salmonella invasion

The massive restructuring of the host cytoskeletal components during Salmonella entry requires many host factors. A Rho subfamily member Cdc 42 mediates bacterial uptake through membrane ruffling (Chen et al., 1996). Evidence abounds that guanidine exchange activity of SopE is responsible for the stimulation of Cdc 42 in the host. The pathogen also activates host PLC upon bacterial contact, leading to the production of two second messengers, which further initiate
signalling events (Ruschkowski et al., 1992). As a consequence, the host cell’s Calcium ions (Ca^{2+}) levels are altered to trigger cytoskeletal rearrangements resulting in Salmonella invasion. Cytoskeletal components involved in invasion include α-actinin, tropomyosin, ezrin and talin (Finlay et al., 1992). However specific roles of these proteins in Salmonella invasion are not defined.

The major surface molecules of Salmonella are important in pathogenesis. The Vi antigen of S. typhi prevents antibody-mediated opsonization, increases resistance to peroxide and confers resistance to complement activation by the alternative pathway and to complement mediated lysis (Looney and Steigbigel, 1986). Vi antigen may therefore function to inhibit phagocytosis of the Salmonella by neutrophilis while not interfering with the induction of phagocytosis by more permissive macrophages and epithelial cells. Moreover, the lipid A component of lipopolysaccharide is a potent toxin for mammalian cells.

Cell mediated immunity (CMI) is important in controlling intracellular pathogens such as Salmonella. The risk of invasive Salmonella is increased in patients with Acquired Immunodeficiency Syndrome (AIDS), organ transplantation and lymphoproliferative disease (Levine et al., 1991). Individuals with a deficiency in interleukin-12 (IL-12) receptors are also extremely susceptible to Salmonella infections (de Jong et al., 1998). IL-12 induces Th-1 type cell responses and interferon-gamma (INF-γ) production, which is important in resistance to
salmonellosis. Patients who have splenic dysfunction, such as those with sickle cell disease, also have an increased incidence of salmonellosis (Workman et al., 1993).

2.3.3 *Vibrio cholerae*

Most of the *V. cholerae* bacteria in the contaminated water that a host drinks do not survive the very acidic conditions of the human stomach (Hartwell et al., 2004). The few bacteria that do survive conserve their energy and store nutrients during the passage through the stomach by shutting down much protein production. When the surviving bacteria exit the stomach and reach the small intestine, they need to propel themselves through the thick mucus that lines the small intestine to get to the intestinal wall where they can thrive. *V. cholerae* bacteria start up production of the hollow cylindrical protein flagellin to make flagella, the curly whip-like tails that they rotate to propel themselves through the mucus that lines the small intestine.

Once the *V. cholerae* bacteria reach the intestinal wall, they do not need the flagella propellers to move themselves any longer. The bacteria stop producing the protein flagellin, thus again conserving energy and nutrients by changing the mix of proteins that they manufacture in response to the changed chemical surroundings. On reaching the intestinal wall, *V. cholerae* start producing the toxic
proteins that give the infected person watery diarrhoea. This carries the multiplying new generations of *V. cholerae* bacteria out into the drinking water of the next host if proper sanitation measures are not in place.

Microbiologists have studied the genetic mechanisms by which the *V. cholerae* bacteria turn off the production of some proteins and turn on the production of other proteins as they respond to the series of chemical environments they encounter, passing through the stomach, through the mucous layer of the small intestine and on to the intestinal wall (DiRita *et al.*, 1991). Of particular interest have been the genetic mechanisms by which cholera bacteria turn on the protein production of the toxins that interact with host cell mechanisms to pump chloride ions into the small intestine, creating an ionic pressure which prevents sodium ions from entering the cell. The chloride and sodium ions create a salt water environment in the small intestines which through osmosis can pull up to six litres of water per day through the intestinal cells creating the massive amounts of diarrhoea. The host can become rapidly dehydrated if an appropriate mixture of dilute salt water and sugar is not taken to replace the blood's water and salts lost in the diarrhoea.

By inserting separately, successive sections of *V. cholerae* DNA into the DNA of other bacteria such as *E. coli* that would not naturally produce the protein toxins,
researchers have investigated the mechanisms by which *V. cholerae* responds to the changing chemical environments of the stomach, mucous layers and intestinal wall. Researchers have discovered that there is a complex cascade of regulatory proteins that control expression of *V. cholerae* virulence determinants. In responding to the chemical environment at the intestinal wall, the *V. cholerae* bacteria produce the TcpP/TcpH proteins, which, together with the ToxR/ToxS proteins, activate the expression of the ToxT regulatory protein. ToxT then directly activates expression of virulence genes that produce the toxins that cause diarrhoea in the infected person and that permit the bacteria to colonize the intestine (DiRita *et al.*, 1991).

The reason for the differences between the symptoms produced by *V. cholerae* 01 and *V. parahaemolyticus*, both of which colonize a similar region of the small intestine, is that they produce different toxins (Miwatani *et al.*, 1988). *V. cholerae* 01 produces a cytotoxic enterotoxin called cholera toxin (CT). *V. parahaemolyticus* produce a lethal toxin referred to as thermostable direct hemolysin (TDH), which at low doses can kill animals within seconds. CT and heat-labile enterotoxin (LT) of Enterotoxin *Escherichia coli* (ETEC) are biologically, immunologically and physiochemically similar in character. CT/LT are both composed of A and B subunits. The A subunit, which consists of A1 and A2 fragments, is enzymatically active while the B subunit binds to the toxin to its ganglioside receptor. Mechanism of induction of diarrhoea by *V. cholerae*
involves: CT binds to ganglioside GM1 receptor through its B subunit, and the A1 fragment causes enzymatic Adenosine Diphosphate (ADP)-ribosylation of the GTP-binding regulatory component (Gs α) of adenylate cyclase. CT thus inhibits the GTPase turn-off reaction, resulting in activation of adenylate cyclase in the cell membrane. The steps after adenylate cyclase activation with consequent accumulation of intercellular cyclic Adenosine Monophosphate (cAMP) are still unknown.

2.4 Clinical features and syndrome

2.4.1 Shigella

Fever and abdominal cramping are considered as signs of neurotoxicity. Shiga toxin is not essential for virulence of S. dysenteriae Type 1 in primates but contributes to severity of disease manifestations, especially bloody diarrhoea/dysentery (Fontaine et al., 1988). Shigellosis typically evolves through several phases and manifestations of Shigella infection vary with the infecting species, the age of the host, the presence of risk factors and the specific immune status of the host. The incubation period ranges from 1 to 4 days, but may be as long as 8 days with S. dysenteriae (Levine et al., 1973).

Shigellosis is an invasive infection of the human colon that affects a spectrum of clinical presentations, from short-lasting watery diarrhoea to inflammatory bowel disease. Clinical disease typically begins within 24-48 hr of ingestion of a few
hundred to a few thousand organisms with constitutional symptoms such as fever, fatigue, malaise, and anorexia. Watery diarrhoea typically precedes dysentery (DuPont et al., 1969) and is often the sole clinical manifestation of mild infection (Taylor et al., 1986). Progression to frank dysentery may occur within hours to days with frequent small volume of bloody, mucoid stools, abdominal cramps and tenesmus. In patients experiencing dysentery, involvement is most severe in the distal colon, and the resulting inflammatory colitis is evidenced in frequent scanty stools reflecting the ileocaecal fluid flow. Patients with severe infection may pass more than 20 dysenteric stools in one day (Mathan and Mathan, 1991).

Dysentery is also characterized by the daily loss of 200-300 ml of serum protein into the faeces. This loss of serum proteins result in depletion of nitrogen stores that exacerbate malnutrition and growth stunting. Depletion of immune factors also increases the risk of concurrent, unrelated infectious disease and contributes to substantial mortality. Anorexia, which is a prominent finding initially, may persist into convalescence and contribute to the deterioration in the patients' nutritional status, which commonly occurs in shigellosis. Large fluid losses and severe dehydration are rare in shigellosis (Butler et al., 1986). A variety of unusual extraintestinal manifestations may occur. The most common is seizures, which usually occur in the presence of fever without associated encephalopathy (Ashkenazi et al., 1987). Microangiopathic haemolytic anaemia can complicate infection with Shiga toxin-producing organisms, manifesting as the haemolytic
uraemic syndrome in children and as thrombotic thrombocytopenic purpura in
adults (Koster et al., 1978). Most episodes of shigellosis in otherwise healthy
individuals are self-limiting and resolve within 5-7 days without sequela.

Acute, life-threatening complications are most often seen in malnourished infants
and young children living in developing countries. These include metabolic
derangements, such as dehydration, hyponatraemia and hypoglycaemia, intestinal
complications such as toxic megacolon, rectal prolapse, intestinal perforation
(Bennish, 1991) and rarely sepsis (Struelens et al., 1985). Shigella bacteremia has
been reported among HIV-infected and other immunocompromised patients
(Kristjansso et al., 1994; Batchelor et al., 1996). Persistent diarrhoea and
malnutrition are the most common chronic sequelae (Black et al., 1982). A rare
post-infectious complication seen primarily in adults following infection with S.
flexneri serotypes is reactive inflammatory arthritis, alone (Sieper et al., 1993) or
as part of a constellation of arthritis, conjunctivitis and urethritis known as
Reiter’s syndrome (Finch et al., 1986).

2.4.2 Salmonella

2.4.2.1 Non-Typhoid Salmonellosis

The incubation period of non-typhoid salmonellosis ranges from 6-8 hrs, but can
occasionally reach 7-12 days. The initial symptoms are nausea and vomiting
followed by periumbilical abdominal pain, cramps in the right lower quadrant and
diarrhoea that is often watery. However, in 50% of cases, severe dysentery with bloody and purulent faeces develops usually accompanied by fever. The diarrhoea disappears after 2-5 days but tends to last longer if the colon is affected frequently persisting for up to 3 weeks (Mandel and Mani, 1976). Hyperaemia, mild edema, granularity, friability and ulceration of the mucosa are witnessed. Potential complications include: intestinal bleedings, septicaemia and toxic megacolon (Bellery and Isaac, 1990). Persistent fever usually suggests metastatic organ infection. The spectrum of metastatic *Salmonella* infections include: meningitis, arteritis, endocarditis, osteomyelitis, spondylodiscitis and septic arthritis.

### 2.4.2.2 Enteric fever

The enteric fever caused by *S. typhi* and *S. paratyphi* is exclusive to man and present primarily as septicaemic disorder. The incubation period ranges from 7-14 days. The classical clinical course lasts for 4 weeks. In the first stage, patients suffer from unspecific symptoms such as low-grade fever, arthralgia and headache. The second stage indicates systemic infection. The body temperature rises up to 40°C, the headaches become more intense and patients suffer from abdominal pain in right upper quadrant. Bowel habits change in 50% of affected individuals, whereby constipation is more common than diarrhoea. The size of lymphoid organs such as the spleen and lymph nodes increases due to hyperplasia of the reticulo-endothelial system. In severe cases, the patient may enter the typhoidal state that is characterised by fatigue, apathy and anorexia. The third stage is
characterised by more frequent bowel movements, declining body temperatures, but more intense abdominal pain in the ileocecal region. The stool resembles a greenish pea soup. Peyer’s patches are hyperplastic and may ulcerate. After 6 weeks, 50% of affected individuals still harbour *Salmonella* in stool. Although this proportion declines to 5-10% at 3-6 months, 1-3% remains chronic carriers of *Salmonella*.

2.4.3 *Vibrio cholerae*

Cholera is characterised by sudden onset of effortless vomiting and profuse watery diarrhoea. The incubation period ranges from several hours to 5 days and depends on the inoculum size. *V. cholerae* infections can be asymptomatic (25-66%), cause slightly watery stools or result in severe dehydrating diarrhoea. In milder forms, the loss of fluid does not exceed 1000 ml per day. The illness is characterised by nausea, vomiting and abdominal distention followed by watery diarrhoea and mild abdominal cramps. The diarrhoea usually lasts 2-4 days.

In severe forms, large volumes of watery stools are evacuated within hours. The stool contains flecks of mucus, known as rice water stool that is associated with a high mortality of 50% in untreated individuals. The major complication of cholera is rapid and extensive dehydration as a result of the massive loss of intestinal fluid. The maximum volume of stool is excreted 24 hr, which can easily reach 500-1000 ml per hour resulting in a daily fluid loss of 15-20 litres. As a consequence,
electrolyte disturbances, hypokalemia and metabolic acidosis may develop. The clinical signs of severe dehydration include: a reduced skin turgor, hoarseness, sunken bulbi, dry mucous membranes, washerwoman’s hands, missing pulses, cold extremities, hypothermia, hypovolemic shock, impaired renal function and change of consciousness.

2.5 Treatment

2.5.1 Shigella

Treatment of shigellosis by appropriate antimicrobial agents has proven efficacious in shortening the duration of fever, diarrhoea and toxaemia and apparently in reducing the risk of lethal complications as well (Askenazi, 1999). Concomitantly, the excretion of the pathogen in stools is shortened significantly, reducing the spread of the infection (DuPont, 1988). Antimicrobial agents currently recommended for the treatment of Shigella infections include ampicillin, ciprofloxacin, norfloxacin, enoxacin, nalidixic acid, pivmecillinam and trimethoprim-sulfamethoxazole. Although initially susceptible to many antimicrobial agents, Shigella isolates resistant to multiple agents have been reported worldwide (Replogle et al., 2000; Lee et al., 2001; McIver et al., 2002; Oh et al., 2003). The changing resistance indicates the need for continuous monitoring of antibiotic resistance in order to update the recommendations for empirical antibiotic therapy of suspected shigellosis (Ashkenazi, 1999).
Pivmecillinam is still effective for most strains of *Shigella* but may not be readily available. Fluoroquinolones such as ciprofloxacin, norfloxacin and enoxacin are often costly and may not be readily available. Fluoroquinolones should be considered only if *Shigella* isolates are resistant to nalidixic acid. For the purpose of developing a treatment policy, the antimicrobial agent chosen should meet the following conditions: be effective against at least 80% of local *S. dysenteriae* Type 1 strains, be given by mouth, be affordable and available locally or able to be obtained quickly (WHO, 2003).

Mobile genetic units are important in the spread of resistant determinants among *Shigella* isolates (Toro *et al.*, 2005). Trimethoprim and sulfamethoxazole resistance is most commonly acquired through a plasmid-encoded variant of the dihydrofolate reductase enzyme (Heikkila *et al.*, 1990). Ampicillin resistance arises as a result of beta-lactamases similar to TEM-1 or OXA-1, whose genes may be located on chromosomes, plasmids, or transposons (Navia *et al.*, 1999). Although resistance to quinolones is commonly mediated through chromosomal mutations rather than mobile genetic units, certain plasmids have been shown to contribute to quinolone resistance by increasing the rate of spontaneous mutation (Ambler *et al.*, 1993).
2.5.2 Salmonella

Early diagnosis of typhoid fever and prompt institution of appropriate antibiotic treatment are essential for optimal management, especially in children. Although most cases can be managed at home with oral antibiotics and regular follow-ups, patients with severe illness, persistent vomiting, severe diarrhoea, and abdominal distension require hospitalisation and parenteral antibiotic treatment. In addition to antibiotics, supportive treatment and maintenance of appropriate nutrition and hydration are crucial.

Appropriate antibiotic treatment (the right drug, dose, and duration) is critical to curing typhoid with minimal complications (Van der Bergh et al., 1999). Standard treatment with chloramphenicol or amoxicillin is associated with a relapse rate of 5-15% or 4-8% respectively, whereas the newer quinolones and third generation cephalosporins are associated with higher cure rates (WHO Department of Vaccines and Biologicals, 2003). The emergence of multidrug resistant typhoid in the 1990s led to widespread use of fluoroquinolones as the treatment of choice for suspected typhoid, especially in South Asia and South East Asia where the disease was endemic (Rowe et al., 1987). In recent years, however, the emergence of resistance to quinolones has placed tremendous pressure on public health systems in developing countries as treatment options are limited (Bhutta, 2006; Frenck et al., 2004).
Studies of short course antibiotic treatment for multidrug resistant typhoid have shown that fluoroquinolones can achieve satisfactory cure rates, but parenteral ceftriaxone was associated with higher rates of relapse. A recent Cochrane review of antimicrobial treatment of typhoid fever concludes that there is little evidence to support administration of fluoroquinolones to all cases of typhoid and that satisfactory cure rates can be achieved in drug sensitive cases with first line agents such as chloramphenicol (Thaver et al., 2006). Although some open studies have suggested that cure rates may be better with oral fluoroquinolones compared with chloramphenicol (Phongmany et al., 2005), these case series also include multidrug resistant cases. Given the signs of rapidly increasing resistance of \textit{S. typhi} to fluoroquinolones, it is imperative that the widespread use of these antibiotics for fever and their availability over the counter are restricted, although it may already be too late (Okeke et al., 2005). However, treatment regimens must restrict as much as possible the use of further second and third line antibiotics for treating typhoid in primary care settings (Okeke et al., 2005).

The prognosis for a patient with enteric fever depends on the rapidity of diagnosis and treatment with an appropriate antibiotic. Other factors include the patient's age, general state of health, nutrition, the causative \textit{Salmonella} serotype and the appearance of complications. Infants and children with underlying malnutrition and those infected with multidrug resistant isolates are at higher risk of adverse outcomes. Although additional treatment with dexamethasone (3 mg/kg for the
initial dose, followed by 1 mg/kg every 6 hours for 48 hours) has been recommended among severely ill patients with shock, obtundation, stupor or coma, this must be done only under strictly controlled conditions and supervision, and signs of abdominal complications may be masked.

Despite appropriate treatment, some 2-4% of infected children relapse after initial clinical response to treatment. Individuals who excrete *S. typhi* for more than three months after infection are regarded as chronic carriers. However, the risk of becoming a carrier is low in children and increases with age, but in general it occurs in less than 2% of all infected children (WHO Department of Vaccines and Biologicals, 2003).

No vaccines are available for non typhoidal salmonella infections. Recent research with recombinant vaccines and live attenuated vaccine vectors may provide better vaccine candidates for testing in humans (Abd El Ghany *et al.*, 2007; Husseiny *et al.*, 2007; Nagy *et al.*, 2006; Negi *et al.*, 2007; Stephens *et al.*, 2006). Immunity and antibody responses are only partially understood and lack of a suitable animal model has hindered vaccine development (Sztein, 2007). Additional investigation of *Salmonella* genes may clarify which genes are likely to be better suited for vaccine development (Boyle *et al.*, 2007). Potential new therapies have employed small molecules that can inhibit type III secretion systems in *Salmonella*,

...
preventing protein secretion, invasion of epithelial cells, and enteritis (Hudson et al., 2007).

By the year 2002, there were at least two effective vaccines available for typhoid, both of which had been licensed for use in the United States. The oral live attenuated vaccine (for use in children aged 6 years and older) and the parental (injectable) capsular polysaccharide vaccine (for use in children aged 2 years and older) each have efficacy of 50-80% and fewer adverse events associated with their use than earlier typhoid vaccines.

2.5.3 Vibrio cholerae

Fluid replacement is the cornerstone of cholera treatment and rehydration therapy is a necessity. Fluids and electrolytes can be replaced rapidly through either oral or intravenous routes. Intravenous therapy is required for patients who are in profound shock or cannot drink. Antimicrobial therapy is helpful although not essential in treating cholera patients. Antimicrobial agents reduce the duration of shedding of Vibrios in the faeces (WHO, 1999). Antimicrobial agents recommended by WHO for treating cholera patients include tetracycline, doxycycline, furazolidone, trimethoprim-sulfamethoxazole, erythromycin or chloramphenicol. Ciprofloxacin and norfloxacin are also effective (WHO, 2003).
Recent studies have documented that licensed vaccines against cholera can be used safely in Africa in refugee populations (Legros et al., 1999) and in adults with HIV infection (Perry et al., 1998). In addition, a recent mass vaccination campaign in a cholera endemic area of Mozambique showed that the two-dose recombinant cholera-toxin B subunit, killed whole cell vaccine was highly effective against clinically significant cholera in an urban sub-Saharan African population with a high prevalence of HIV infection (Lucas et al., 2005). Although cholera vaccines are not yet appropriate tools for long-term cholera prevention and control in Africa because of their high cost and short duration of protection, they may have a useful role to play in specific situations (WHO, 2001). Currently available cholera vaccines are not licensed for use in children under 2 years old.
CHAPTER THREE
MATERIALS AND METHODS

3.1 The study area

The study was carried out in Budalang’i area of Busia district in Western Province of Kenya, a rural community bordering Lake Victoria. It covers a total area of 312 square kilometers, which includes 120 square kilometres of water surface area. The area experiences two rainy seasons:- long (March-May) and short (August-October) rains. Poor drainage of soils, which are mainly of deep firm and clay type, compounded with the frequent outburst of river Nzoia and river Yala banks occasioned by heavy rainfalls in the upstream predisposes this region to frequent floods. The high incidence of various diseases, among them HIV/AIDS, malaria, diarrhoeal and other water-borne diseases has led to loss of many lives. Most of these diseases have their genesis in poverty, lack of treated water supply, poor sewerage and drainage systems, food contaminations, low standards of personal hygiene and environmental sanitation. Accessibility to the area is difficult because of numerous streams and undulating topography that constrain the development of roads (District Commissioners Office, Busia 1996).

3.2 Inclusion criteria

Stool samples from all patients presenting with diarrhoea at Budalang’i Health Centre and had consented to the study.
3.3 Exclusion criteria

Stool samples from patients presenting with diarrhoea who had received antibiotics treatment over a period of not less than two weeks. Patients presenting with diarrhoea but did not consent to the study.

3.4 Sample size

Enteric bacterial pathogens isolation rate of 30% (Saidi et al., 1997) and a level of significance of 5% were assumed. This yielded a minimum sample size required using the formula of Harper, (1980).

\[
N = \frac{Z^2 (1 - a) P (1 - P)}{d^2}
\]

\[
N = \frac{1.96^2 \times (1-0.05) \times 0.3 \times (1-0.3)}{0.05 \times 0.05} = 336.
\]

Where: 
- \(N\) = minimum sample size
- \(Z = 1.96\) (standard errors from the mean)
- \(P = 0.30\) (isolation rate of bacterial pathogens in stool)
- \(d = 0.05\) (5% absolute precision)
- \(a = 0.05\) (5% significance level).

Hence 336 stool samples were collected in this study.
3.5 Methodology

3.5.1 Sample collection

Patients attending out-patient department of Budalang’i Health Centre with complaint of diarrhoea were given clean containers (polypots) of sufficient size with tight fitting leak proof lid and requested to provide their fresh diarrhoea stool.

3.5.2 Preparation of filter paper media

Three sets of Whatman filter paper were cut using a pair of scissors to 5 x 5 square centimetres, following the procedure of Mutwewingabo et al., (1984). The papers were sterilised at 100°C in air steam. One set of the filter papers was impregnated with formula 1 (Appendix I). The second set was impregnated with formula 2 only (Appendix I) and the third set of filter papers received no impregnated material hence acted as the control.

3.5.3 Inoculation of specimen for transportation

3.5.3.1 Filter paper media

Ten drops of freshly collected diarrhoeal stool (mucus and shreds preferred) were dropped on each of the three sets of filter papers, labelled and left to air dry at room temperature. After air-drying, the filter papers were placed inside plastic pouches, sealed using a cello tape and kept in a box for onward transportation to Centre for Microbiology Research (CMR), Kenya Medical Research Institute (KEMRI) laboratories in Nairobi for bacterial isolation and identification.
3.5.3.2 Gold standard transport media

The same stool samples were placed in Cary-Blair transport medium by inserting a cotton-tipped sterilised swab into the collected stool, rotated and then placing it all the way to the bottom of the bottle containing the medium. The portion of the stick touching the finger was broken off, the screw cap replaced tightly; the bottle labelled and then stored with the inoculated medium in a cool dark place (to keep it as cool as possible). The transport tubes (bottle) were wrapped carefully and packed in a suitable, durable container and then sealed for onward transportation to Centre for Microbiology Research, KEMRI, Nairobi for bacteriological isolation and identification.

3.5.4 On Site Identification

Xylose Lactose Desoxycholate (XLD) and Salmonella Shigella (SS) agar plating were performed at the collection site and used as growth controls.

3.5.5 Specimen processing

3.5.5.1 Inoculation of stool specimens from filter paper

The bacteria on each of the filter papers was recovered by dividing the paper into three pieces and then dipping one piece in 10 ml of normal saline for 2 hrs, the second one in 10 ml of distilled water for 2 hrs (control) and the third one in 10 ml of Alkaline Peptone Water (APW) for 6 hrs. The test tubes were then vortexed and a flamed sterilised wire loop (50 μl) used to make primary inoculums on XLD, SS,
MacConkey (MAC) and Thiosulfate Citrate Bile Salt (TCBS) agars. The same wire loop was used to spread the primary inoculum to the four quadrants of the plate by successively streaking with a back and forth motion into each quadrant and turning the plate at 90°. The wire loop was flame sterilised between each successive quadrant streaks. The purpose of this technique was to dilute the inoculum sufficiently enough on the surface of the agar medium so that well isolated colonies of bacteria could be obtained from colony forming units (cfu). Ten µl was taken from the normal saline and inoculated into Selenite-F-broth (SEL) medium to act as an enrichment medium for *Salmonella*. The inoculated plates and Selenite-F-broth were then incubated at 37°C for 24 hrs.

3.5.5.2 Inoculation of swabs from Gold standard transport media

Stool samples in the Cary-Blair was inoculated using swabs on the primary media (MAC, SS, XLD and TCBS agars) by making a heavy inoculum on the media of high selectivity and a light inoculum on the media of low selectivity (two centimetres in diameter) and then streaked using a flame sterilised wire loop as described in 3.5.5.1 above. Using the same swab, the stool was inoculated in 10 ml of SEL for 16 hrs and in 10 ml of APW for enrichment of *Vibrio* and incubated for 6 hrs at 37°C before inoculating on primary media (TCBS).

Inoculants of SEL and distilled water were sub-cultured on MAC, SS and XLD using a flamed sterilised wire loop as explained above and then incubated at 37°C.
for 24 hrs.

3.5.6 Biochemical typing (Biotyping)

Well-separated colonies of typical appearance (Appendix I) were identified and transferred from each of the plating media for further testing by making a mark on the bottom of the petri dish. Five non lactose fermenting (NLF) colonies were picked from each plating media and inoculated into separate tubes of Triple Sugar Iron agar (TSI), Sulfide-Indole-Motility (SIM), Simmon citrate agar, Methyl Red Voges Proskauer (MRVP), and Urea agar. To avoid picking up the contaminants that might have been on the surface of the agar, a flamed sterilised straight inoculating needle was used to lightly touch, only the very centre of the identified colony. TSI was inoculated by stabbing the butt and then streaking the slant with a zigzag configuration. SIM was inoculated by making a single stab about 1-2 cm down into the medium. Simmon citrate agar was inoculated by streaking the surface of the slant with a zigzag configuration. MRVP was inoculated by dipping the inoculating needle into the medium. Urea agar was inoculated by stabbing into the medium five times. To avoid anaerobic conditions existence in the test tubes, the caps were loosened before their placement in the incubator. The test tubes were then incubated at 37°C for 24 hrs.

Isolates from the primary media were examined for NLF colonies; positive cultures were biotyped as explained above.
All the biochemical results were interpreted as per “Biochemical reaction chart for Enterobacteriaceae, *Aeromonas* and *Plesiomonas*” Krieg and Holt, (1984) (Appendix II).

Biotyping tubes from SEL sub-cultures were examined and potential pathogens processed as explained in 3.5.6.

### 3.5.7 Confirmation

API 20E strip consisting of 20 cupules containing dehydrated substrates was used. The strips were prepared by; first preparing an incubation box (tray and lid) and then dispensing about 5 ml of distilled water or demineralised water into each of the honeycombed wells of the tray to create a humid atmosphere. Strain reference was recorded on the elongated flap of the tray, removed the strip from its packaging and placed in the incubation box. 0.5M McFarland Turbidity Standard of the test isolates were prepared in normal saline (Appendix). Using a pipette, both the tube and cupule of the test CIT, VP and GEL were filled. The other tests were inoculated by filling the tube only. Anaerobic conditions were created in tests ADH, LDC, ODC, H₂S and URE by overlying them with mineral oil. The incubation box was then closed and incubated at 37°C for 24 hrs.

Using Pasteur pipette, one drop of DA reagents was added to each test, one drop of James reagent to IND test and one drop each of VP1 and VP2 reagents added to
VP test. The results were read and identification obtained by referring to the Analytical Profile Index (Appendix III) or using the Identification Software.

3.5.8 Antimicrobial sensitivity test

Antimicrobial sensitivity test was done using disk diffusion method modified by Kirby-Bauer technique that has been carefully standardised by National Committee on Clinical Laboratories Standards (NCCLS, 2002). This is an international interdisciplinary, non-profit, educational organisation that develops updated consensus standards and guidelines for the healthcare community on an annual basis. The antimicrobial tested included:

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Symbol</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>amp</td>
<td>10 µg</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>c</td>
<td>30 µg</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole (cotrimoxazole)</td>
<td>sxt</td>
<td>25 µg</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>gn</td>
<td>10 µg</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>te</td>
<td>30 µg</td>
</tr>
<tr>
<td>Cefutaxime</td>
<td>ctx</td>
<td>10 µg</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>cip</td>
<td>10 µg</td>
</tr>
</tbody>
</table>

Mueller-Hinton (MH) agar medium was used.

3.5.8.1 Preparation of inoculum

The isolates were streaked onto MH agar medium to obtain fresh growths. After incubation at 37°C overnight, an inoculating loop was used to transfer the growth
to a tube of sterile normal saline and vortexed thoroughly. Using adequate light, each tube was read against a background with a contrasting black line to compare it with the 0.5M McFarland Turbidity Standard. The turbidity was adjusted by either adding sterile normal saline or bacterial growth. The final concentration was equivalent to $10^8$ cfu.

3.5.8.2 Inoculation procedure

Plates were inoculated within 15 minutes after adjusting turbidity of the inoculum. A sterile non-toxic cotton swab on a wooden applicator was dipped into the bacterial inoculum suspension; excess inoculum was removed by pressing and rotating the swab firmly against the side of the tube above the level of the liquid. The swab was then streaked all over the dried surface of the MH agar plate medium that had been brought to room temperature, 3 times rotating the plate through an angle of 60° after each application and then finally applying the swab all around the edge of the agar surface. This was done to ensure an even distribution of the inoculum. The lid of the petri-dish was replaced and allowed 5 to 15 minutes for the surface of the agar to dry before adding the antibiotic disks.

3.5.8.3 Application of antibiotic disks

The antibiotic disks were placed manually, on the inoculated plates in zones properly divided and marked on the back of petri-dish, using a pair of sterile forceps. The disks, which had been allowed to warm to room temperature, were
placed at least 15 mm from the rim of the petri-dish with approximately 20 mm of space between the disks. This was to avoid overlapping of zones of growth inhibition or extension of a zone to the edge of the dish. Each disk was then gently pressed to the agar surface with the point of the forceps or an applicator stick shortly after they were placed to ensure that firm contact was made with the agar.

The prepared antibiotic susceptibility plates were then placed in a 37°C incubator without increased atmospheric carbon dioxide for 18 hrs. Incubation was done within 15 minutes of applying the disks. The plates were placed in the incubator upside down so that any moisture or condensation that collected under the lid did not fall onto the agar surface.

3.5.8.4 Measurement of zones

All final measurements were made at exactly 18 hrs as recommended by NCCLS 2002. This is the time when the reactivity between the growing organisms and the inhibitor effects of the antibiotic are optimal and the zone margins of growth are most distinct. Zones of complete growth inhibition around each of the disks were carefully measured using a ruler to the nearest millimetres, to include the diameter of the disk. All measurements were made with the unaided eye, while viewing the back of the petri-dish with reflected light against a black, non-reflecting background. Plates were viewed from a directly vertical line of sight to avoid any parallax that may result from misreading.
3.5.9 Minimum inhibition concentration (MIC) test

This is defined as the lowest concentration of antimicrobial that will inhibit the visible growth of a micro organism after overnight incubation (16-18 hrs).

3.5.9.1 Preparation of stock solution

The powders were removed from the freezer (-20°C) and allowed to come to room temperature before use to avoid condensation of water. Stock solutions were prepared using the formula below;

\[ W = \frac{1000 \times V(\text{ml}) \times C (\mu g \text{ ml}^{-1})}{P (\mu g \text{ ml}^{-1})} \]

Where \( P \) = potency given by manufacturer in relation to base

\[ V = \text{volume required in ml (20 ml)} \]

\[ C = \text{final concentration of the solution (multiple of 1000)} \]

\[ W = \text{weight of antimicrobial in mg to be dissolved in V} \]

Appropriate amount of antibiotic was weighed and reconstituted in appropriate liquid before being dissolved in a suitable diluent (Appendix V) to top it to 20 ml.

3.5.9.2 Preparation of the working stock solutions

Eleven sterile universal test tubes each containing 10 ml of sterile water was labelled for each of the 3 drugs ampicillin, chloramphenicol and tetracycline as follows: 1280, 640, 320, 160, 80, 40, 20, 10, 5, 25, 1.25 whereas for gentamicin, only 8 sterile universal test tubes were labelled starting from 160 to 1.25μg/l. In
the first test tube labelled 1280 was added 10 ml of the stock solution using a sterilised pipette, mixed well and 10 ml of the resulting solution was pipetted and added to the next tube labelled 640. The procedure was repeated for the stock solution of each drug. The sterilised petri dishes were labelled with the abbreviations of the drug and the containing content as follows: (128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, and 0.125) μg/l.

Two ml of the working stock solution of each drug was pipetted onto individual petri dish before adding 18 ml of MH agar (sterilised in autoclave at 112°C for 15 minutes and temperature maintained at 60°C in a water bath) and the content thoroughly mixed to obtain an equally distributed solution. This gave a 1:9 dilution that was left to solidify on a flat bench before storage at 4°C. Eight (8) agar plates without antibiotics were prepared to act as controls.

3.5.9.3 Inoculation of the plate

Fresh sub-cultures of isolates were used to prepare suspensions equivalent to the 0.5M McFarland Turbidity Standard (1 x 10⁸ colony forming units per ml) as described in 3.5.8.1. Using a sterilised micropipette, each prepared suspension of the isolate was placed into it’s corresponding well in an aluminium seed plate (template) each containing 27 wells. Each template also contained, an E. coli ATCC 25922 as control. A multipoint, an instrument with a spring loaded head fitted with 27 flat surface inoculating pins each about 3mm in diameter, was used
to inoculate the plates. The inoculating pins were first sterilised by dipping them in 80% alcohol, flaming and then cooling them to room temperature. The template, each containing 27 bacterial suspensions, was then properly aligned with the guide at the base of the replication. The head of the multipoint was lowered so that the pins extended fully into each of the wells thereby sampling approximately $3 \times 10^{-3}$ ml of each bacterial suspension on the surface of each inoculating pin. The head was then raised and the template removed. Next, agar plate without antibiotic (control) followed by agar plates containing antibiotics were each placed beneath the inoculating head, which in turn was lowered so that the flat surface of each inoculating pin just touched the agar surface. The head was raised, and the inoculated agar plate removed and replaced with another in succession. Agar plate without antibiotic was inoculated last to check the growth viability of the isolates. All the plates were incubated upside down within 15 minutes of inoculation at 37°C for 16-18 hrs.

Concentrations that inhibit visible growth of microorganism after overnight incubation was recorded and interpreted per NCCLS guidelines of 2002.

3.5.10 Plasmid profiles analysis

Plasmid extraction was done for all isolates. Modified procedure of Birnboim and Doly (1979) was followed as described below:
3.5.10.1 Bacterial growth

Test isolates were inoculated into 4 ml of Lauria Bertani (LB) broth and incubated in a water bath at 37°C for 16-20 hrs with shaking at 120 shakes per minute. The shaking was important in order to achieve maximum aeration of the culture to increase the yield.

3.5.10.2 Harvesting and extraction

Both solutions 1 (B-I) and solution II (B-II) were prepared fresh before use (Appendix VI). One and a half (1.5) ml of the culture was transferred to an Eppendorf microtube and centrifuged at 15000 rpm for 30 seconds. The tubes were then removed from the centrifuge and the supernatant discarded as much as possible to leave the bacterial pellet at the bottom of the microtube. The bacterial pellet was resuspended in 100 μl of ice-cold solution 1 (B-I), vortexed to mix well and incubated on ice for 10 minutes. Two hundred (200) μl of solution II (B-II) was then added, mixed by vortexing and incubated on ice for 5 minutes. One hundred and fifty (150) μl of 3M sodium acetate, pH 5.2 (B-III), was added, mixed by inverting the microtube several times and then incubated on ice for 10 minutes. The mixture was centrifuged at 15000 rpm for 10 minutes. About 400 μl of supernatant was transferred to a fresh microtube, 100 μl of cold ethanol added to it and the content mixed by inverting the microtube severally. The tubes were incubated at -80°C for 10 minutes then centrifuged at 15000 rpm for 10 minutes. The supernatant was discarded. The pellet was dissolved in 200 μl of Tris ethylene
diamine-tetra-acetic acid (TE) buffer and 100 µl of 7.5M Ammonium acetate added. The mixture was incubated on ice for 30 minutes.

The mixture was centrifuged at 15000 rpm and 300 µl of the supernatant transferred to a new microtube. Eight hundred (800) µl of cold ethanol was added, the contents mixed by inverting the microtube severally and incubated at -80°C for 10 minutes. The mixture was then centrifuged at 15000 rpm for 10 minutes and the supernatant discarded.

The DNA pellets were then rinsed with one ml of 80% ethanol, centrifuged at 15000 rpm for 5 minutes and the supernatant discarded by aspiration. This step was repeated once. The DNA pellet was then dried in the 37°C incubator for 30 minutes and dissolved in 50 µl of TE buffer by vortexing and stored at -20°C until analysed.

3.5.11 Agarose gel electrophoresis

The DNA were removed from storage at -20°C and allowed to thaw at room temperature. Meanwhile, 1% agarose gel was prepared by dissolving 1g of agarose in 100 ml of 1 x TE buffer (working buffer) and boiling in a microwave oven while shaking gently. The agarose was cooled to about 45°C, about 25 ml measured and poured into the gel container with appropriate combs in place. The gel was left to settle for 30 minutes.
Tris Borate ethylene diamine-tetra-acetic acid (TBE) electrophoresis buffer was prepared in the ratio 1:10 solution and poured into the electrophoresis tank and the gel introduced. A micropipette was used to pick 50 µl of DNA, and diluted in 100 µl bromophenol dye (loading dye). About 140 µl of the resulting mixture was then loaded into each well of the gel. The first well (lane 1) was loaded with plasmid DNA of *E. coli* R39 which has molecular sizes 147, 63, 43.5, and 6.9 kDa. The last well (lane 17) was loaded with plasmid DNA of *E. coli* V517 strains with molecular sizes 53.7, 7.2, 5.6, 3.9, 3.0, 2.7 and 2.1. These two were used as molecular size markers. The gel was allowed to run for approximately 1.5 hrs.

The gel was stained with ethidium bromide (10 mg/ml) in a plastic tray for about five minutes and then washed with running tap water. The gel was photographed with a Polaroid instant camera.

3.5.12 Studies on transfer of resistance genes (r-factor)

*In vitro* conjugation tests were performed according to the method of Walia *et al.*, (1987). Single discreet colonies of each donor bacterial strain and recipient *E. coli* K-12 (F Na') strain were sub-cultured into 5 ml Brain Heart Infusion (BHI) broth and allowed to multiply to logarithmic phase (ca. 10^8 cells) by incubating on a shaker (250 rpm) at 37°C for 2 hours.

The culture was then centrifuged at 15000 rpm for 30 seconds and the media
discarded as much as possible. The pellet was washed with normal saline (0.85% sodium chloride solution in distilled water) and then centrifuged at 15000 rpm for 30 seconds. This step was repeated once. This was to remove any β-lactamases that might have leaked into the media from the bacterial cells and which might give a false resistance. One ml of normal saline was added to each pellet. The donor and recipient bacterial broth cultures were then diluted in 1:10 in fresh BHI broth and mixed in equal proportion to make a resulting mixture of 5 ml broth culture. This was incubated at 37°C overnight to allow conjugation to take place. In order to select transconjugants, 3 μl samples were drawn from the overnight culture and inoculated on MAC agar plates containing 32 μg/ml of nalidixic acid and 32 μg/ml of ampicillin using multiple inoculator (Sakura Japan). The agar plates were then incubated at 37°C overnight.

The isolates were picked and identified as *E. coli* by biotyping. To determine what antibiotic resistances co-transferred to recipient *E. coli* K-12 strain, antimicrobial disc susceptibility tests for each of the transconjugants were performed using the range of antibiotic discs previously described. *E. coli* isolates were picked from the sensitivity discs, sub-cultured into LB broth before proceeding with plasmid extraction of the transconjugants.
CHAPTER FOUR

RESULTS

4.1 Study population

During the five months period, the stools from 336 diarrhoeal patients aged birth to 85 years were cultured and 50 (14.9%) samples presented bacterial growth and were included in this study. Each sample was isolated on three different transport media thus giving a study population of 150 specimens. Out of the total positive cultures, 30 (60%) isolates were from female patients while the rest 20 (40%) were from male patients. An association between sex of the patient and isolated pathogen species was sought but this was not statistically significant (P value = 0.103) for sex of patients.

The age distribution of the study population is illustrated in figure 1 below. More than a half of the population was less than 9 years of age. Those in the age group (10-19) and (30-39) had equal frequency while very few individual were ≥40 years of age. Again, there was no statistical difference between the two variables of age and frequency of infection [P<.103 (Pearson χ² = 18.430)].
Figure 1: Age distribution of the study population and frequency of infection

4.2 Stool contigency

Three types of stool were identified as shown in Table 1 below. The stool was recorded as mucoid, bloody or watery. Mucoid stool was most frequent (54%) followed by bloody and watery with 26% and 20% respectively.
Table 1. Stool characteristic and frequencies

<table>
<thead>
<tr>
<th>Stool Type</th>
<th>Frequency</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bloody</td>
<td>13</td>
<td>26</td>
</tr>
<tr>
<td>Mucoid</td>
<td>27</td>
<td>54</td>
</tr>
<tr>
<td>Watery</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>50</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>

4.3 Prevalence of the enteric bacterial pathogens isolated

Salmonella species was the most frequent enteric bacterial pathogen encountered and was found in 56 (37.3%) cultures while Shigella and Vibrio species had equal frequency of 19 (12.7%) as shown in table 2 below.

Table 2. Isolation rates of enteric bacterial pathogens

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Frequency</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>56</td>
<td>37.3</td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td>56</td>
<td>37.3</td>
</tr>
<tr>
<td><em>Shigella</em></td>
<td>19</td>
<td>12.7</td>
</tr>
<tr>
<td><em>Vibrio</em></td>
<td>19</td>
<td>12.7</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>150</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>
4.4 Transport media

Two transport media: Cary-Blair (CB) and Impregnated Filter Paper (IFP) were tested against a control comprising of a blank filter paper (not impregnated). The media were chosen on the standpoint that the categories “Gold standard”, new method and control were adequately represented.

4.5 Comparison of recovery rates of isolates by transport media

Recovery rates of enteric bacterial pathogens from transport media within the medium type is shown in figure 2 below. CB was superior as transport media for both *Salmonella* (52%) and *Shigella* (20%). IFP was the best media for isolation of *Vibrio* (16%). FP performed fairly well for isolation of *Vibrio* (12%) but poorest for both *Salmonella* (18%) and *Shigella* (4%).
There was a statistically significant relationship between species of enteric bacterial-pathogen isolated and transport media type used \([P<0.001 \ (\chi^2 = 31.252)]\).

### 4.6 Comparison of isolates by stool type

The relationship between recovered isolates and stool types is illustrated in figure 3. The chances of recovering species of enteric bacterial pathogens from bloody stool was highest for *Salmonella* (41%), followed by *Shigella* (23.1%) but was not detected for *Vibrio*. The likelihood of recovering isolates from mucoid stool was most common in *Salmonella* (43.2%), followed by *Shigella* (12.3%) but very rare
for *Vibrio* (3.7%). Watery stool was best for the recovery of *Vibrio* (53.3%), very unlikely for *Salmonella* (16.1%) but highly unlikely for *Shigella* (<10).

![Figure 3. Percentages of isolates recovered against types of stool](chart)

The relationship between type of stool and the enteric bacterial pathogens isolated was highly significant \([P<0.001\ (\text{Pearson } \chi^2 = 61.418)]\).

### 4.7 Antibiotic resistant profiles for the enteric bacterial pathogens isolated

The antibiotic resistant patterns of the isolates tested against seven antimicrobials is shown in figure 4 below. Among *Shigella* isolates, total resistance was noted to ampicillin (100%), tetracycline (100%) and high levels of resistance to co-trimoxazole (94.7%), cefotaxime (68.4%) and chloramphenical (68.4%). *Salmonella* isolates demonstrated total resistance to tetracycline, high levels of
resistance to ampicillin (75%), co-trimoxazole (73.2%) and moderate level of resistance to chloramphenical (50%). Otherwise, *Vibrio* isolates presented very low resistance rates varying from 52.6% for tetracycline to 26.3% for chloramphenical. Cefutaxime resistance was relatively low to both *Salmonella* and *Vibrio* isolates. All isolates were totally susceptible to ciprofloxacin (100%).

---

**Figure 4.** Antimicrobial resistance profiles of enteric bacterial isolates tested against seven antimicrobial drugs. Te-tetracyline; Amp-ampicillin; Sxt-co-trimoxazole; Cl-chloramphenicol; Ctx-cefotaxime; Gm-gentamicin; Cip-ciprofloxacin
4.8 Minimum inhibitory concentration profiles of isolates tested against four antibiotics

4.8.1 Minimum inhibitory concentration profiles of *Shigella* isolates

The relationship between *Shigella* isolates and their MICs for the four antibiotic tested is illustrated in figure 5 below. All the *Shigella* isolates demonstrated 100% resistance to all the four antibiotics tested: Ampicillin (the CLSI breakpoint MIC ≥ 8 µg/ml); Te (MIC ≥ 2 µg/ml) Cl (MIC ≥ 8 µg/ml) and Gm (MIC ≥ 1 µg/ml).

![Figure 5. Minimum inhibitory concentration of *Shigella* isolates to the four antibiotics tested. Amp-ampicillin; Cl-chloromphenicol; Te-tetracyline; Gm-gentamicin](image-url)
4.8.2 Minimum inhibitory concentration profiles of *Salmonella* isolates

The relationship between *Salmonella* isolates and their MICs for the four antibiotic tested is shown in figure 6 below. All the *Salmonella* isolates demonstrated total resistance to ampicillin, chloramphenicol gentamicine and tetracycline.

![Figure 6. Minimum inhibitory concentration of *Salmonella* isolates to the four antibiotics tested. Amp-ampicillin; Cl-chloromphenicol; Te-tetracyline; Gm-gentamicin](image-url)
4.8.3 Minimum inhibitory concentration profiles of *Vibrio cholerae* isolates

The relationship between *Vibrio* isolates and their MICs for the four antibiotic tested is illustrated in figure 7 below. All *Vibrio* isolates demonstrated total resistance to both ampicillin and tetracycline, 73% were resistant to chloramphenicol and 89.5% to gentamicine.

![Graph showing MIC concentrations of Vibrio isolates to four antibiotics: Ampicillin (Amp), Chloramphenicol (Cl), Tetracycline (Te), and Gentamicin (Gm). The x-axis represents MIC concentrations in mg/ml, ranging from 1 to 256, and the y-axis represents % of isolates with resistance levels at various concentrations.]

Figure 7. Minimum inhibitory concentration of *Vibrio* isolates to the four antibiotics tested. Amp-ampicillin; Cl-chloromphenicol; Te-tetracyline; Gm-gentamicin
4.9 The antimicrobial resistant types (Resistypes) for the enteric bacterial pathogens isolated

The association between antimicrobial resistype for the isolates tested is as shown in table 3 below. Multidrug resistant (MDR) was observed in 17 (18.1%) of the isolates. The highest frequency of MDR was noted in *Salmonella* 14(25%), followed by *Vibrio* 2(10.5%) and *Shigella* 1(5.3%). All the 17 MDR isolates were subjected to conjugation experiments to determine whether they were plasmid-mediated. A total of 6 resistypes were observed. The most common resistypes were ampicillin, co-trimoxazole, tetracycline and ampicillin, chloramphenicol, co-trimoxazole, tetracycline with a frequency of 5. Ampicillin, co-trimoxazole, tetracycline was noted in all species as follows: 3 *Salmonella*, 1 *Shigella* and 1 *Vibrio*. Apart from ampicillin, tetracycline which occurred in both *Salmonella* and *Vibrios* with equal frequency 1 (1.2%), the rest were confined to *Salmonella* with tetracycline observed in only 1 (1.2%) isolate.
### Table 3. Antibiotic resistant type of the enteric bacterial pathogens isolated

<table>
<thead>
<tr>
<th>Resistant type</th>
<th><em>Salmonella</em> n=56</th>
<th><em>Shigella</em> n=19</th>
<th><em>Vibrio</em> n=19</th>
<th>Total n=94</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>42</td>
<td>18</td>
<td>17</td>
<td>77 (81.9%)</td>
</tr>
<tr>
<td>Amp, sxt, te</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>5 (5.3%)</td>
</tr>
<tr>
<td>Amp, c, sxt, te</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>5 (5.3%)</td>
</tr>
<tr>
<td>Amp, c, te</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2 (2.1%)</td>
</tr>
<tr>
<td>Amp, te</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2 (2.1%)</td>
</tr>
<tr>
<td>C, te</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2 (2.1%)</td>
</tr>
<tr>
<td>Te</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1 (1.2%)</td>
</tr>
</tbody>
</table>

Total 14 (25%) 1 (5.1%) 2 (10.5%) 17 (18.1%)

Amp-Ampicillin Sxt-Co-trimoxazole C-Ciprofloxacin Te-Tetracycline

### 4.10 Plasmid profiles

Plasmid sizes ranging from 7MDa to 194 MDa were identified and their frequencies are shown in table 4 below. Plasmids were extracted and analysed from 27 (28.7%) of the 94 isolates. Plasmid size 10 MDa occurred most frequently on overall 9 (9.6%). Plasmid size 7 MDa and 54 MDa occurred with equal frequency of 8 (8.5%). Plasmid size 195 MDa occurred only in *Vibrio* and once. In some cases two plasmid sizes were observed in the same isolate. Plasmid size 58 MDa accompanied with 10 MDa were observed in all the three species while 47 MDa accompanied by 10 MDa appeared in both *Salmonella* and *Shigella* with equal frequency. Plasmid sizes 140 MDa and 47 MDa occurred in both *Salmonella*
and *Vibrio* with equal frequency whereas plasmid size 30 MDa accompanied by 7 MDa occurred only in *Salmonella* and once.

Table 4. Plasmid sizes in MDa and frequencies of occurrence

<table>
<thead>
<tr>
<th>Plasmid size (MDa)</th>
<th><em>Salmonella</em> n=56</th>
<th><em>Shigella</em> n=19</th>
<th><em>Vibrio</em> n=19</th>
<th>Total n=94</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. plasmid</td>
<td>25</td>
<td>13</td>
<td>12</td>
<td>50 (53.0%)</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>8 (8.5%)</td>
</tr>
<tr>
<td>10</td>
<td>8</td>
<td>0</td>
<td>1</td>
<td>9 (9.6%)</td>
</tr>
<tr>
<td>30</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>3 (3.2%)</td>
</tr>
<tr>
<td>30, 7</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1 (1.1%)</td>
</tr>
<tr>
<td>47</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1 (1.1%)</td>
</tr>
<tr>
<td>47, 10</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2 (2.1%)</td>
</tr>
<tr>
<td>49</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2 (2.1%)</td>
</tr>
<tr>
<td>54</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td>8 (8.5%)</td>
</tr>
<tr>
<td>58</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2 (2.1%)</td>
</tr>
<tr>
<td>58, 10</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>5 (5.4%)</td>
</tr>
<tr>
<td>140, 47</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2 (2.1%)</td>
</tr>
<tr>
<td>195</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1 (1.1%)</td>
</tr>
</tbody>
</table>
4.11 Transfer of r-factors (resistant genes)

Plasmid sizes ranging from 7 MDa to 47 MDa of the transconjugants and their frequencies are shown in table 5 below. Fifteen isolates that were MDR were subjected to conjugation experiment. Several resistotype were successfully transferred. Amp, sxt, te and amp, te were both transferred by a 10 MDa plasmid in *Salmonella* and *Vibrio* respectively. Te was transferred by a 30 MDa plasmid while amp, c, sxt, te was transferred by a 47 MDa alongside a 7 MDa; both observed only in *Salmonella*.

Table 5. Plasmid sizes of the transconjugants

<table>
<thead>
<tr>
<th>Transconjugant in (MDa)</th>
<th><em>Salmonella</em> n=56</th>
<th><em>Shigella</em> n=19</th>
<th><em>Vibrio</em> n=19</th>
<th>Total n=94</th>
</tr>
</thead>
<tbody>
<tr>
<td>No transconjugants</td>
<td>44</td>
<td>18</td>
<td>17</td>
<td>79(84.0%)</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td>10(10.6%)</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1(1.1%)</td>
</tr>
<tr>
<td>30</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1(1.1%)</td>
</tr>
<tr>
<td>32, 7</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2(2.1%)</td>
</tr>
<tr>
<td>47, 7</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1(1.1%)</td>
</tr>
</tbody>
</table>
CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

Cary-Blair transport medium is the gold standard in the transportation of stool samples to laboratories for the isolation and identification of etiologic agents of diarrhoea. This medium is both expensive and unavailable in most resource poor countries. In addition, where it is available, there is usually poor recovery rates of etiologic agents due to non-viability of the causative bacteria in this medium. This has consequently led to poor diagnosis with no bacteria being detected at all, resulting in overall unchecked spread of morbidity and mortality. The dried filter paper method for sending diarrhoeal specimens to a laboratory for bacteriologic examination was developed by Lie Kian Joe in 1948 at the laboratory of Tropical Hygiene of Leyden. The method was based on the observation of Dold and Ketterer (1943) that *Shigella* may survive from one to two months in faecal material dried on filter paper.

In this study, the method was modified by impregnating steam sterilized Whatman filter paper with 0.1% glucose. This was based on the fact that enteric bacterial pathogens are able to utilise glucose as a source of energy and a supply of carbon and other elements required for cell structure and function. Freshly collected stool sample was dropped on the developed media, left to air dry at room temperature and placed in a plastic pouch to help maintain the sample as near its original state
as possible with minimum deterioration. This greatly simplified method, lacking in sophisticated instrumentation should produce acceptable results for surveying the status and identification of diarrhoeal diseases in geographical areas deficient in laboratory facilities.

The results showed that impregnated dry filter paper can successfully be used to transport all the three enteric bacterial pathogens with varying isolation rates. That the most effective media in this study for isolation of *Vibrio* is impregnated dry filter paper. These results are substantial improvement on results of Joe (1950) who isolated both *Shigella* and *Salmonella* from non-impregnated filter paper but did not detect *Vibrios*. However, for increased recovery of enteric bacterial pathogens, especially *Vibrio* from suspected stool specimens, the inoculated impregnated dry filter paper must not be completely dried. *Vibrios* are known to survive for a longer period in moist condition. In addition, other microorganisms are known to overgrow *Vibrios* in other media (WHO, 1999) hence, hinder their recovery. That *Vibrios* were effectively recovered from impregnated dry filter paper may only demonstrate how these microorganisms are likely to be inhibited in the developed medium. While it is not known for certain what inhibited the growth of these microorganisms in impregnated dry filter paper, it can be argued that the absence of other ingredients such as protein hydrolysates and other carbohydrates probably minimized the survival of those pathogens which utilize them as sources of energy and supply of other essential elements giving a higher
chance for Vibrios to survive.

The recovery of both Shigella and Salmonella from impregnated dry filter paper was almost similar. Of much interest is Shigella, which is known to be fastidious and therefore die easily during transport (Niyogi, 2005). Moreso, Shigella are found in low numbers in diarrhoeal stool specimen (Mayor, 2002). The results demonstrate that Shigella were viable in the developed medium which may be attributed to the 0.1% glucose added to the filter paper and proper handling of specimens to minimize adverse conditions that would otherwise deleteriously affect their survival. On the other hand, Salmonella does not pose a lot of challenge during transportation. Salmonella are robust organisms quite competent to survive and remains detectable under almost any circumstances (Welton et al., 1975).

Cary-Blair transport medium is the preferred medium for transporting many bacterial pathogens, including Shigella, Salmonella and Vibrio cholerae (WHO, 2003), which was confirmed in this study. Cary-Blair was the most effective medium in the recovery of both Salmonella and Shigella but poorest in recovery of Vibrios. Since Cary-Blair is a medium of choice for most diarrhoeal pathogens, it can be argued that, other organisms could have possibly overgrown Vibrios hence the poor performance. This may be supported by the fact that unimpregnated filter paper was able to recover higher number of Vibrio isolates as opposed to the gold
standard. This study was limited to the isolation and identification of only enteric bacterial pathogens and therefore we could not establish the existence of multiple infections.

Whereas Cary-Blair remained superior in isolation of both *Salmonella* and *Shigella*, impregnated dry filter paper was best in the recovery of *Vibrios*. The recovery rates of both *Salmonella* and *Shigella* from the two media were nearly the same. The relationship between the recovery rates of the isolates from the two transport media was not statistically significant. This means that impregnated dry filter paper is as good as Cary-Blair in the recovery of these enteric bacterial pathogens. However, the choice of the media to be used will depend on the cost and availability. Cost-effective and more readily available medium would be more appropriate in remote and impoverished areas without immediate accessability to laboratory facilities.

Furthermore, in situations where all the two media are present, it would be advisable to use both for the purpose of increasing the recovery rates of the suspected enteric bacterial pathogens and in particular, strengthening the chances of recovering *Vibrios*. Indeed, Mutwewingabo *et al.*, (1984) showed that when more than two media are used to transport stool specimens, the recovery rates are likely to be higher. The findings of the present study will go a long way in improving the level of accurate diagnosis and/or help to check the spread of these
diseases in outbreak areas. Advantages of impregnated dry filter paper over Cary-Blair include: inexpensive, easily available and can be prepared with minimum laboratory equipment. In addition, impregnated dry filter paper does not require refrigeration, which is nonexistent in most outbreak areas, nor is it likely to spill like Cary-Blair and thus pose greater risks to unsuspecting individuals during transportation.

In developing countries, diarrhoea continues to be an important cause of morbidity and mortality especially among children mainly due to poor socio-economic factors and sanitary conditions (Guerrant et al., 1990; Kariuki et al., 2006). The isolation rate of *Shigella* from 12.7% of outpatients with diarrhoea in this study was higher than the 7.7% isolation rate from Djibouti (Mikhail et al., 1990) and 7.1% to 11% from Addis Ababa, Ethiopia (Mache et al., 1997). The isolation rate of *Salmonella* from 37.3% and *Vibrio cholerae* from 12.7% of the patients in this study were far higher than the 7.3% and 0.5% respectively from Malindi (Saidi et al., 1997) and 2% and < 1% respectively from Asembo Bay (Brooks et al., 2003). This higher prevalence of enteric bacterial pathogens in Budalang’i may reflect their endemicity in the study area. Since the samples were obtained from patients staying in camps, these results may also help to emphasize the importance of enteric bacterial pathogens as potential causes of diarrhoeal disease in overcrowded and unhygienic conditions (Totaro et al., 2004; Lee et al., 2003).
It is generally agreed that in suspected bacterial enteric diseases, the specimens of choice is a freshly passed stool (WHO, 2003). Rectal swabs may also be used, provided sample collection is done properly. However, as this study shows, the type of stool is likely to have a bearing on the type of isolate recovered. Comparison of isolates by stool type revealed that *Salmonella* was most likely to be recovered from mucoid stool as opposed to bloody or watery stool. This is in agreement with clinical presentation of gastroenteritis caused by *Salmonella*. The recovery of some *Salmonella* isolates from bloody stool types in this study may suggest either classical cases of typhoid fever or mixed infections in the study area. However, definitive identification of the causative strain of typhoid fever and/or determination of multiple infections is important for conclusive statement to be made. The current study however did not address this.

Generally, enteritis caused by *Shigella* species tend to vary in severity, with the diarrhoea ranging from watery to dysenteric (bloody) (WHO, 1999). In this study, *Shigella* was isolated with almost equal frequencies from either mucoid or bloody but not detected from watery stool. This may signify high prevalence rate of both *S. dysenteriae* Type 1 and *S. flexneri* in the study area. These are the strains of *Shigella* normally associated with bloody diarrhoea and commonly found in developing countries (Subekti *et al.*, 2001; WHO, 2003; Navia *et al.*, 1999). Both *S. boydii* and *S. sonnei* are known to cause infections that result in watery diarrhoea (Keusch and Bennish, 1989).
Watery stool was best for the recovery of *Vibrios*, very unlikely for *Salmonella* but highly unlikely for *Shigella*. These results reinforce observation that watery stool is usually associated with *V. cholerae* which produces a powerful enterotoxin that affect the bowel mucosa in such away that there is profuse outpouring of fluids (Mugoye *et al.*, 2008; Gaffga *et al.*, 2007). The relationship between stool type and the isolates recovered was highly significant. In this regard, the type of stool may act as a presumptive diagnosis where laboratory facility is unavailable a waiting definitive identification of the causative pathogen.

Antimicrobial resistance in bacterial diarrhoea is a significant public health problem throughout the developing world (Opintan *et al.*, 2008; WHO, 2001). The findings of the current study that more than 75% of all pathogens were not susceptible to locally available antibiotics demonstrates the magnitude of the problem. Resistance to commonly used antimicrobial agents among enteric bacterial pathogens has been reported worldwide (Niyogi, 2005; Ashkenazi *et al.*, 2003; Egah *et al.*, 2003; Maraki *et al.*, 2003; MoezArdalan *et al.*, 2003). Apart from increasing the cost of treating diarrhoeal illness, especially relative to per capita income in Kenya, ineffective treatment is likely to contribute significantly to the development of further resistance of both enteric and non-enteric bacterial pathogens (Shapiro *et al.*, 2001). This will tremendously limit the treatment options available for potentially fatal epidemics of *Shigella dysenteriae* Type 1 and *Vibrio cholerae* 01 particularly in the developing world (Malakooti *et al.*, 1997).
No observation was made on resistance to ciprofloxacin and minimal resistance to cefotaxime. Other studies have also found little resistance to cefotaxime and/or no resistance to ciprofloxacin among enteric bacterial pathogens in East Africa (Legros et al., 1998; Materu et al., 1997) and in other parts of the world (Iwalokun et al., 2001; Pitman et al., 1996; Aarestrup et al., 2003). Ciprofloxacin resistance among enteric bacteria was not seen in these isolates and therefore its resistance remains rare. However chromosomally acquired ciprofloxacin resistance in \textit{S. typhi} and \textit{S. paratyphi} has been recently described in various parts of Asia, possibly related to the widespread and indiscriminate use of quinolones (Renuka et al., 2005; Shirakawa et al., 2006). Therefore, progressive resistance to both ciprofloxacin and cefotaxime may cause concern. Close continued monitoring of resistance rates to all these drugs among enteric bacteria is warranted.

Therapeutic options for enteric bacteria in developing countries such as Kenya are extremely limited (Kariuki et al., 2005). No widely available antimicrobial agent is useful for treating these illnesses and therefore, the increasing prevalence of enteric bacterial pathogens resistance to commonly prescribed antimicrobials, especially in developing countries poses a major concern in the management of these infections (Kariuki and Hart, 1997). In the current study, observation of high drug minimum inhibitory concentrations in almost all isolates to locally available and most frequently prescribed antibiotics suggests that these drugs may have no reasonable role in the empiric treatment of these infections. In many health centers
in sub-Saharan Africa, the repertoire of first-line drugs is limited to ampicillin, chloramphenicol, erythromycin, gentamicin, penicillin, tetracycline and trimethoprim-sulphathoxazole. The available second line antibiotics vary with locality but will often include amikacin, amoxicillin-clavulanic acid, cefuroxime, ciprofloxacin and nalidixic acid (Fasehun, 1999; Hart and Kariuki, 1998).

It is particularly disconcerting to note that a number of countries do not have a broad enough selection of second-line drugs and so would have difficulty managing resistant infections (Fasehun, 1999). Even when not available for individualized patient care, surveillance susceptibility testing remains a valuable tool for cost-effective customization of empiric antibiotic therapy. The cost of resistance is difficult to compute and generally underestimated. More serious and more difficult to quantify are the costs arising from mortality. These devastating consequences of resistance are seen increasingly more frequently during outbreaks of life-threatening diseases such as cholera and dysentery (Malakooti et al., 1997).

Multidrug resistance in enteric bacterial pathogens has substantial public health and economic impact. In the present study, Salmonella isolates had the highest frequency of multidrug resistance. Other studies have highlighted broadening of antibiotic resistance spectra in Salmonella species (Glynn, 1998; Wolfgang et al., 2001; Prager et al., 1999). Shigella and Vibrio isolates resistant to multiple agents have also been reported (Replogle et al., 2000; Lee et al., 2001; McLver et al.,
Although different resistance patterns are observed at different locations, resistance to trimethoprim-sulfamethoxazole, ampicillin, and tetracycline has been observed worldwide (Kariuki et al., 2006; Kelly and Farthing, 1997; Sack et al., 1997). In the present study, six (6) resistype were determined with the most frequent isolated resistype being ampicillin, chloramphenicol, cotrimoxazole and tetracycline thus confirming the above findings. It is difficult to ascertain the selective pressures for maintenance of the different resistance patterns.

Bacterial strains that are resistant to antimicrobial can produce enzymes that inactivate the drug, are impermeable to it, actively export it from the cell or bypass the cellular target the agent interferes with. Bacteria acquire the ability to do this by altering their own genes (through mutation) or, more commonly, by horizontally acquiring ‘ready made’ resistance genes from other organisms. Horizontally acquired resistance genes are often carried on transmissible plasmids, small rings of DNA that are efficiently transferred from one cell to another by mating or conjugation. The present study demonstrated the presence of plasmids in these enteric bacteria with plasmid size 10 MDa occurring most frequently on overall and in Salmonella but not in Shigella. Most of the plasmids observed were of the small sizes ranging from 7 MDa to 195 MDa. In some Salmonella and Shigella isolates large size plasmid appeared accompanied with a smaller size.
The ability of bacteria to acquire and disseminate exogenous genes via mobile genetic elements has been the major factor in the development of multidrug resistant strains (Rowe-Magnus et al., 2002). The use of antibiotics and the transmission of resistance plasmids are factors that affect plasmid profiles (Hadfield and Monson, 1995). This study, through genetic mating experiments, further demonstrated the organisms' ability to transfer plasmids and subsequently resistance to *E. coli* K-12 (F NA\(^+\)). Small molecular weight plasmids of approximately 7 MDa, 10 MDa, 30 MDa and 47 MDa were transferred in the conjugation experiments thus implicating them in multidrug resistance.

This research has also shown that both *Shigella* and *Vibrio* species have the capacity to acquire plasmid-mediated resistance to multiple antibiotics, which previously have been used successfully against them. Mobile genetic units are important in the spread of resistant determinants among *Shigella* isolates (Toro et al., 2005). Trimethoprim and sulfamethoxazole resistance is most commonly acquired through a plasmid-encoded variant of the dihydrofolate reductase enzyme (Heikkila et al., 1990). Ampicillin resistance arises as a result of beta-lactamases similar to TEM-1 or OXA-1, whose genes may be located on chromosomes, plasmids, or transposons (Navia. et al., 1999). Such resistance coupled with rapid transmission from person to person and their high attendant mortality rates make these enteric bacteria formidable pathogens even in this era of antibiotics. Other
genetics studies have shown that resistance in *V. cholerae* is encoded on an H 11 incompatibility plasmid and is transferable (Shanahana *et al.*, 1998).

Lack of microbiological laboratories to perform routine analyses is one of the main factors that have been documented to favour the development of bacterial resistance to antibiotics in developing countries (Gason *et al.*, 2000). Most clinicians in the third world countries usually have no information about either the etiology of the infectious diseases or antimicrobial susceptibility which are both essential for clinical practice. Consequently, bacterial infections are often treated empirically with broad-spectrum antibiotics. Specific and periodic studies regarding the etiology and antimicrobial susceptibility of some important infectious disease agents would help clinicians decide on which antibiotics to choose to treat such infections. Moreso, selection of effective antimicrobial agents, when indicated as part of optimal case management, could be improved by expanding laboratory-based antimicrobial susceptibility testing at selected sentinel sites. This can only be possible where a cost-effective, readily available and more reliable transport medium is.

Further to providing selection pressure, humans have encouraged resistance among bacteria by creating conditions suitable for bacterial multiplication and the exchange of genetic material. Warm, moist and unclean environments are conducive for the spread of pathogens, but they also encourage the spread of
resistant organisms that may not be pathogenic. These organisms often carry resistance genes that can be spread to pathogens and therefore constitute a hidden reservoir of antibiotic resistance. Surveys of healthy people have shown that they often carry large proportions of resistant *Escherichia coli* in their gastro-intestinal tracts (Okeke *et al.*, 2000; Iwalokun *et al.*, 2001). The rising prevalence of resistance in these organisms over time has been correlated to resistance in clinical *Salmonella* or *Shigella* enteritis in the same environment. Furthermore, hygiene, sanitation and infection control programs help to prevent the spread of resistant agents, reducing the need for antibiotics in the first place.

5.2 Conclusions

i. Impregnated dry filter paper is as good as Cary-Blair when used as transport media for recovery and diagnosis of enteric bacteria usually implicated in diarrhoea.

ii. There was an association between the type of isolate and the type of media used and this association resulted in increased recovery rates.

iii. There was an association between the stool type and the isolates which can be utilized to increase the recovery rates of isolates and/or act as a presumptive diagnosis where laboratory facility is unavailable.

iv. Most isolates were resistant to locally available antibiotics and this resistance was transferable.
5.3 Recommendations

i. Impregnated dry filter paper may be used to transport stool samples suspected for enteric bacterial pathogens in impoverished areas in absence of conventional media and/or with conventional media to increase the recovery rates of enteric bacterial pathogens.

ii. A more elaborate study is required to compare recovery rates of enteric bacteria pathogens from these media at serotype level.

iii. The time taken for an enteric pathogen to survive on this new media before isolation is done needs to be investigated.

iv. Other than enteric bacteria, other pathogens that could be transported by these new media need to be investigated.

v. Further studies on the antibiotic resistance mechanism and genetic relatedness of enteric bacterial pathogens are required to understand the progression of antibiotic resistance.


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PROTOCOLS FOR MEDIA AND REAGENTS PREPARATION

Quality control
This included the selection of satisfactory raw materials, the preparation of media according to approved formulation by the manufacturers and the use of well characterised (reference) strains to check the growth-supporting capabilities of the prepared media.

Media and reagents

- Sterile petri-dishes (3 ml petri-dishes per 100 ml) were used.
- Media were prepared according to the manufacturers' instructions.
- Dehydrated formulation were used to avoid lot-to-lot variations.
- Biotyping media were distributed in 5 ml volumes in suitable tubes (13 x 100 mm to 15 x 125mm).
- All test tubes containing media were autoclaved with their caps loosened, thereafter, were tightened, dated/labeled and stored in a cool place (4°C) before use. This was to maintain stability and avoid drying out.
- Media (agar) was poured in sterile petri-dishes to a uniform depth of 4mm on a flat surface in an area with minimum amount of traffic and air contents.
- Media was allowed to solidify with covers of plates partially removed. The lids were then replaced, the plates put in plastic bags, sealed, labeled/dated and stored in an inverted position in a refrigerator at 4°C before use. This
was to prevent rapid dehydration and extend the shelf life of the medium.

- Autoclaving was done at 121°C for 15 minutes.

**Transport media**

**Formula 1**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polypeptone peptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Yeast extracts</td>
<td>2 g</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>1 g</td>
</tr>
<tr>
<td>Sodium Citrate</td>
<td>20 g</td>
</tr>
<tr>
<td>Sodium Desoxycholate</td>
<td>5 g</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>3 g</td>
</tr>
</tbody>
</table>

Made to 1 litre with distilled water.

pH 7.0

**Formula 2**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextrose</td>
<td>0.1 %</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1 litre</td>
</tr>
</tbody>
</table>

**Cary Blair Medium**

Dehydrated formulations was used (133g in 1 litre) and dissolved in appropriate amount of distilled water while heating in a boiling water bath. The medium was allowed to cool to 50°C. Four and a half (4.5) ml of freshly prepared calcium chloride (CaCl₂) was added to it and well mixed. The pH of the medium was adjusted to 8.4 using 0.1 mol/L Sodium hydroxide (NaOH). Seven (7) ml was
dispensed using pipette in screw cap bottles (Bijou bottles 13 x 100 mm). The medium was sterilised by steaming at 100°C for 15 minutes.

**Enrichment broth**

**Selenite-F-broth (SEL).**

This medium was used as an enrichment for *Salmonellae* species

With care, the dry ingredients were mixed thoroughly in water in the order given and heated to 70-80°C to dissolve (placed the flask in a container of hot water). The pH was adjusted to 7.0 by phosphates. 10 ml was distributed by pippeting in screw cap universal type bottles and sterilised by steaming at 100°C for 20 minutes.

**Alkaline peptone water (APW)**

The ingredients (20g) were weighed and added to the water and the pH adjusted to 9.0-9.2 with concentrated sodium hydroxide (NaOH) solution. Five (5) ml was dispensed using a pipette into screw cap universal type bottles and then autoclaved.

**Normal saline**

8.5g were dissolved in 1 litre of distilled water. 5 ml was dispensed using a pipette into screw caps universal type bottles then autoclaved.

**Primary media**

**MacConkey Agar (MAC)**

MAC agar is a differential and low selectivity medium, which was used to distinguish lactose fermenters from non-lactose fermenting (NLF) bacteria. *Shigella* species on MAC appears as convex, colourless colonies about 2-3 mm in
diameter. *S. dysenteriae* Type 1 colonies may be smaller. *S. typhi* colonies are flat, colourless and usually 2-3 mm in diameter.

**Preparation:** 52 gm was weighed and dissolved in 1 litre of deionised water in a conical flask, heated for about 10 minutes while swirling to mix well. The medium was autoclaved, left to cool to about 50°C, mixed well and poured into sterile petri-dishes. The lids were left ajar for about 20 minutes to allow the surface of the agar to dry.

**Quality control**

*E. coli* produce pink to red colonies with excellent growth

**Salmonella Shigella (SS) Agar**

SS agar is a highly selective medium for isolation of *Salmonellae* and *Shigella* but not *S. dysenteriae* Type 1 because some strains are inhibited. *S. typhi* produces smooth, colourless, transparent or translucent colonies that may have black centres indicating Hydrogen Sulphide (H2S) production.

**Preparation:** 63gm was weighed and dissolved in 1 litre of distilled water, mixed well by shaking and heated with great care to boiling with frequent agitation to obtain complete solution. The medium was cooled to 50°C-55°C, mixed well and then dispensed in sterile petri-dishes.

**Quality control**

*Salmonella* produce good growth, colourless colonies with or without black centres.

*E. coli* grow poorly and appear as pink colonies.
Xylose Lysine Desoxycholate Agar (XLD)

XLD is a selective differential medium suitable for isolation of *Shigella* and *Salmonella* from stool specimens. Differentiation is accomplished by xylose and lactose fermentation, Lysine decarboxylation and hydrogen sulfide production. *Shigellae* species appear as transparent, pink or red smooth colonies 1-2 mm in diameter. *S. dysenteriae* Type 1 colonies are frequently very tiny, unlike other *Shigella* species. *Salmonella* species are usually red (delayed lysine decarboxylation) with black centres but can also be yellow (xylose utilisation) with black centres.

**Preparation:** 53 gm was weighed and mixed thoroughly in 1 litre of distilled water. The medium was heated with agitation just until the medium boils. Care was taken not to over-heat nor allow the medium to cool too long as these would cause precipitation of the medium. The flask was cooled under running water until just cool enough to pour. It was again well-mixed and dispensed aseptically into sterile petri-dishes while leaving the lids ajar.

**Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS)**

TCBS is a selective medium used to isolate *V. cholerae* from faecal specimens.

Bromthymol blue (0.2% solution) 20.0 ml

Thymol blue (1% solution) 4.0 ml

Bromthymol blue solution: Add 2.5 ml of 0.1N NaOH to 47.5 ml water, and then add 0.1g of indicator.

Thymol solution: Add 2.2 ml of 0.1 N NaOH to 7.8 ml of water, and then add 0.1g
of thymol blue. The suspension was completely dissolved. The agar was cooled in a water bath until cool enough to pour (50-55°C); then poured into sterile petri-dishes while the lids were left ajar for about 20 minutes so that the surface of the agar could dry.

**Quality control**

*V. cholerae* 01 show good growth of yellow colonies. *E. coli* have none to poor growth of translucent colonies.

**Biotyping media**

**Triple Sugar Iron (TSI) Agar**

**Principle:** TSI is a carbohydrate containing screening medium used to differentiate lactose fermenters from non-lactose fermenters and contains H$_2$S indicator. H$_2$S producing organisms cause the blackening of the medium in TSI. TSI contain glucose, lactose and sucrose. Organisms that ferment either lactose or sucrose produce an acid (yellow) slant while organism that ferment neither carbohydrate will have an alkaline (red) slant. Glucose fermenters produce an acid (yellow) reaction in the butt (sometimes with gas production).

**Preparation:** 49g of the powder was weighed and dispensed in 1 litre of deionised water, heated to bring to boil with frequent swirling to dissolve and then 5 ml of the medium dispensed in screw-capped 15 x 150 mm tubes. The screw caps were left loose, the medium autoclaved thereafter, the slants were allowed to solidify in a manner such that the medium in the butt of the tube was about 2.5 cm and the slant was about 2.5 cm long.
Quality control

*E. coli* give an acid slant and butt with production of gas but no \( \text{H}_2\text{S} \).

*S. flexneri* give an alkaline slant, acid butt without gas or \( \text{H}_2\text{S} \).

\( \text{H}_2\text{S} \) producing *Salmonella* may be used to control the reaction.

**Sulfide Indole Motility (SIM)**

SIM combines three tests in a single test tube: Sulphide production, Indole production and motility.

**Principle:** Indole test is based on the formation of a red colour complex when indole reacts with the aldehyde group of \( \text{P-dimethylaminobenzaldehyde} \), the active chemical in Kovac’s reagent.

**Kovac’s Indole reagent content.**

- Pure Amyl or Isoamyl alcohol or Butyl alcohol \( 150.0 \text{ ml} \)
- \( \text{P-dimethylaminobenzaldehyde} \) (CR) \( 10.0 \text{g} \)
- Concentrated \( \text{HCl} \) \( 50 \text{ ml} \)

**Preparation:** 30g of dehydrated medium was weighed and suspended in 1 litre of distilled water. The medium was boiled to dissolve completely. Five (5) ml volumes were dispensed into screw-capped tubes and then autoclaved.

**Preparation of Kovac’s Indole reagent**

The aldehyde was dissolved in the alcohol and the acid slowly added. The reagent was stored in a dark brown glass stoppered bottle in a refrigerator.

**Quality control**

*E. coli* is Indole positive, \( \text{H}_2\text{S} \) negative and motility positive. \( \text{H}_2\text{S} \) producing
Salmonella strain was used to control the H$_2$S reaction, likely to be motile and indole negative. Shigella are both motile and H$_2$S negative but are variable for the indole reaction.

**Simmon's Citrate agar**

**Principle:** Sodium citrate is a salt of citric acid, a simple organic compound found as one of the metabolites in Tri-Carboxylic Acid (TCA) cycle. Some bacteria obtain energy by utilising citrate as the sole source of carbon. Any medium used to detect citrate utilisation by test bacteria must be devoid of protein and carbohydrate as a source of carbon. Citrate utilisation is detected in citrate medium by the production of alkaline by-products. The medium includes; sodium citrate anion, as the source of carbon and ammonium phosphate as the sole source of nitrogen. Bacteria that can utilise citrate also can extract nitrogen from the ammonium salt with production of ammonia (NH$_3$), leading to alkalinization of the medium from the conversion of the NH$_3$ to Ammonium hydroxide (NH$_4$OH). Bromthymol blue which appears yellow below pH 6.0 and blue pH 7.6 is the indicator.

**Preparation:** 23g of dehydrated agar was dissolved in 1000ml of distilled water and then boiled to dissolve completely. 2ml was dispensed in test tubes, sterilized by autoclaving and then set to cool in a slanting position.

**Positive control**- *K. pneumoniae* ATCC 13883

**Negative control**- *E.coli* ATCC 25922
Methyl Red Voges Proskauer broth (MRVP)

**Principle:** Methyl red is a pH indicator with a range of both 6.0 (yellow) and 4.4 (red). The pH at which methyl red detects acid is considerably lower than the pH for which other indicators used in bacteriologic culture media do. For a colour change to appear, the test organism must produce large quantities of acid from the carbohydrate substrate being used. Methyl red test is a quantitative test for acid production, requiring positive organisms to produce strong acids (lactic, acetic, formic) from glucose through the mixed acid fermentation pathway.

**Preparation of methyl red pH indicator**

Methyl red 0.1g in 300ml of 95% ethyl alcohol

Distilled water 200ml.

**Preparation:** 17g was dissolved in 1 litre of distilled/deionised water, dispensed in screw cap tubes and then sterilised by autoclaving.

**Urease Test**

**Principle:** Urea is a diamide of carbonic acid. All amides are easily hydrolysed with the release of NH$_3$ and carbon dioxide CO$_2$. Urease is an enzyme possessed by many species of micro-organisms that can hydrolyse urea to produce carbon dioxide, water and ammonia. The NH$_3$ reacts in solution to form ammonium carbonate (NH$_4$)$_2$CO$_3$, resulting in alkalinization and an increase in the pH of the medium.

**Preparation:** 2.4g were suspended in 95ml of distilled water. The solution was brought to the boil to dissolve completely, sterilised by autoclaving and cooled to
50°C. Five (5) ml of sterile 40% urea solution (SR 20) was introduced aseptically (Urea is heat labile), mixed well and then 10 ml amounts distributed into sterile screw-capped tubes.

Positive control: *Proteus* species

Negative control: *E. coli*

Mueller-Hinton (MH) Agar

MH agar is the NCCLS-recommended medium used for standardised antimicrobial susceptibility testing of certain bacteria.

Preparation: 38g of powder was weighed and dispensed in 1 litre of deionised water. The solution was allowed to soak for 10 minutes, swirled to mix completely and then sterilised by autoclaving. After cooling to 50°C, 20 ml per plate was measured and dispensed into 15 x 100 mm sterilised petri-dish. The agar was poured into flat-bottomed glass or plastic petri-dish on a level-pouring surface to a uniform depth of 4mm. Agar deeper than 4mm are likely to cause fake resistance results, whereas, agar less than 4mm deep may be associated with a false susceptibility report. Do not leave the lids ajar because the medium is easily contaminated.

Quality control

*E. coli* ATCC 25922 standard strain for antimicrobial susceptibility testing was used for each new lot prepared. pH of each new lot of MH should be between 7.2 and 7.4; if not the batch should be discarded.
Brain Heart Infusion

Preparation: 37g were dissolved in 1000 litre of distilled water, mixed well, distributed into the final containers and sterilized by autoclaving.

Negative control: Uninoculated media

API 2OE

Formula

IDA – Ferric chloride in water

VPI reagents 40 g of potassium hydroxide in 100 ml of H₂O.

VP2 reagents - 6 g of (-naphthanol in 100 ml of ethanol).

0.5M McFarland Turbidity Standard

A half (0.5) ml of 0.048M BaCl₂ 1.175% w/v BaCl₂ 2H₂O) was added to 99.5 ml of 0.36 (NH₃)₂SO₄ (1% v/v). The resulting solution was mixed using a magnetic stirrer and 5ml of the standard distributed in screw capped tubes of the same size as those used in growing the broth culture. The tubes were sealed to prevent loss by evaporation and stored in the dark at room temperature. The turbidity standard was vigorously agitated on a mechanical vortex mixer just before use.
## APPENDIX II

### THE REACTION OF ENTEROBACTERIACEAE, AEROMONS AND PLESIOMONES (KREIG AND HOLT, 1984)

<table>
<thead>
<tr>
<th>Organism</th>
<th>Slant/butt</th>
<th>Gas</th>
<th>H₂S</th>
<th>MOT</th>
<th>IND</th>
<th>M.R</th>
<th>V.P</th>
<th>S.C</th>
<th>URE</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia</em></td>
<td>d/+</td>
<td>+/-</td>
<td>-</td>
<td>d</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Citrobacter freundii</em></td>
<td>d/+</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>d</td>
</tr>
<tr>
<td><em>C. diversus</em></td>
<td>+/-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>C. amalonaticus</em></td>
<td>+/-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Edwardsiella tarda</em></td>
<td>+/-</td>
<td>+</td>
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<td>+</td>
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<td>d</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td><em>S. Abortusovis</em></td>
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<td>-</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>d</td>
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<td>+</td>
<td>d</td>
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<td>+</td>
<td>+</td>
<td>d</td>
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<tr>
<td><em>Pleiosmonas shigelloides</em></td>
<td>+/-</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
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</tr>
</tbody>
</table>

*Enter pathogenic;*
In TSI Agar  

-/+, ferment glucose only; +/+, ferment glucose and lactose or sucrose or both; d/+, ferment glucose and fermentation of lactose or glucose shows different reactions; GAS, gas from glucose.

SIM; MOT, motility; IND, indole; M.R, methyl red; V.P, voges-proskauer; S.C, Simmon citrate; URE, urease; -, negative; +, positive; d, different reactions; +l, rarely negative; -lI, rarely positive
## APPENDIX III

### REACTIONS INTERPRETATION TABLE FOR APPAREILS ET PROCEDES D’IDENTIFICATION 20 E

<table>
<thead>
<tr>
<th>TESTS</th>
<th>SUBSTRATES</th>
<th>REACTIONS/ENZYMES</th>
<th>RESULTS NEGATIVE</th>
<th>POSITIVE</th>
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<tbody>
<tr>
<td>ONPG</td>
<td>Ortho-nitro-phenyl-galactoside</td>
<td>Beta-galactosidase</td>
<td>Colourless</td>
<td>Yellow (1)</td>
</tr>
<tr>
<td>ADH</td>
<td>Arginine</td>
<td>Arginine dehydrogenase</td>
<td>Yellow</td>
<td>Red/Orange (2)</td>
</tr>
<tr>
<td>ADC</td>
<td>Lysine</td>
<td>Lysine decarboxylase</td>
<td>Yellow</td>
<td>Orange</td>
</tr>
<tr>
<td>ADC</td>
<td>Ornithine</td>
<td>Ornithine decarboxylase</td>
<td>Yellow</td>
<td>Red/Orange (2)</td>
</tr>
<tr>
<td>CIT</td>
<td>Sodium citrate</td>
<td>Citrate utilization</td>
<td>Palegreen/Yellow</td>
<td>Blue-green/green (3)</td>
</tr>
<tr>
<td>H₂S</td>
<td>Sodium thiocyanate</td>
<td>H₂S production</td>
<td>Colourless/grayish</td>
<td>Black deposit/thin line</td>
</tr>
<tr>
<td>URE</td>
<td>Urea</td>
<td>Urease</td>
<td>Yellow</td>
<td>Red/Orange (2)</td>
</tr>
<tr>
<td>TDA</td>
<td>Tryptophane</td>
<td>Tryptophane deaminase</td>
<td>JAMES Immediate</td>
<td>Dark brown</td>
</tr>
<tr>
<td>IND</td>
<td>Tryptophane</td>
<td>Indole production</td>
<td>Pale green-yellow</td>
<td>Pink</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IND Yellow</td>
<td>JAMES Red ring</td>
</tr>
<tr>
<td>VP</td>
<td>Sodium pyruvate</td>
<td>Acetoin production</td>
<td>VPI + VP 2/10 min</td>
<td>Pink/Red</td>
</tr>
<tr>
<td>GEL</td>
<td>Kohn’s gelatin</td>
<td>Gelatinase</td>
<td>No diffusion of</td>
<td>Diffusion of black pigment</td>
</tr>
<tr>
<td>GLU</td>
<td>Glucose</td>
<td>Fermentation/Oxidation (4)</td>
<td>Blue/blue-green</td>
<td>Yellow</td>
</tr>
<tr>
<td>MAN</td>
<td>Mannitol</td>
<td>Fermentation/Oxidation (4)</td>
<td>Blue/blue-green</td>
<td>Yellow</td>
</tr>
<tr>
<td>INO</td>
<td>INOSITOL</td>
<td>Fermentation/Oxidation (4)</td>
<td>Blue/blue-green</td>
<td>Yellow</td>
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<tr>
<td>SOR</td>
<td>SORBITAL</td>
<td>Fermentation/Oxidation (4)</td>
<td>Blue/blue-green</td>
<td>Yellow</td>
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<td>RHAMNOSE</td>
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<td>Blue/blue-green</td>
<td>Yellow</td>
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<tr>
<td>SAC</td>
<td>SACRUCOSE</td>
<td>Fermentation/Oxidation (4)</td>
<td>Blue/blue-green</td>
<td>Yellow</td>
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<td>MEL</td>
<td>MELIBIOSE</td>
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<td>AMY</td>
<td>AMYGDALIN</td>
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<td>Blue/blue-green</td>
<td>Yellow</td>
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<tr>
<td>ARA</td>
<td>ARABINOSE</td>
<td>Fermentation/Oxidation (4)</td>
<td>Blue/blue-green</td>
<td>Yellow</td>
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<tr>
<td>OX</td>
<td>ON FILTER PAPER</td>
<td>CYTOCHROME OXIDASE</td>
<td>OX /1-2min</td>
<td>Colourless</td>
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</tbody>
</table>

### Notes:

1. Avery pale yellow should also be considered positive.
2. An orange colour after 24 hours of incubation must be considered negative.
3. Reading made in cupule (aerobic).
4. Fermentation begins in the lower portion of the tubes, oxidation begins in the cupule.
### APPENDIX IV

**ANTIMICROBIAL SUSCEPTIBILITY TEST RANGES (NATIONAL COMMITTEE ON CLINICAL AND LABORATORY STANDARDS, 2002)**

<table>
<thead>
<tr>
<th>Antimicrobial Agents</th>
<th>Code</th>
<th>R</th>
<th>I</th>
<th>S</th>
<th>Control</th>
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<tbody>
<tr>
<td>Amoxycillin/Clavulanic</td>
<td>AMC-30</td>
<td>≤13</td>
<td>14-17</td>
<td>≥18</td>
<td>19-25</td>
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<tr>
<td>Ampicillin</td>
<td>AMP-30</td>
<td>≤13</td>
<td>14-16</td>
<td>≥17</td>
<td>16-22</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>CTX-30</td>
<td>≤14</td>
<td>15-22</td>
<td>≥23</td>
<td>29-35</td>
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<tr>
<td>Cefuroxime</td>
<td>CXM-30</td>
<td>≤14</td>
<td>15-17</td>
<td>≥18</td>
<td>20-26</td>
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<tr>
<td>Chloramphenicol</td>
<td>C-30</td>
<td>≤12</td>
<td>13-17</td>
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<td>21-27</td>
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<td>Ciprofloxacin</td>
<td>CIP-5</td>
<td>≤15</td>
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<td>Gentamicin</td>
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<td>≤12</td>
<td>13-14</td>
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<td>19-26</td>
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<td>Nalidixi Acid</td>
<td>NA-30</td>
<td>≤13</td>
<td>14-18</td>
<td>≥19</td>
<td>22-28</td>
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<td>Nitrofurantoin</td>
<td>F/M-300</td>
<td>≤14</td>
<td>15-16</td>
<td>≥17</td>
<td>20-25</td>
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<tr>
<td>Trimethoprim Sulfamethoxazole</td>
<td>SXT 1.25/23.75</td>
<td>≤10</td>
<td>11-15</td>
<td>≥16</td>
<td>24-32</td>
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<td>Tobramycin</td>
<td>NN-10</td>
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<td>13-14</td>
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<td>Ceftazidime</td>
<td>CAZ-30</td>
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<tr>
<td>Tetracycline</td>
<td>TE-30</td>
<td>≤14</td>
<td>15-18</td>
<td>≥19</td>
<td>18-25</td>
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</table>

**Quality control**

*E. coli* ATCC 25922 was used.
### APPENDIX VI

**SOLVENTS AND DILUENTS OF VARIOUS ANTIBIOTICS**

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<thead>
<tr>
<th>Antibiotic</th>
<th>Reconstitute</th>
<th>Diluent</th>
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<tr>
<td>Ampicillin (2560)</td>
<td>PBS 0.1M pH 8.0</td>
<td>PBS 0.1M pH 6.0</td>
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<td>Ethanol</td>
<td>Sterilised distilled water</td>
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<tr>
<td>Gentamicin (320)</td>
<td>PBS 0.1M pH 8.0</td>
<td>Sterilised distilled water</td>
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<tr>
<td>Tetracycline (2560)</td>
<td>Sterilised Distilled water</td>
<td>Sterilised distilled water</td>
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</table>
APPENDIX VII

REAGENTS FOR PLASMID EXTRACTION AND CHARACTERIZATION

Lauria Bertini broth (LB)

Bacto Tryptone 10g
Bacto yeast extract 5g
Sodium chloride 10g
Distilled water 1000ml

The media was distributed into 4ml portions in large test tubes and sterilized by autoclaving at 121°C for 15 minutes.

10N NaOH

80g of sodium hydroxide pellets were dissolved in 200 ml deionised water.

20% SDS

40g SDS was dissolved in 160 ml deionised water, warmed, mixed and filled up to 200 ml with deionised water.

0.5M EDTA, pH 8.0

37.22g Na₂EDTA.2H₂O was added to 160 ml of deionised water, pH adjusted to 8.0 with ≤ 4g sodium hydroxide pellets, filled up to 200 ml with deionised water and then sterilised by autoclaving.

20% Glucose

40g anhydrous dextrose was dissolved in 200 ml of deionised water, sterilized by filtration through a 0.22 μm disposable filter and stored at 4°C.
1M Tris. HCl, pH 8.0

121.1g Tris base was dissolved in 800 ml of deionised water, pH adjusted to 8.0 with HCl, filled up to 1 litre and sterilized by autoclaving.

3M Sodium acetate, pH 5.2 (B-III)

204.12g NaOAC.3H2O was dissolved in 400 ml of deionised water, pH adjusted to 5.2 with ≤ 100 ml acetic acid, filled up to 500 ml with deionised water and then sterilised by autoclaving.

7.5M Ammonium acetate

289.05g ammonium acetate was dissolved in 500 ml deionised water and then sterilised by autoclaving.

20 X TE buffer (1 X 10 mM Tris, 1 mM EDTA)

24.11g Tris base and 7.45g Na2EDTA.2H2O were dissolved in 800 ml deionised water, pH adjusted to 8.0 with HCl and then filled up to 1 litre with deionised water.

Stop mix (0.07% BPB, 7% SDS and 20% Ficoll)

70 mg bromophenol blue, 7 g SDS and 20g Ficoll type were dissolved in 80 ml deionised water, warmed, mixed and filled up to 100 ml with deionised water.

5 X TBE Electrophoretic buffer (Use 1: 10 solution in the bath)

(1 X is 89 mM Tris, 89 mM boric acid and 2.8 mM EDTA)

53.89g Tris base, 27.51g boric acid and 5.21g Na2EDTA.2H2O were dissolved in 1 litre of deionised water.
10 mg/ml ethidium bromide

0.5g ethidium bromide was dissolved in 50 ml deionised water in a dark bottle and stored at 4°C.

80% ethanol

160 ml ethanol was mixed with 40 ml sterilized deionised water in a sterilized bottle.

**Preparation of solution I (B-I) and solution II (B-II)**

**Solution 1 (B-I)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme</td>
<td>20 mg</td>
<td>(2 mg/ml)</td>
</tr>
<tr>
<td>1M Tris.HCl, pH 8.0</td>
<td>0.25 ml</td>
<td>(25 mM)</td>
</tr>
<tr>
<td>0.5M EDTA, pH 8.0</td>
<td>0.20 ml</td>
<td>(10 mM)</td>
</tr>
<tr>
<td>20% Glucose</td>
<td>0.45 ml</td>
<td>(50 mM)</td>
</tr>
<tr>
<td>Distilled water</td>
<td>9.10 ml</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>10.00 ml</td>
<td></td>
</tr>
</tbody>
</table>

**Solution 11 (B-11)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10N NaOH</td>
<td>0.4 ml</td>
<td>(0.1N)</td>
</tr>
<tr>
<td>20% SDS</td>
<td>1.0 ml</td>
<td>(1.0%)</td>
</tr>
<tr>
<td>Distilled water</td>
<td>18.6 ml</td>
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</tr>
<tr>
<td><strong>Total</strong></td>
<td>20.0 ml</td>
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</table>