ISOLATION AND CHARACTERISATION OF *AEROMONAS* SPECIES FROM BOREHOLES AND WELLS IN SELECTED TOWNS OF KENYA

BY

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DECLARATION

I declare that this thesis is my own original work and has not been presented for a degree in any other university or any other award.

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To my wife Fardowsa, children Shuraim, Fatuma and Zuhayla and my brothers Mahamud Ali and Ali Gabow who gave me the inspiration to accomplish this study.

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LIST OF ABBREVIATIONS

API – Appareils et Procedes d' identification

BCIP – Bromo –4- chloro 3 idolyl phosphate salt

CMR - Center for Microbiology Research

DNA – Deoxyribonucleic acid

DVBD- Division of Vector Borne Diseases

EDTA – Ethylene diamine tetra- acetic acid

GEL - Gelatine

KEMRI – Kenya Medical Research Institute

MIC – Minimum Inhibitory Concentration

NaOH – Sodium hydroxide

NAS – NAS Airport Services (Nairobi)

NBT – Nitro blue tetrazolium

NPHLS – National Public Health Laboratory Services

RT – Room temperature

SDS - Sodium dodecyl sulphate

SSC - Sodium citrate

TE - Tris ethylene diamine tetra-acetic acid

Tris HCl – Tris hydrochloric acid

WHO – World Health Organization

ABSTRACT

Aeromonas species are recognized as causative agents of various infections in humans, including gastroenteritis, wound and systemic infections. The pathogenicity of *Aeromonas* species is potentially supported by several virulence factors such as production of toxins and their ability to adhere to and invade the epithelial cells. Evidence shows that *Aeromonas* infections in humans are often related to water exposure. This study isolated and characterized *Aeromonas* species from water wells and boreholes in Lamu, Ongata Rongai and Wajir towns. In addition the antimicrobial susceptibility profiles and toxin assays (haemolysis and enterotoxins) were determined. The ability of *Aeromonas* to harbor and transfer resistance plasmids to *Escherichia coli* was determined through conjugation assays. Out of the 87 *Aeromonas* species isolated from the three locations, 51 (59 %) were *A. hydrophila* while 21 (24.1 %) and 15 (17.2 %) were *A. caviae* and *A. sobria,* respectively. *A. hydrophila* was more prevalent than either *A. caviae* or *A. sobria* ($P< 0.05$). All the *Aeromonas* species were resistant to ampicillin, while resistance levels of *A. hydrophila* to nalidixic acid, chloramphenicol, cefotaxime, ceftazidime, tetracycline and co-trimoxazole were significant ($P < 0.05$). However, *A. sobria* varied significantly in resistance to nalidixic acid, chloramphenicol, cefotaxime, ceftazidime and ciprofloxacin ($P<0.05$) but not to tetracycline and co-trimoxazole ($P > 0.05$). Fifty (57.5 %) isolates of *Aeromonas* species had plasmids of different sizes while 56 (84, 8 %) isolates of *E. coli* $K12F^{-}NA^{r}$ Lac^{+} transconjugants had plasmids after transconjugation. Forty nine (96. 1%) *A. hydrophila* isolates, 12 (80 %) *A. sobria* and 16 (76.2 %) *A. caviae* were haemolytic. Only 10 (11.5 %) of the *Aeromonas* isolates produced enterotoxins, 6 (60 %) produced heat labile while 4 (40 %) produced heat stable enterotoxin. The results obtained illustrate *Aeromonas* species as important potential waterborne pathogens in Ongata Rongai, Wajir and Lamu where ground water constitutes large proportions of water source for the respective communities. The observation that there is increased resistance of *Aeromonas* species to the commonly used antibiotics, and the presence of virulence factors such as enterotoxins and haemolysins further strengthens the cases for their pathogenic potential.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

1.1.1 Background information

Members of the genus *Aeromonas* are gram-negative, oxidase positive, facultative anaerobic rod-shaped bacteria of the family Vibrionaceae (Gluskin *et al.*, 1992). *Aeromonas* measure 1.0 to 3.5 μm long and 0.3 to 1.0μm wide and most of the species possess polar, usually monotrichious flagella with a wavelength of 1.7 μm (Carnaham and Altwegg, 1996; Popoff, 1984). The maximum temperature range at which growth occurs is 38°C to 41°C (McGowan and Del Rio, 1990).

The motile species of the genus *Aeromonas* are characteristic of aquatic ecosystems and are commonly isolated both on surface (Nishikawa and Kishi, 1998; Araujo *et al.*, 1989; Bernagozzi *et al.*, 1995; Parveen *et al.*, 1995) and ground waters (Burk *et al.*, 1984; Havelaar *et al.*, 1990; Krovacek *et al.*, 1992) as well as in waste-water (Monfort and Baleux, 1990; Araujo *et al.*, 1991; Poffe and Op de Beeck, 1991). The concentration of Aeromonas spp. in the water environment appears to be related to the presence of nutrients such as nitrogen, dissolved phosphorus or particulate organic substances (Van Der Kooji and Hijnen, 1988; Van Der Kooji, 1991).

Aeromonas spp. are associated with a wide variety of diseases in warm and cold-blooded vertebrates including frogs, fish, reptiles, sharks, birds and humans (Farmer *et al.*, 1992). There is increasing evidence that *Aeromonas spp* infections in humans are often related to water exposure (Schubert, 1991; Wadstrom and Ljungh, 1991). Over the last three decades these organisms have also emerged as opportunistic pathogens responsible for gastroenteritis, skin and soft tissue infections and a variety of clinical syndromes in immunologically compromised patients (Jones and Wilcox, 1995). The prevalence of *Aeromonas* associated gastroenteritis has been reported worldwide and its incidences in the developed countries has been reported to be relatively low compared with that in the developing countries (Altwegg and Geiss, 1996). *Aeromonas hydrophila, A. caviae* and *A. sobria* are most commonly associated with diseases in man. The gastroenteritis affects both children and adults (George *et al.,* 1985). The highest seasonal incidence occurs during summer months when higher temperatures presumably result in higher number of organisms being present in both contaminated food and water (Gomez *et al.,* 1996). A number of virulence factors have been associated with these organisms and may be responsible for their enteropathogenicity. These include the production of cytotoxins, enterotoxins and haemolysins (Gracey *et al.*, 1982; Singh and Sanyal, 1992). Studies have shown that haemolytic *Aeromonas* are also enterotoxigenic. This suggests that the detection of haemolytic activity is sufficient to incriminate enterotoxigenic *Aeromonas* species as pathogenic (Majeed and MaCrae, 1994).

Although *Aeomonas*-associated disease in the very young, the old and the immuno-compromised often requires antimicrobial therapy, reports on the susceptibility of these organisms to antimicrobial agents are rare (Ghenghesh *et al.*, 2001). Antibiotic sensitivity of clinical isolates of *Aeromonas* spp. has been extensively studied (Chang and Bolton, 1987) but less is known about environmental strains (Morita *et al.*, 1994; Montoya *et al.*, 1992). Antiseptic and disinfectant susceptibility of this genus has received little attention except for chlorine (Sisti *et al.*, 1998). However, ground waters receive human and animal wastewater discharges, which are expected to contain antimicrobial agents likely to exert a selective pressure and commensally resistant bacteria which are capable of transferring their resistance to other environmental bacteria within their vicinity (Ghenghesh *et al.*, 2001).

1.2 Literature Review

1.2.1 Classification of *Aeromonas* species

Aeromonas are Gram-negative, non-spore forming, rod-shaped facultatively anaerobic bacteria that occur ubiquitously and autochthonously in aquatic environments (Popoff, 1984). On the basis of these characteristics they were listed as belonging to the family Vibrionaceae (Baumann and Schurbet, 1984). However, as molecular genetic evidence emerged, it was proposed that *Aeromonas* be classified in a separate family, Aeromonadaceae (Colwell *et al.*, 1986), with four phenotypically separable *Aeromonas* spp; *A. hydrophilla, A. caviae, A. sobria* and *A. salmonicida. Aeromonas salmonicida* has three subspecies, which are not of clinical importance (Baumann and Schurbet, 1984).

Thereafter, several new phenotype species and DNA hybridisation groups (HGS) have been described and include *A. media, A. eucrenophila, A. veronii, A. trota, A. allosaccharophila, A. popoffii,, A. schubertii* and *A. jandaei* (Janda, 1991).

Aeromonas salmonicida is a fish pathogen and has not been associated with human infection. Conversely, the mesophilic species have been associated with a wide range of infections in humans (Janda and Abbott, 1998). Although members of the genus have classically been divided into three biochemically differentiated groups (typified by *A. hydrophila, A. caviae* and *A sobria),* these contain a number of genospecies to which new species have been added (Carnaham and Altwegg, 1996).

1.2.2 Environmental occurrence of *Aeromonas* species

Aeromonads are ubiquitous in aquatic environments and are readily isolated from both nutrient-rich and nutrient-poor environments (Holmes *et al.*, 1996). As *Aeromonas spp* are autochthonous to fresh and marine waters their recovery is to be expected. However, increasing levels of pollution may result in substantially greater populations and may also affect distribution of the organisms (Holmes *et al.,* 1996). Several studies have shown that *A. caviae* tends to predominate in waters with a high degree of organic loading (Araujo *et al.,* 1991; Stecchini and Domenis, 1994); *A. caviae* and *A. hydrophila* are almost equally distributed in less polluted waters, and *A. sobria* becomes more frequent in unpolluted and brackish waters (Holmes *et al.,* 1996). Aeromonad densities have also

been related to trophic status, and populations in some waters have a seasonal variation, with highest numbers occurring in the warmer months. Relationships between aeromonad densities and parameters relating to trophic status or sewage contamination will vary according to the site, season and region (Rhodes and Kator, 1994).

Although species of mesophilic *Aeromonas* are commonly resident in drinking water distribution systems, few data exists on the factors affecting their occurrence. However, it is generally reported that higher rates of isolation and larger populations occur during the warmer months and at the peripheries of distribution systems (Havelaar *et al.,* 1990; Stelzer, 1992; Holmes *et al.,* 1996). For chlorinated water, Burke *et al;* (1984) reported that the occurrence *of Aeromonas spp* was positively correlated with water temperature and negatively correlated with residual chlorine levels. A seasonal variation in mean *Aeromonas* counts closely paralleled mean water temperature in samples that were either unchlorinated or had free chlorine values consistently below 0.3mg/l. Isolations of *Aeromonas* spp. from drinking water lacking chlorine was generally associated with water temperatures greater than 14 °C.

1.2.3 Clinical importance of *Aeromonas* spp in man

The mesophilic aeromonads have been commonly isolated from patients with gastroenteritis although their role in disease causation remains unclear. They are also associated with sepsis and wounds and with eye, respiratory tract and other systemic

infections (Janda and Abbott, 1998; Nichols, 1996). Many of the systemic infections arise following contamination of lacerations and fractures with *Aeromonas*-rich waters. The species principally associated with gastroenteritis are *A. caviae*, *A. hydrophila*, and *A. veronii* biovar sobria (Joseph, 1996); *A. caviae* is particularly associated with infections in young children (under 3 years of age). Many studies have resulted in the isolation of several species of *Aeromonas* from patients with gastroenteritis and these have been extensively reviewed (Altwegg and Geiss, 1989; Janda, 1991; Joseph, 1996). There has been considerable debate as to whether the mesophilic aeromonads are primary enteropathogens, prompted largely by failure to establish significant infection in volunteer studies. In a study in which 57 volunteers were challenged using five strains of *A. hydrophila* with doses ranging from 10^4 to 10^{10} organisms, only two individuals developed diarrhoea, one had mild diarrhoea after receiving a dose of 10^9 organisms and the other developed moderate diarrhoea following administration of a dose of 10^7 organisms (Gosling, 1996). The value of these data is limited, as the strains used were poorly characterized and some were not demonstrably enterotoxigenic (Gosling, 1996). However, there have been reports of laboratory-acquired infections in investigators who (unintentionally) ingested significant doses of *Aeromonas spp* and developed self-limiting diarrhoea (Joseph, 1996).

Understanding the clinical significance of enteric isolates of *Aeromonas* has further been complicated by the fact that some studies have demonstrated similar isolation frequencies from symptomatic and asymptomatic adults (Altwegg and Geiss, 1989), while others have shown significant correlations between diarrhoea and enterotoxin-producing

Aeromonas spp. (Gracey *et al.,* 1982; Bloom and Bottone, 1990). Seasonal variations in isolation of *Aeromonas spp* from stools have also been reported, with highest recoveries during the warmer months (Burke *et al.*, 1984). The available evidence indicates that people are generally unaffected by enteric *Aeromonas* and that Aeromonads may be a natural part of the gut flora, either transiently or in the longer term. A number of factors including age, immunocompetence, infection dose, underlying illness, and expression of sufficient virulence factors by the infecting organism affect the ability of *Aeromonas* spp. to cause disease (Nichols, 1996).

Aeromonas hydrophila has also been implicated in skin and soft tissues infections. The condition is characterised by necrotizing myositis and is often associated with wounds contaminated by water or soil and usually involving the lower limbs (Khadori and Fainstein, 1988). This phenomenon is due to the high diffusion capacity of Aeromonas toxins. Other localized forms usually arising from asepticemic status are meningitis, osteomyelitis, peritonitis, intra-abdominal abscesses, urinary tract infection and otitis media (Khardori and Fainstein, 1988). The prevalence of respiratory infections evolving into pneumonia, lung abscess or empyema is reportedly increasing (Janda and Abbott, 1998).

1.2.4 Pathogenicity of *Aeromonas* species

The pathogenicity of *Aeromonas* infections still remains poorly understood. Mesophilic *Aeromonas* spp. can express a range of virulence factors including attachment mechanisms and production of a number of toxins. Several studies have demonstrated that strains of *A. hydrophila* produce lectins and adhesins that enable adherence to epithelial surfaces and gut mucosa (Gosling, 1996). Additionally, two types of pili have been characterized from Hep-2-adherent *A. hydrophila* (Carrello and Lamb, 1988; Gosling, 1996), and invasion of Hep-2 cells by faecally derived *A. hydrophila* has also been reported (Lawson *et al.*, 1985).

Aeromonas spp capable of expressing a number of extra cellular toxins and enzymes. Early characterization of the toxins, however, resulted in confusion regarding their number and activities. The primary toxins produced are haemolysins, of which the most significant is aerolysin, expressed by many strains of *A. hydrophila* and *A. sobria* (Janda, 1991; Howard *et al.,* 1996). This is a heat-labile beta-haemolysin, which exhibits phospholipase A and C activity. It is a pore-forming cytolysin able to insert into the cell membrane bilayer causing leakage of cytoplasmic contents. Haemolytic enterotoxins have been reported by some authors (Chopra *et al.,* 1991; Gosling, 1996). A weak haemolysin, glycerophospholipid: cholesterol acyltransferase (GCAT) has been characterized from *A. hydrophila* and *A. salmonicida* (Howard *et al.,* 1996); other haemolysins may also exist, but need to be isolated and purified before haemolytic activity can be confirmed. In addition, at least one cytotonic enterotoxin with

similar activity to cholera toxin has been demonstrated (Ljungh *et al.,* 1982; Gosling *et al.,* 1996). Evidence for plasmid-encoded expression by *A. hydrophila* and *A. caviae* of a cytotoxin similar to Shiga-like toxin 1 has been reported (Haque *et al.,* 1996). Species of *Aeromonas* also produce a range of cell-surface and secreted proteases that probably enhance virulence (Gosling, 1996). Expression of virulence factors including haemolysins and proteases by aeromonads has been shown to be influenced by environmental temperature (Mateos *et al.,* 1993). There is abundant evidence to suggest associations between mesophilic aeromonads and diarrhoea, and production of enterotoxins has been demonstrated. Further work is needed to clarify the pathogenic mechanisms of *Aeromonas* spp. and substantiate the causative role of these organisms in gastroenteritis. There is also a need for reliable data on human infective doses for well-defined strains of putative enteropathogenic *Aeromonas spp* pending the establishment of an appropriate animal model for the study of *Aeromonas*-associated diarrhoea.

In the Nyanza province of Kenya, Muthotho *et al;* (1986) isolated *Aeromonas* from community water sources and four strains from diarrhoeal patients with similar enteropathogenic properties. Thereafter, Zhi-Dong *et al*; (2002) isolated *Aeromonas* among international travelers with diarrhoea in Mombasa, Kenya. The problem of adverse environmental sanitation due to inadequate and often unsafe methods of waste disposal may play a major role in perpetuating infections. In many different parts of the world, it has been demonstrated that there is a close association between inadequate environmental sanitation, high prevalence of diarrhoeal diseases and certain enteric pathogens (Henry, 1981).

1.2.5 Virulence factors associated with Aeromonads

Several studies have demonstrated that many mesophilic aeromonads isolated from drinking water can exhibit toxigenic factors. Millership *et al;* (1986) found that cytotoxicity was demonstrated by 28% of *Aeromonas* isolates (mainly *A. hydrophila*) from chlorinated and unchlorinated drinking water but by none of the strains of *A. caviae* (which represented 50 % of the isolates). Recently Holmes *et al;* (1996) found that 20 % of *Aeromonas* isolates exhibited phenotypic characteristics associated with enterotoxicity; of these, 75 % were *A. hydrophila*, 14 % *A. sobria*, 9 % *A. caviae*, and the remainder were *A. schubertii*. In contrast, Burke *et al*; (1984) reported that 61 % of aeromonads isolated from an unchlorinated municipal water supply in Australia were enterotoxigenic, and 64 % produced haemolysins. Other studies have shown that all of 26 *A.hydrophila* isolated from drinking-water and 9 of 22 isolates of *A. sobria* exhibited the haemolytic enterotoxin Asao toxin and cytotoxicity to Vero cells, while none of 14 isolates of *A. caviae* produced toxins (Burke *et al.*, 1984). Similarly, Krovacek *et al.,* (1992) found that all A. *hydrophila* and 70 % of *A. sobria* in Swedish chlorinated and unchlorinated drinking-water were haemolytic, but that less than 30 % of the isolates were enterotoxigenic. Kirov, (1994) found that 53.6 % of isolates of *A. hydrophila* hybridization group 1 (HG1) and 55.9% of HG3 from water expressed two or more virulence factors.

1.2.6 Epidemiology of Aeromonads

Despite the association of virulence factors in Aeromonads from drinking-water, there is increasing evidence that strains isolated from the environment generally belong to different groups from strains associated with gastroenteritis. Havelaar *et al*; (1992) characterised 187 *Aeromonas spp* isolated from human diarrhoeal stools and 263 from drinking water. There was little similarity between the strains from stools and those from drinking water. This was particularly true of *A. caviae*, which was the dominant aeromonad in both sets of samples. Other studies have indicated that *A. hydrophila* prevalence may be related to hybridization groups. Both Kirov (1994) and Hšnninen *et al*; (1995) found that HG1 was associated with clinical specimens, while HG3 and to a lesser extent HG2 are predominant in water and environmental samples. It appears that this may be reflected in the maximum growth temperatures (t_{max}) of the homology groups. Kirov (1994) reported that hybridization groups of *Aeromonas* associated with clinical samples (HGs 1, 4, 9/10 and 13) generally had a (t_{max}) of 40–44 °C, while isolates from freshwater (HGs 3 and 11) had t_{max} values between 36.5 °C and 37.5 °C.

It has been claimed that drinking-water supplies are responsible for the increased incidence of *Aeromonas* associated gastroenteritis. Ghanem *et al;* (1993) considered that, since 90 % of the domestic water supplies in Cairo were positive for aeromonads, and that 56 % of isolates produced enterotoxins, the supplies were a major source of *Aeromonas* infections. Investigating a case of long-term diarrhoea in a child aged 18

months, Krovacek *et al*; (1992) concluded that the cause was *A. hydrophila* from a private, unchlorinated well in which counts ranged from 70 CFU/100ml to 6.4 X 10^4 CFU/100ml. The majority of isolates were enterotoxin-producers. Although these reports indicate a possible relationship between *Aeromonas* in drinking water and increased incidence of aeromonad-related illness, the evidence is tenuous. In one case comparing the typing of faecal and water isolates, the two groups proved to be unrelated (Moyer, 1996). Following a number of cases of diarrhoea in children using a small community water supply, *Aeromonas* was isolated from water-treatment and distribution samples; ribotyping and DNA hybridization showed that isolates from faeces were of different ribotypes and DNA hybridization groups (HGs 1 and 4) from drinking water isolates were predominantly HGs 2, 3 and 5A (Moyer, 1996).

1.2.7 Control of Aeromonads in drinking water

Aeromonas in drinking water in distribution systems has been controlled by increased disinfection and it appears that free cells of *Aeromonas* are relatively susceptible to the common chlorine-based disinfectants. Knichel and Jeppessen (1991) found that strains of *A. hydrophila, A. sobria, A. caviae* and *A. veronii* were generally more susceptible to pseudomonads. Medema (1991) found that laboratory-grown and environmental *Aeromonas* spp were also susceptible to chlorine compounds. Despite this relative susceptibility to chlorine-based disinfectants, controlling the numbers of Aeromonads in a distribution system may require some considerable time and chlorine concentrations in excess of 0.2 mg/l (Edge and Finch, 1987).This is probably due to association of the

organisms with biofilm. MacKerness *et al;* (1991) found that *A. hydrophila* became readily established within a mixed heterotrophic bacterial biofilm and was unaffected by the addition of 0.3mg/l monochloramine

1.2.8 Health risk assessment

Although Aeromonads are frequently isolated from drinking-water systems, and some strains may exhibit enterotoxigenic properties, further epidemiological studies are required to ascertain any significance in relationships between cases of *Aeromonas*-associated diarrhoea and presence of these organisms in drinking water. Current evidence indicates that the predominant aeromonads typically found in drinking water do not belong to the same DNA homology groups as those isolated from cases of gastroenteritis. It also appears that, if *Aeromonas* species are primary enteropathogens, high numbers are required to initiate disease. As numbers in drinking water are generally low compared with those found in foods (10^3–10^5 CFU/g), treated drinking water probably represents a very low risk. The virulence of enterotoxigenic *Aeromonas* for risk groups (newborn infants and immunocompromised individuals), however, remains to be ascertained. There is no firm evidence yet that direct transmission occurs via drinking water, but in the absence of more definitive proof of their public health significance it would be advisable to control excessive numbers of aeromonads in drinking-water supplies.

1.2.9 Risk management strategies

The mesophilic aeromonads are a ubiquitous component of the natural bacterial flora of aquatic environments. When temperature and nutrient conditions allow, they can rapidly proliferate in unchlorinated drinking water supply systems and where chlorine residuals tend to be low (for example, in the extreme parts of extensive distribution systems). The key factors in controlling *Aeromonas* proliferation are temperatures below 14 °C (although the organisms are capable of growth at 4 °C), free chlorine residuals above 0.1–0.2 mg/l, and the limitation of organic carbon compounds that would serve as nutrients. Control of the development of biofilms within water supply systems will reduce, but not prevent, the proliferation of *Aeromonas* (WHO, 2002). As *Aeromonas spp* are associated with biofilm development, significant increases in numbers in a drinking-water supply are indicative of a general deterioration of bacteriological quality. The increasing use of granulated activated carbon in water treatment may allow proliferation and dissemination of *Aeromonas spp*. limiting the numbers of aeromonads released into distribution systems thus requires effective management of filter beds and maintenance of adequate final chlorination. Control of aeromonads in piped distribution systems is achieved primarily by limiting regrowth possibilities, this will also limit the numbers of heterotrophic bacteria and improve the efficacy of chemical disinfection in the distribution system (WHO, 2002).

1.2.10 Horizontal transfer of drug resistance

The ease with which bacteria become resistant to currently used antimicrobial agents has been and continues to be of concern to clinicians, public health officials and researchers. Transferable drug resistance represents a major threat to the treatment of infectious diseases in both humans and animals, including fish farms. The use of antimicrobial agents in both human and veterinary medicine exerts a strong selective pressure inducing resistance to antimicrobial agents among bacteria (Levy, 1992; Young, 1993; Linton, 1986). Generally, bacteria with the highest level of resistance are isolated from environments contaminated with antimicrobial agents, such as hospitals, fish farms, sewage effluents and waste-waters (Gauthier and Breittmayer, 1990; Sandaa *et al.,* 1992). However, the importance of investigating plasmid transfer in natural environments has recently been emphasized by the increasing spread of multi-resistant bacterial pathogens (Davis, 1994) and the extensive use of antibiotic derivatives as growth promoters for domestic animals (Witte, 1998). In this context, transfer of genes encoding antibiotic resistance within the complex bacterial flora of the animal gut is of particular interest.

In spite of the large number of communications describing plasmid transfer in natural environments (Kruse and Sorumm, 1994; Rang, *et al.,* 1996), there are no studies which systematically compare conjugation and transfer of resistance plasmids (R plasmids) under different conditions and address more general questions about the mechanisms important for transfer processes

1.2.11 Treatment of Aeromonas infections

For the management of acute diarrhoea due to *Aeromonas*, rehydration will generally suffice. Protracted colitis may benefit from co-trimoxazole or a fluoroquinolone. Those antibiotics can also be used for urinary tract infections. For the treatment of severe invasive infections (Septicaemia) excellent activity can be expected from cephalosporins III, chloramphenicol, fluoroquinolones and aminoglycosides. Some strains may be resistant (WHO, 1993).

1.2.12 Justification

The provision of an adequate supply of safe water was one of the eight components of primary health care identified by the International Conference on Primary Health Care in Alma-Ata in 1978, where Kenya was a signatory (WHO, 1978). In most countries, Kenya included, the principal risks to human health associated with the consumption of polluted water are microbiological in nature (although the importance of chemical contamination should not be underestimated). An estimated 80 % of all diseases and over one-third of deaths in developing countries are caused by the consumption of contaminated water and on average as much as one tenth of each person productive time is sacrificed to water-related diseases (WHO 1997).

Most people in rural Kenya use untreated water directly from available sources and are therefore, at risk of exposure to a variety of water related diseases. Human pathogenic

microorganisms that are transmitted by water include; *Salmonella* spp, *Shigella* spp, Pathogenic *Escherichia coli, Vibrio* spp and *Aeromonas* spp. Most of these microorganisms transmitted by water usually proliferate in the human intestinal tract and reach the environment through faecal contamination.

Considerable worldwide epidemiological, microbiological and clinical investigation have shown that some strains of the different motile Aeromonads are of increasing enteropathogenic significance, especially in children, the elderly and the immuno-compromised individuals (Golik *et al.*, 1990). *Aeromonas spp* have been associated with human gastroenteritis. Several studies in different parts of the world have demonstrated the association between the presence of *Aeromonas* in water sources and a high degree of gastroenteritis in people who consume the waters (Janda and Abbot, 1998). A study carried out by Muthotho *et al*; (1986) indicated the presence of the organisms in different water bodies in Kenya. A study carried out in Mombasa by Zhi-Dong Jiang *et al*; (2002) indicated the presence of *Aeromonas* among international travelers with diarrhoea. Another study carried out by Basil *et al*; (2002) in Nigeria reported that the causative agents for gastroenteritis in children and young adults were due to *A. hydrophila, Salmonella* spp, *Shigella* spp and enteropathogenic *E. coli*.

In Kenya, few studies have been carried out on *Aeromonas spp* as possible causative agents of waterborne gastroenteritis. In view of the transmission of *Aeromonas,* wells and boreholes are vulnerable to contamination especially in rural areas where improper

disposal of human and animal waste is rampant. Improvements in the quality of drinking water may result in substantial reduction in disease prevalence. Due to multiplicity of transmission routes, improvement in the quality and availability of water, excreta disposal, and the enhancement of good hygiene practices are all important factors in reducing diarrhoeal morbidity and mortality.

In Kenya, there are many cases of diarrhoea from which the causative agents are not isolated in diagnostic laboratories because of lack of appropriate media and the focus on isolation of traditional diarrhoea causing bacteria. The aim of this study was to determine the prevalence, species distribution, haemolytic activities, enterotoxin produced, antibiotic resistance patterns, plasmid content, transferability of resistance and some specific chemical parameters in *Aeromonas* found in ground water in three different geographical areas in Kenya.

1.2.13 Hypothesis

Ground water (wells and boreholes) in Ongata Rongai, Lamu and Wajir are not contaminated with pathogenic *Aeromonas* species

1.2.14 Objectives

1.2.14.1 General objective

To isolate and characterize *Aeromonas* species causing gastroenteritis from wells and boreholes water in Ongata Rongai, Lamu and Wajir.

1.2.14.2 Specific objectives

1. To isolate and identify *Aeromonas* species from wells and boreholes in the three locations.
2. To determine the antibiotic susceptibility patterns of *Aeromonas* isolated from the ground water.
3. To determine the virulence factors (heamolysin and enterotoxin) in *Aeromonas* species.
4. To determine the contamination potential of wells and boreholes by faecal *E. coli* in ground water.
5. To assess the chemical properties of sampled ground waters from the three locations.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Study sites

This study was carried out in three towns in Kenya with different physiographic and natural conditions. The towns were Wajir (North Eastern Province), Lamu (Coast Province) and Kajiado (Rift Valley Province). All the three provinces face severe water shortage and clean water for drinking is a major problem. Sub-surface water forms the bulk of the water supply. The sources are unprotected and water for domestic use is fetched from the same sources where the livestock are watered. This situation has led to poor health standards and frequent outbreaks of waterborne diseases in the three districts. Appendix 7 shows the geographical location of the three sites.

Wajir has a population of 391,266 (Kenya population census, 1999). The district lies within the sahelian climatic region that is characterised by long dry spells and short rainy seasons. The mean temperatures soar above 35°C and sometimes reach 40°C. The inhabitants in the district are basically nomadic and move from place to place in search of pasture for their livestock. The town of Wajir has no natural rivers and is served by unprotected shallow wells which are either located at residential homes or in open fields. The depths of the wells usually range from 3 meters to 10 meters because the water tables are high.

Lamu is an ancient Swahili town with a population of 72,686 (Kenya population census, 1999) is generally flat and lies between zero and 50 meters above sea level. The main source of water for the people in Lamu is shallow unprotected wells. The depth of the wells range between 3 to 10 meters and the water table is high. Temperatures generally range from 25 °C to 34 °C throughout the district. The main economic activities are coconut growing, fishing and tourism.

Kajiado with a population of 406,054 (Kenya population census, 1999) is characterised by plains and occasional volcanic hills. The area has a bimodal rainfall pattern. The lower part of the district is warmer with temperatures ranging from 25 °C to 35 °C while the upper part is hilly and has temperature ranging between 20 °C to 25 °C. The inhabitants lead either a nomadic life or are small-scale farmers or urban dwellers. The main sources of water in the area are seasonal rivers, boreholes and wells, which are used for both livestock and humans. The water table is low as the rocks are generally porous and allow water to percolate to great depth into the ground (District planning and Development 2003 – 2004). Boreholes are drilled to depth varying between 60 and 175 meters.

2.2 Site identification

The criteria for choosing the sample site were determined after initial field visits. Selection was based on the level of daily activities observed around the communal water points. Twenty wells or boreholes were identified from each site. Two samples were

collected, one for bacteriological analysis and the other for chemical analysis. A preliminary study of boreholes and wells was conducted to profile the bacteria content and to establish the presence of *Aeromonas* spp. Isolated strains that could not be identified at the genus level were disregarded.

2.2.1 Inclusion criteria

1 .Boreholes and wells that were located in open fields and used as communal water points for both domestic and animal use.

2. Water points that were close to sanitation.

2.2.2 Exclusion criteria

Boreholes and wells those were private, protected, and shallow or dry at the time of sampling.

2.3 Sample collection

The water samples were collected from each site into two clean sterile containers one with 0.2 ml 10 % sodium thiosulphate (treated sample only) in which 200 ml of water was added for bacteriological analysis while one litre of water was collected and put in

another bottle for chemical analysis. The water bottles were labelled and a questionnaire. The questionnaire had the following important information (Appendix 1); Name of person doing the sampling, date and time of sampling, location, source of sample, proximation to septic tank, latrine, depth of the well and if the water is treated or untreated (WHO, 1997). All samples were placed in an insulated cool box and sent to the National Public Health Laboratory Services (NPHLS) for analysis within 4 hours of collection.

2.3.1 Sampling from pump outlet

Sampling was done from the pump outlet into 200 ml sterile glass bottles into which 0.1 ml of sodium thiosulfate was dispensed before sterilization. Sterile gauze was used to clean the taps to remove dirt prior to sampling. The tap was turned on to maximum flow and water let to run for 2 minutes. It was then turned off and sterilized using a flame (an ignited alcohol-soaked cotton-wool swab). The tap was carefully turned on and water left to flow for 2 minutes at medium flow rate without re-adjusting the flow. The sterilized bottles were opened by carefully unscrewing the caps, then, holding the caps and protective cover face downward in order to prevent contamination during the sampling. The bottles were immediately held under the water jet and filled (Fig. 1). A small air space was left to allow for shaking before analysis. The bottled caps were screwed back and a foil protective cover fixed in place.

Figure 1: A picture showing a typical borehole

2.3.2 Sampling from dug wells

A sterile sample bottle was attached to a clean weight with a piece of string. The cap was aseptically unscrewed from the bottle and the bottle tied to a clean 20 meter length string. The bottle was lowered, weighted down by the weight, into the well avoiding contact with the sides of the well (Fig 2 and 3). The container was immersed into the water below the surface without hitting the bottom or disturbing any sediment. Once the bottle was filled, the attached string was pulled to bring up the bottle. Some water was discarded from completely filled up bottle to provide airspace. The cap was screwed back onto the bottle and the foil protective cover fixed in place.

Figure 2: A picture showing a typical unprotected well

Figure 3: A picture showing a typical partially protected well

2.4 Water analysis

2.4.1. Isolation of *Aeromonas*

On receipt of the water samples, the foil protective covers were removed. The sample number, sites and questionnaire forms were verified and details entered into the laboratory water analysis register. Before analysis, the water samples were brought to room temperature, mixed by inverting the bottle and was enriched in alkaline peptone water broth pH 8.6. This was done aseptically by pipetting 50 ml of water sample into 50 ml of the broth. After thorough mixing the bottles were then incubated aerobically at 37 °C for between 18 to 24 hours.

The alkaline peptone water broth from day 1 incubated at 37 °C aerobically overnight was removed from the incubator. Using a flame sterilized wire loop and without agitating the bottle, an inoculum from the pellicle (upper area) of the culture broth was sub-cultured onto Thiosulfate Citrate Bile Sucrose agar (TCBS) and MacConkey Tween 80 by streaking onto the plates with flaming after each streaking that would ensure that, discrete colonies were obtained after overnight incubation. All procedures were carried out aseptically. The inoculated plates were then incubated in an inverted position aerobically at 37 °C for between 18 to 24 hours.

The TCBS and MacConkey Tween 80 agar plates from day 2 were removed from the incubator and examined for medium sized yellow colonies while the MacConkey Tween

80 plate was re-incubated further at room temperature for two hours and was examined for non-lactose fermenting colonies with a halo appearance typical of *Aeromonas* spp. Characteristic colonies were picked and inoculated into biotyping media, Sulphur Indole Motility (S.I.M.) Simmons medium, citrates agar, Urea agar, Methyl red Voges proskauer (MRVP) medium and Triple Sugar Iron (T.S.I) as follows; Sulphur Indole Motility (S.I.M) was inoculated by stabbing in a single up-and-down motion in the centre of the agar keeping the wire as vertical as possible and without touching the bottom of the medium. Simmons citrate medium was inoculated by streaking the slanted surface of the agar. Methyl Red Voges Proskauer medium (MRVP) was inoculated using a sterile wire loop with a smooth colony of the test isolate. Urea agar tube was inoculated by stabbing 2-3 times into the agar and lastly, T.S.I agar slants in a tube were inoculated with test organisms. The butts were first stabbed with a single stab inoculation with up and down motion. After stabbing, slanted surface of agar was immediately streaked and the tube loosely capped. All the biochemical test tubes were loosely capped and incubated at 37°C aerobically overnight. The results were all interpreted using the Biochemical reaction chart for Enterobacteriaceae, *Aeromonas* and *Plesiomonas* (Krieg and Holt, 1984).

2.5 Confirmation of *Aeromonas* species

Further identification of the phenospecies of *Aeromonas* was done using API 20 NE (*appareils et procedes d'identification montalieu vercien,* France) system. The API 20 NE commercial test system is an identification system for Aeromonadaceae and other non-fastidious, non enteric gram negative rods not belonging to Enterobacteriaceae. It

uses 23 miniaturized biochemical tests and database. The strip consists of 20 microtubes containing dehydrated media substrates. The media are inoculated with a bacterial test suspension. In the incubation period the bacterial metabolism produces colour changes that are either spontaneous or revealed by the addition of specific reagents. The assimilation tests are inoculated with test organisms in the suspension medium and incubated at 37°C for between 18 to 24 hours.

The strips were removed the following day and read using the reference interpretation table (Appendix 2). All spontaneous reactions were recorded on the report sheet. One drop of James reagent was added to the TRP test. The immediate development of a pink colour in the whole cupule indicated a positive indole reaction, which was recorded on the result sheet. The reactions were read according to the interpretation table (Appendix 2) and the pattern of the reactions obtained was then coded into a numerical profile as indicated in the instruction manual. The strip was quality controlled by use of stock culture standard organisms (*Pseudomonas aeruginosa* ATCC 27853 and *Vibrio cholerae* ATCC 14035).

2.5.1 Cytochrome oxidase test

On a filter paper moistened with a drop of distilled water on a petri dish lid, a loopful of suspected colony was smeared into the reagent zone of the filter paper. A drop of OX (oxidase) reagent was then added onto the piece of filter paper. A deep purple colouration that appears within 10 to 60 seconds time period indicates a positive reaction. *Aeromonas*

are oxidase positive. The positive control included *Pseudomonas aeroginosa* ATCC 27853 while the Negative control was *E. coli* ATCC 25922.

2.6 Presumptive coliform test

All water samples were subjected to the presumptive coliform test (WHO, 1997). The foil protective cover was removed from the bottle and with the cap in position the bottle was shaken vigorously to achieve a homogeneous dispersion of bacteria. Using a sterile pipette, 50 ml of water sample containing 50 ml of MacConkey broth (double strength). Then, with a sterile 10 ml pipette, 10 ml volume of water sample was inoculated into each of 5 tubes containing 10 ml of MacConkey broth purple (double strength). Fifty millilitre of sample was added to a tube containing 50 ml of MacConkey broth purple (double strength) and 1 ml of water into each of the five tubes containing 5 ml MacConkey broth purple (single strength). Tubes were gently shaken to distribute the sample evenly ensuring that the Durham tubes were devoid of any air bubbles.

The tubes were inoculated at 37 °C for 24 hrs. After 24 hr incubation, each Durham tube was examined for presence of a gas due to fermentation as indicated by change of broth colour from purple to yellow. The number of positive tubes was recorded and negative tube re-incubated for a further 24 hrs period after which they were checked again for fermentation and gas production. Coliform organisms are able to ferment lactose at 37 °C with the production of acid, gas and indole within 24 hours. Therefore, acid and gas

production at the end of the 24 hours of incubation was presumed to be due to the presence of coliforms. The number of positive tubes after 24 hours was recorded and the result interpreted using McCrady's Probabilities Table (Appendix 3). Presence of coliform in chlorinated supplies was taken as indication of treatment failure or post treatment contamination (WHO, 1997).

2.6.1 Eijkman test for confirmation of faecal *E. coli*

All the tubes positive for the presumptive coliform test were subjected to the Eijkman test. Unlike other coliforms, *E. coli* is capable of fermenting lactose with subsequent production of gas and indole from tryptophan at 44 °C (WHO, 1997). Based on this a drop of broth was transferred from each presumptive positive tube into a tube containing 5ml single strength MacConkey broth and to a tube containing tryptone water, respectively. A different sterile pipette was used for each positive tube. The sub-cultured samples were incubated for 24 hours at 44 ± 1 °C. At the end of the 24 hours incubation, each tube was examined for fermentation and gas production (positive test) and the results recorded. To each of the tryptone water tubes, a drop of Kovac's reagent was added and gently mixed. The presence of *E. coli* was indicated by a red colour in the Kovac's reagent forming a film over the aqueous phase of the medium and indicated a positive indole reaction. Final identification of *E. coli* was done using its respective homologous antisera. McCrady Probability Table was used to interpret result and counts were expressed per 100 ml. The presence of *E. coli* was taken as an indication of recent

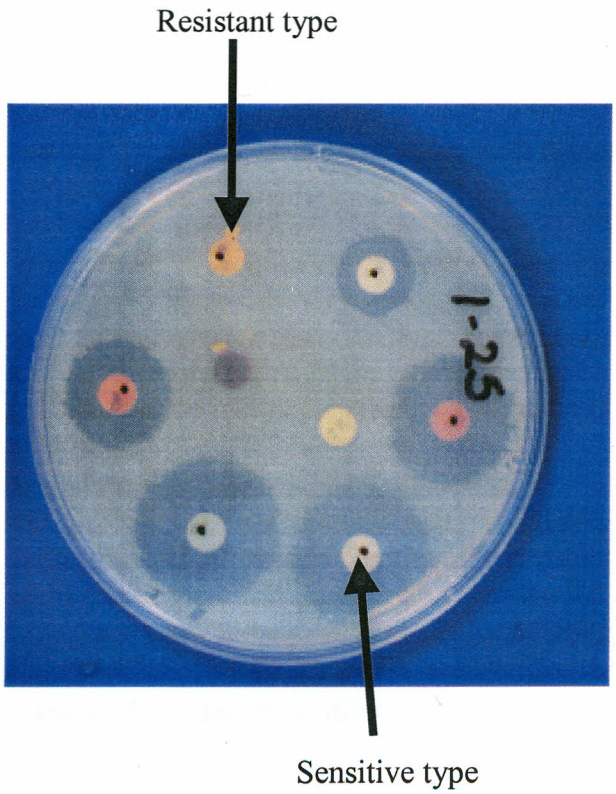
faecal contamination of either human or animal origin. *E. coli* was used as a positive control (WHO, 1997).

2.7 Antimicrobial Susceptibility Testing

2.7.1 Antibiotic sensitivity disks

The Kirby-Bauer (Bauer *et al.,* 1966) disk diffusion method was employed to test locally available antibiotics. Well isolated colonies from an agar plate culture, were touched with a sterile wire-loop and transferred to sterile saline to obtain turbidity visually comparable to that of Macfarland 0.5 standard and diluted ten times to a density of approximately 10^5 cfu/ml. A sterile cotton swab was dipped into the inoculum suspension and rotating the swab with firm pressure on the inside wall of the tube to remove excess fluid. The dried surface of Mueller-Hinton agar plate that had been brought to room temperature was inoculated by streaking the swab three times over the entire agar surface, rotating the plate at an approximate angle of 60 degrees to ensure an even distribution of the inoculum. The surface of the agar was allowed to dry before adding the antibiotic disks. Antimicrobial containing disks were gently placed and pressed to the agar surface by use of sterile forceps to ensure that firm contact was made with the agar. The inverted plate was incubated at 37 °C overnight and the result interpreted according to National Committee for Clinical Laboratory Standards (NCCLS 2003) as shown in Figure 4. The panel of antimicrobials were chosen because they are locally recommended, wide spread use for treatment of enteric fever in Kenya and importance in the treatment of Gram-

negative bacterial infection. The antibiotics included ampicillin, nalidixic acid, chloramphenicol, tetracycline, cefotaxime, co-trimoxazole, ceftazidime and



ciprofloxacin.

Figure 4: An agar culture plate showing sensitive and resistant types of *Aeromonas* isolates

2.7.2 Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of antimicrobials against the bacterial isolates was determined by the agar dilution technique as described by the special

standards (Isenberg, 1992; NCCLS, 2003). Pure antimicrobial powder of co-trimoxazole, tetracycline, ampicillin, chloramphenical, gentamicin and nalidixic acid were used to prepare doubling dilutions of the antibiotics in Mueller-Hinton agar (NCCLS, 2003). The concentrations to be tested were determined by the interpretive breakpoints as provided by NCCLS. MIC was the lowest concentration of the antimicrobials to prevent visible growth of the bacteria. The weight of pure powder to be used was calculated as:

$$\text{Weight (mg)} = \frac{\text{Volume (ml)} \times \text{Concentration } (\mu g/ml)}{\text{Assay potency } (\mu g/ml)}$$

The concentrations were prepared as doubling dilutions from the highest (256µg/ml) concentrations to the lowest (0.032 µg/ml). The stock solution was prepared as X 10 strength. Two millilitres of the stock solutions of the drugs was added to 18 ml of sterile Mueller-Hinton agar, cooled to 45 ^{0}C to give a final concentration of x1 as required. Serial two fold dilutions were then prepared in normal saline and 2 ml added to 18 ml of M-H agar as above. The plates were left on the bench overnight to control for media contamination. The isolates were grown in 2 ml of M-H broth at 37 ^{0}C overnight and 0.5 MacFarland turbidity standard prepared for each isolate in sterile normal saline. These were introduced to the multiple inoculating chambers and inoculated into the plates with the antimicrobials. The plates were then incubated at 37 ^{0}C overnight. The next day the plates were removed from the incubator and the minimum inhibitory concentration read

as the plate containing the lowest concentration of the respective antibiotic showing no visible growth.

2.7.3 Detection of haemolysis production

The production of the haemolysis by the isolated *Aeromonas* strains was tested on the following media: Rabbit blood agar, Sheep blood agar, Horse blood agar and Human blood agar all prepared at a concentration of 5 %. Haemolysin production were assayed by inoculating the test organism in Brain Heart Infusion broth for between 4 to 5 hours and incubated at 37 °C aerobically. Determination of the haemolysis was done by inoculating each isolate on all the 4 media and incubating the plates in an inverted position at 37 °C aerobically for 24 hours. The plates were removed from the incubator and examined for the presence of haemolysis around the colonies. Known positive (*Vibrio cholerae*) and negative (*Enterococcus faecalis*) strains of bacteria were included as control.

2.7.4 Toxin assay using DNA hybridization test

Enterotoxin assay was done according to the procedures described by Tamatsukuri (1991). Test organisms were inoculated into 4 ml of Lauria Bertani (LB) broth (Appendix 5) and incubated at 37 °C for 16 - 20 hours with shaking to achieve maximum aeration of the culture to increase the yield. One and a half millilitres of the culture was then

transferred to a micro tube. This was centrifuged at 15,000 rpm for 30 seconds. The tube was removed from the centrifuge and the supernatant discarded completely from the tube. The Bacterial pellet was re-suspended into 50 µl of sterilized de-ionised water and 5µl spotted on nylon membrane then air-dried. In a water bath set at 50 °C, 0.5 N NaOH, 1 % SDS and 5 X SSC were warmed. The nitrocellulose membrane was placed on Whatman 3 mm filter paper wetted with 0.5 N NaOH 1 % SDS. This was kept for 10 minutes to lyse the cells. The membrane was then transferred to Whatman 3mm filter paper wetted with 1 M Tris HCl pH 7.4 and kept for 1 minute to neutralize alkaline pH. This step was repeated twice with timing of 1 minute and 10 minutes. The cell debris from the membrane was gently rubbed with sponge in 5 X SSC 1 % SDS. The membrane was dried at room temperature for 1 hour and placed into a hybridization bag. The hybridization buffer and 2 X SSC 1 % SDS were warmed in a water bath at 50 °C. Two millilitre of the working hybridization buffer were prepared by adding 10 µl of the probe to 2 ml of hybridisation buffer (5µl of probe/ml). The working hybridization buffer was poured into a hybridization bag. The bubbles were removed and the hybridization bag sealed by heat and incubated in a water bath at 50 °C for 15 minutes. Then, the hybridization bag was removed from the water bath, opened and the membrane removed using forceps. The membrane was transferred to 100 ml of 2 X SSC 1 % SDS then incubated at room temperature for 10 minutes with gentle shaking (first washing) the membrane was transferred to about 100 ml of 1 X SSC 0.5 % Triton X 100 and then incubated at room temperature for 10 minutes with gentle shaking (the second washing). The substrate buffer 7.5 ml was prepared by adding 33 µl of NBT solution and 25 µl of BCIP solution to 7.5 ml of alkaline phosphates buffer. The membrane was then placed

into hybridization bag and the substrate buffer poured into the hybridization bag. The bubbles were removed and hybridisation bag sealed with heat. The sealed bag was incubated at 37 °C for 30 – 60 minutes to detect positive and negative reactions. The bag was removed from the incubator, opened, then the membrane washed with de-ionised water and kept dry.

2.7.5 Plasmid Analysis

2.7.5.1 Plasmid DNA extraction and detection

Plasmid DNA extraction and profiling was done for all the *Aeromonas* isolates from the 3 sites according to the method described by Birnboim and Doly (1979) (Appendix 4). The Aeromonads were inoculated into 4 ml of Lauria Bertani (LB) broth and incubated at 37 °C for 16 - 20 hours with a shaking at 120 cycles per minute. The shaking was used to provide maximum aeration to the culture to increase the yield. Solution 1 (B-1) and solution 11 (B-11) were prepared fresh before use (Appendix 4). One and a half millilitre of the sample was transferred to an Eppendorf microfuge. This was then centrifuged at 15,000 rpm for 30 seconds. The tube was removed from the centrifuge and the supernatant discarded to leave the bacterial pellet at the bottom of the microfuge. The pellet was re-suspended in 100 µl of ice-cold solution 1 (B-1) vortexed and then incubated on ice for 10 minutes. A 200 µl of solution 11 (B-11) was added, mixed by vortexing and incubated on ice for 5 minutes. A 150 µl of 3 M Sodium acetate, pH 5.2 (B-111) was added to the microfuge and contents mixed by inverting the microfuge

several times and incubated on ice for 10 minutes and centrifuged at 15000 rpm for 10 minutes. The supernatant (about 400 µl) was transferred to a fresh sterile microfuge leaving a small pellet in the tube and 1000 µl of cold ethanol added to precipitate the DNA. The content was mixed by inverting the microfuge severally. The tube was incubated at – 80 °C for 10 minutes and centrifuged at 15,000 rpm for 10 minutes. The supernatant was discarded and the pellet dissolved in 200 µl of Tris ethylene diamine-tetra-acetic acid (TE) buffer and 100 µl of 7.5 M Ammonium acetate added and incubated on ice for 30 minutes. The mixture was centrifuged at 15,000 rpm for 10 minutes and the supernatant (300 µl) transferred to a new microfuge. Cold ethanol (800 µl) was added, the content mixed by inverting the tubes severally and incubated at –80 °C for 10 minutes. The mixture was removed and immediately centrifuged at 15,000 rpm for 10 minute and the supernatant discarded. The DNA pellet was rinsed with 1 ml of 80 % ethanol and centrifuged at 15,000 rpm for 5 minutes. The supernatant was gently discarded by aspiration. The DNA pellet obtained was dried at 37 °C incubator for 30 minutes and dissolved in 50 µl TE buffer by vortexing and stored at -20°C until analysed.

2.7.5.2 Gel electrophoresis

The DNA was removed from the – 20 °C and allowed to thaw at room temperature. A 1 % agarose gel was prepared by dissolving 1g of agarose in 100 ml of 1 X TE buffer. The agarose was cooled to about 45 °C and poured into the gel container with the combs in place. The gel was left to set for 30 minutes. A 1:10 solution of Tris Borate ethylene diamine-tetra-acetic acid (TBE) electrophoresis buffer was prepared and poured into the

electrophoresis tank and the gel introduced. A 10 µl plasmid DNA solution and 5 µl stop mix were loaded into each well of the gel. Lanes 1 and 14 of the gel were loaded with the plasmid DNA of *Escherichia coli* V517 and *E. coli* 39R861 strains (Macrina *et al.,* 1978) respectively, which are plasmids molecular markers of known molecular sizes. The gel was allowed to run for approximately 1.5 hours. The gel was stained with ethidium bromide (10 mg/ml) in a plastic tray for 5 minutes and washed with running tap water visualised under UV and photographed with a Polaroid instant camera.

2.7.5.3 Transconjugation assays

In vitro conjugation tests on transferable antimicrobial resistance were performed according to the method of Walia *et al*; (1987). Single discreet colonies of each donor bacterial strain and recipient *E. coli* K12 (F^-Na^r Lac^+) strain were sub cultured into 5 ml Tryptic Soy Broth (Difco) and allowed to multiply to the logarithmic phase (ca. 10 cells) by incubating on a shaker (250 rpm) at 37 °C for 2 hours. The donor and the recipient bacterial broth cultures were diluted 1:10 in fresh Tryptic Soy Broth and mixed in equal proportions to make a 5 ml broth culture. The mixture was incubated at 37 °C and conjugation allowed to take place overnight without shaking. The culture was flush centrifuged (15,000 rpm for 5 seconds) and media discarded. The pellet was washed once with normal saline (0.85 % sodium chloride solution in distilled water). This was used to wash out any β-Lactamases which might have leaked into the media from the bacterial cell and which might give false resistance. To select transconjugants, 3 µl samples were drawn from the overnight culture and plated onto MacConkey agar plate, containing 32

μg/ml nalidixic acid and 32 μg/ml ampicillin, using a multiple inoculator. The agar plates were then incubated at 37 °C overnight. In order to determine what antibiotic resistances co-transferred to recipient *E. coli* K 12 strain, antimicrobial disk susceptibly tests for each of the transconjugants were performed as described on section 2.7.1.

2.8 Chemical Analysis

2.8.1 Sodium

The concentration of sodium was determined using an Atomic Absorption Emission Spectrophotometer (AAS). The flame emission was used since the element emits characteristic flame of various intensities.

2.8.2 Chloride

The MOHR method was used whereby the chloride solution containing Chromate was titrated with Silver nitrate. Silver chloride precipitates at end point. Known volume (50 ml) in porcelain dish with 1 ml 5% potassium chromate and titrated with standard silver nitrate solution (1 ml $AgNo_3$ = 1 mg chloride) until the slightest reddish colour appears (due to excess formation of silver chromate).

2.8.3 Nitrate

Nitrate reacts with phenoldisulphonic acid to produce 6-nitrophenoldisulphonic acid which upon conversion to the alkaline salt (using 10% ammonium hydroxide) yields a yellow colour which was determined colorimetrically using a nitrate disk in a lovibond comparator.

2.8.4 Fluoride

Fluoride exerts a decolourising action proportional to the quantity of fluoride on the complex of zirconium and alizarin, which was determined colorimetrically.

2.8.5 Lead

This element was determined using AAS (Atomic absorption Emission Spectrophotometer) absorption method test.

2.8.6 Total Dissolved Solids / Electrical conductivity

The electrical conductivity of water was related to its concentration of dissolved mineral matter (T.O.S.). A lovibond datromix conductivity meter was used and reading in siemens.

2.9 Data analysis

Descriptive data such as variation and prevalence of *Aeromonas* species within the three locations was analyzed using Chi-square SPSS (Statistical Package for the Social Sciences) version 11.5. The sensitivity profiles and the quantitative data such as variation in minimum inhibition concentrations among *Aeromonas* species were analyzed using student t test in SPSS.

CHAPTER THREE

RESULTS

3.1 Distribution of *Aeromonas* species

A total of 87 *Aeromonas* species were isolated from different ground water sampled from Ongata Rongai, Lamu and Wajir. Forty (46 %) of the 87 *Aeromonas* species were isolated from Wajir while 27 (31 %) and 20 (23 %) were isolated from Ongata Rongai and Lamu, respectively. Three *Aeromonas* species were identified; these were 51 (58.6 %) *Aeromonas hydrophila* and 20 (74.1 %) of these were isolated from Ongata Rongai, 10 (50 %) and 21 (52.5 %) were from Lamu and Wajir, respectively. Twenty one (24.1 %) of the isolates were *A. caviae* distributed as follows; 4 (14.8 %), 3 (15 %) and 14 (35 %) from Ongata Rongai, Lamu and Wajir, respectively. Fifteen (17.2 %) of the isolates were *A. sobria*, 3 (11.1 %) were isolated from Ongata Rongai, while 7 (35 %) and 15 (17.2 %) were from Lamu and Wajir, respectively (Table 1). *Aeromonas hydrophila* were more prevalent species. Ground water from Wajir had highest prevalence of *Aeromonas* species ($\chi^2 = 9.997$; df = 4; P = 0.04).

Table 1: The distribution and prevalence of *Aeromonas* species in the three study locations

ISOLATES	LOCATION			Total
	Ongata Rongai	Lamu	Wajir	
A. sobria	3 (11.1 %)	7 (35 %)	5 (12.5 %)	15 (17.2 %)
A. caviae	4 (14.8 %)	3 (15 %)	14 (35 %)	21 (24.1 %)
A. hydrophila	20 (74.1 %)	10 (50 %)	21 (52.5 %)	51 (58.6 %)
TOTAL	27 (31%)	20 (23%)	40 (46%)	87 (100%)

3.1.1 Antimicrobial sensitivity profile for *A. hydrophila* isolates

All isolates of *A. hydrophila* from Ongata Rongai, Lamu and Wajir, were sensitive to nalidixic acid, chloramphenicol, cefotaxime, ceftazidime and ciprofloxacin. However, 18 out of 20 (90 %), 9 out of 10 (90 %) and 20 out of 21 (95.2 %) *A. hydrophila* isolated from Ongata Rongai, Lamu and Wajir respectively, were sensitive to tetracycline and the sensitivity patterns were significantly different within the three location ($t = 4.631$; $df = 2$; $P = 0.044$). Similarly, 18 (90 %), 9 (90 %) and 18 (85.7 %) *A. hydrophila* from Ongata Rongai, Lamu and Wajir respectively, were sensitive to co-trimoxazole. The sensitivity patterns were significantly different within the three locations ($t = 5.0$; $df = 2$; $P = 0.038$).

All the *A. hydrophila* isolated from the three locations were resistant to ampicillin. However, 2 (10 %), 1 (10 %) and 1 (4.7 %) *A. hydrophila* isolated from Ongata Rongai, Lamu and Wajir respectively were resistant to tetracycline. However the resistance patterns were not significantly different within the three locations (t = 4; df = 2; P = 0.057). Similarly 2 (10 %), 1 (10 %) and 3 (14.3 %) *A. hydrophila* isolated from Ongata Rongai, Lamu and Wajir respectively, were resistant to co-trimoxazole. However, the resistance patterns were again not significantly different within the location of isolation (t = 3.464; df = 2; P = 0.074) as shown in Figure 5.

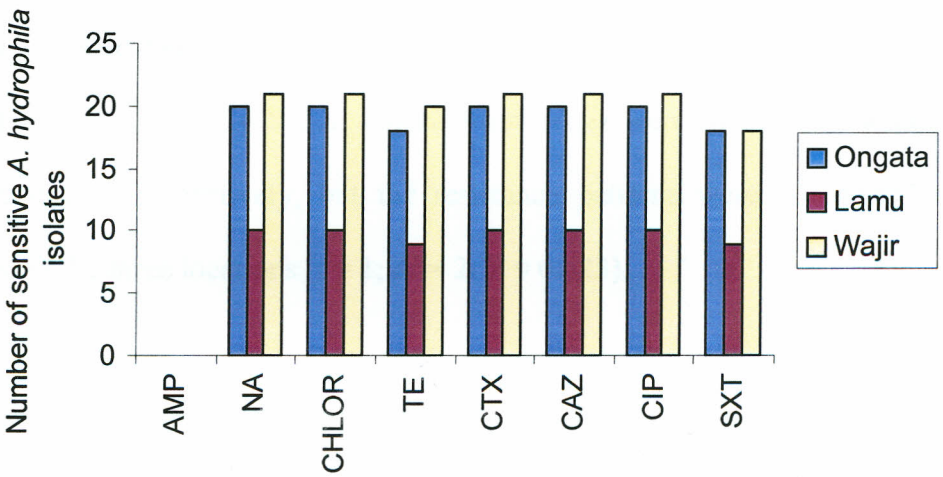


Figure 5: The sensitivity profile of *A. hydrophila* isolates obtained from Ongata Rongai, Lamu and Wajir. (Amp- Ampicillin, NA- Nalidixic Acid, CHLOR-Chloramphenicol,TE-Tetracycline,CTX-Cefotaxime,CAZ-Ceftazidime,CIP-Ciprofloxacin, SXT- Sulphamethaxazole/Trimethroprim)

3.1.2 Antimicrobial sensitivity profile for *A. caviae* isolates

Four (100 %), 3 (100 %) and 14 (100 %) *A. caviae* isolated from Ongata Rongai, Lamu and Wajir respectively, were sensitive to nalidixic acid, chloramphenicol, cefotaxime, ceftazidime and ciprofloxacin. Four (100 %) and 3 (100 %) and 11 (78.6 %) *A. caviae* isolated from Ongata Rongai, Lamu and Wajir respectively were sensitive to tetracycline and co-trimoxazole as shown in Figure 6. The sensitivity patterns were not significantly different among the three locations ($t = 2.384$; $df = 2$; $P = 0.14$).

All the *A. caviae* isolated from the three locations were resistant to ampicillin. Three (21.4 %) and 4 (28.6 %) *A. caviae* from Wajir were resistant to tetracycline and co-trimoxazole respectively, but the resistance patterns were not significantly different among the three locations ($t = 1$; $df = 2$; $P = 0.423$).

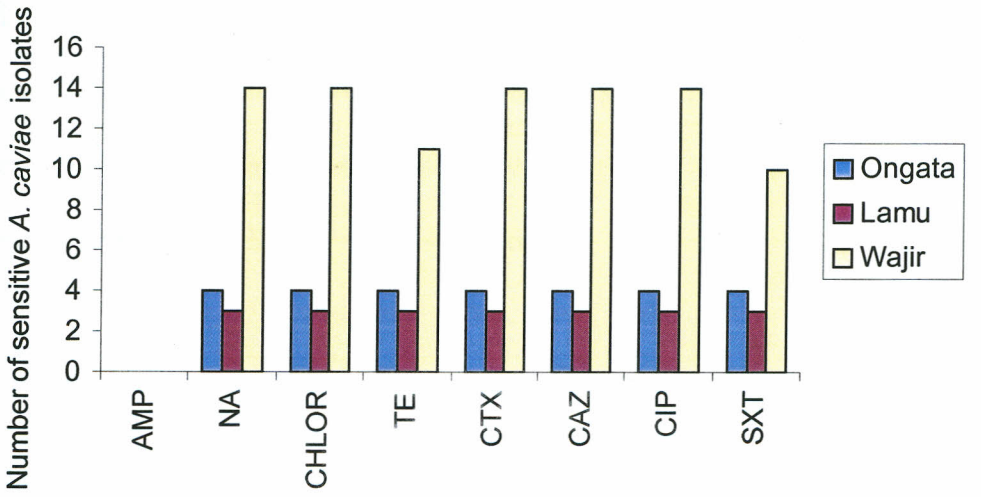


Figure 6: The sensitivity profile of *A. caviae* isolates obtained from Ongata Rongai, Lamu and Wajir. (Amp- Ampicillin, NA- Nalidixic Acid, CHLOR-Chloramphenicol,TE-Tetracycline,CTX-Cefotaxime,CAZ-Ceftazidime,CIP-Ciprofloxacin ,SXT- Sulphamethaxazole/Trimethroprim)

3.1.3 Antimicrobial sensitivity profile for *A. sobria* isolates

Figure 7 shows the sensitivity profiles of *A. sobria* from the three locations. The 3 (100 %), 7 (100 %) and 5 (100 %) *A. sobria* isolated from Ongata Rongai, Lamu and Wajir respectively, were sensitive to nalidixic acid, chloramphenical, cefotaxime, ceftazidime and ciprofloxacin. Two (28.6 %) and 5 (100 %) *A. sobria* isolated from Lamu and Wajir respectively, were sensitive to tetracycline and co-trimoxazole. The sensitivity pattern was not significantly different among the three locations ($t = 1.606$; $df = 2$; $P = 0.250$).

All the *A. sobria* isolated from the three locations were resistant to ampicillin. Three (100 %) and 5 (71 %) *A. sobria* from Ongata Rongai and Lamu respectively, were resistant to tetracycline and co-trimoxazole. The patterns were not significantly different among the three locations (t = 1.835; df = 2; P = 0.208).

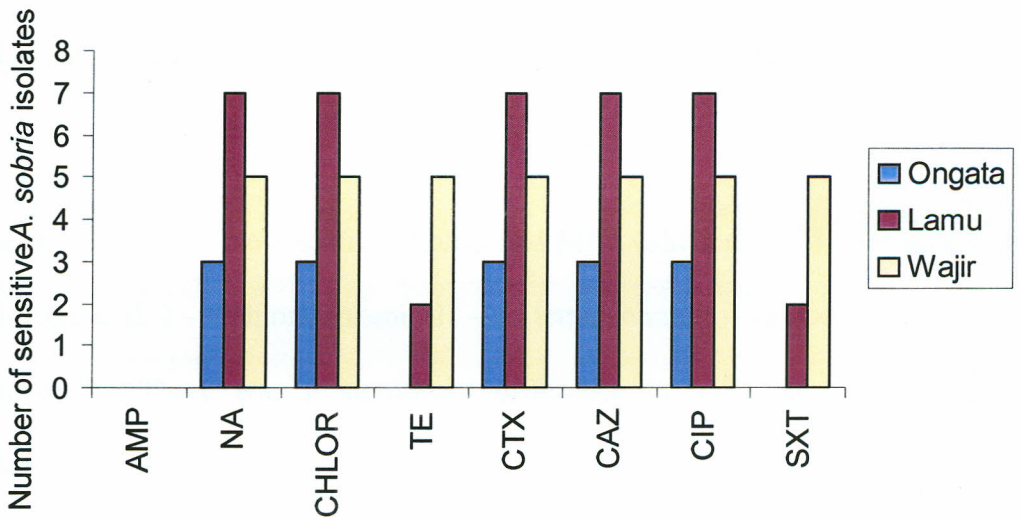


Figure 7: The sensitivity profile of *A. sobria* isolates obtained from Ongata Rongai, Lamu and Wajir. (Amp- Ampicillin, NA- Nalidixic Acid, CHLOR-Chloramphenicol,TE-Tetracycline,CTX-Cefotaxime,CAZ-Ceftazidime,CIP-Ciprofloxacin ,SXT- Sulphamethaxazole/Trimethroprim

3.2 Minimum Inhibitory Concentration

3.2.1 Minimum Inhibitory Concentration for *A. hydrophila* isolates

The MIC (mg/ml) of ampicillin for *A. hydrophila* isolated from Ongata Rongai were all > 256, 0.5 – 8 nalidixic acid, 2 – 8 chloramphenical, 2 – 6 tetracycline, 4 – 128 co-trimoxazole and 0.25 – 4 gentamicin. The MIC values were significantly different against nalidixic acid, chloramphenical, tetracycline, co-trimoxazole, and gentamicin ($t = 5.785$; $df = 19$; $P = 0.0001$), ($t = 7.764$; $df = 19$; $P = 0.0001$), ($t = 2.218$; $df = 19$; $P = 0.039$), ($t = 2.256$; $df = 19$; $P = 0.036$), and ($t = 9.181$; $df = 19$; $P = 0.0001$), respectively.

The *A. hydrophila* isolated from Lamu had MIC values of > 256 for ampicillin, 2 – 4 nalidixic acid, 2 – 8 chloramphenical1 – 64 tetracycline, 8 – 128 co-trimoxazole and 1 – 4 gentamicin. There was a significant difference in MIC values for nalidixic acid, chloramphenical and gentamicin ($t = 6.328$; $df = 9$; $P = 0.0001$), ($t = 5.477$; $df = 9$; $P = 0.0001$), and ($t = 8.5$; $df = 9$; $P = 0.0001$) respectively. However, the MIC values for *A. hydrophila* from Lamu against tetracycline and co-trimoxazole were not significantly different ($t = 1.45$; $df = 9$; $P = 0.181$) and ($t = 1.667$; $df = 9$; $P = 0.181$).

The MIC values of *A. hydrophila* isolated from Wajir were > 256 for ampicillin, 1 – 8 nalidixic acid, 2 – 64 chloramphenical 4 – 128 tetracycline, 4 – 128 co-trimoxazole and 2 – 4 gentamicin (Table 2). The MIC values were significantly different for nalidixic acid, chloramphenical co-trimoxazole and gentamicin ($t = 11.827$; $df = 20$; $P = 0.0001$), ($t =$

3.061; df = 20; P = 0.006), (t = 3.446; df = 20; P = 0.02), and (t = 11.533; df = 20; P = 0.063), respectively. However, no significant difference in MIC values for tetracycline was observed (t = 1.963; df = 20; P = 0.063).

Table 2. The MIC (mg/ml) values for various antibiotics tested against *A. hydrophila* from the three study locations

Antibiotic	MIC values (mg/ml)		
	Ongata Rongai	**Lamu**	**Wajir**
Ampicillin	> 256	> 256	> 256
Nalidixic acid	0.5 – 8	2 – 4	1 – 8
Chloramphenical	2 – 8	2 – 8	2 – 64
Tetracycline	2 – 64	1 – 64	4 – 128
Co-trimoxazole	4 – 128	8 – 128	4 – 128
Gentamicin	0.25 - 4	1 - 4	2 - 4

3.2.2 Minimum Inhibitory Concentration for *A. sobria*

The MIC (mg/ml) values for ampicillin for *A. sobria* isolated from Ongata Rongai were > 256, 1 – 4 nalidixic acid, 2 – 8 chloramphenical 4 – 64 tetracycline, 8 – 128 co-trimoxazole and 2 – 4 gentamicin. There was no significant difference in the MIC values against nalidixic acid, chloramphenical, tetracycline, co-trimoxazole and gentamicin ($t = 2$; $df = 2$; $P = 0.184$), ($t = 2$; $df = 2$; $P = 0.184$), ($t = 2.2$; $df = 2$; $P = 0.159$), ($t = 2.2$; $df = 2$; $P = 0.159$) and ($t = 1.429$; $df = 2$; $P = 0.289$), respectively.

The MIC values for ampicillin for A. *sobria* isolated from Lamu were > 256, 4 – 8 nalidixic acid, 4 – 8 chloramphenical 2 – 256 tetracycline, 4 – 128 co-trimoxazole and 2 – 4 gentamicin. There was a significant difference in MIC values against nalidixic acid, chloramphenical, tetracycline, co-trimoxazole and gentamicin ($t = 8$; $df = 6$; $P = 0.001$) ($t = 7.071$; $df = 6$; $P = 0.0001$), ($t = 2.526$; $df = 6$; $P = 0.048$), ($t = 4.048$; $df = 6$; $P = 0.007$) and ($t = 7.778$; $df = 6$; $P = 0.0001$), respectively.

The MIC values for ampicillin against *A. sobria* isolated from Wajir were > 256, 4 – 8 nalidixic acid, 2 – 8 chloramphenical 4 tetracycline, 8 – 128 co-trimoxazole and 2 – 4 gentamicin (Table 3). The MIC values were significantly different against nalidixic acid, chloramphenical and gentamicin ($t = 3.667$; $df = 3$; $P = 0.035$), ($t = 4.333$; $df = 3$; $P = 0.023$), and ($t = 5$; $df = 3$; $P = 0.015$) respectively. However the MIC values were not significantly different against co-trimoxazole ($t = 1.267$; $DF = 3$; $P = 0.295$).

Table 3. The MIC (mg/ml) values for various antibiotics tested against *A. sobria* from the three study locations

Antibiotic	MIC values (mg/ml)		
	Ongata Rongai	**Lamu**	**Wajir**
Ampicillin	> 256	> 256	> 256
Nalidixic acid	1 – 4	4 – 8	4 – 8
Chloramphenical	2 – 8	4 – 8	2 – 8
Tetracycline,	4 – 64	2 – 256	4
Co-trimoxazole	8 – 128	4 – 128	8 – 128
Gentamicin	2 - 4	2 - 4	2 - 4

3.2.3 Minimum Inhibitory Concentration for antibiotics tested against *A. caviae*

The MIC (mg/ml) values for ampicillin against *A. caviae* isolated from Ongata Rongai were > 256, 2 – 4 nalidixic acid, 4 – 8 chloramphenical 2 – 4 tetracycline, 4 – 8 co-trimoxazole and 2 – 4 gentamicin. The MIC values for nalidixic acid, chloramphenical, tetracycline, co-trimoxazole and gentamicin were significantly different ($t = 6.708$ $df = 5$; $P = 0.001$), ($t = 7$; $df = 5$; $P = 0.0001$), ($t = 7.906$; $df = 5$; $P = 0.001$), ($t = 7.906$; $df = 2$; $P = 0.001$) and ($t = 6.708$; $df = 5$; $P = 0.001$) respectively.

The MIC value for ampicillin against *A. caviae* isolated from Lamu were > 256 , 1 – 4 nalidixic acid, 2 – 4 chloramphenical 4 tetracycline, 8 co-trimoxazole and 4 gentamicin. The MIC values were not significantly different against nalidixic acid ($t = 5$; $df = 2$; $P = 0.184$). However, the MIC values differed significantly against chloramphenical ($t = 5$; $df = 2$; $P = 0.007$).

The MIC values for ampicillin against *A. caviae* isolated from Wajir were > 256 , 1 – 8 nalidixic acid, 1 – 8 chloramphenical 4 – 128 tetracycline, 4 – 8 co-trimoxazole and 2 – 4 gentamicin as shown in Table 4. The MIC values were significantly different against nalidixic acid, chloramphenical and gentamicin ($t = 3.667$; $df = 3$; $P = 0.035$), ($t = 4.333$; $df = 3$; $P = 0.023$), and ($t = 5$; $df = 3$; $P = 0.015$), respectively. However, the MIC values were not significantly different against co-trimoxazole ($t = 1.267$; $df = 3$; $P = 0.295$).

Table 4. The MIC (mg/ml) values for various antibiotics tested against *A. caviae* from the three study locations

Antibiotic	MIC values (mg/ml)		
	Ongata Rongai	Lamu	Wajir
Ampicillin	> 256	> 256	> 256
Nalidixic acid	2 – 4	1 – 4	1 – 8
Chloramphenical	4 – 8	2 – 4	1 – 8
Tetracycline,	2 – 4	4	4 – 128
Co-trimoxazole	4 – 8	8	4 – 128
Gentamicin	2 - 4	4	2 - 4

3.3 Antibiotic susceptibility patterns of the *Aeromonas* isolates

Frequency of resistance in *Aeromonas spp* was highest against ampicillin which accounted for 66 (77.9 %) of all the *Aeromonas isolates*.Thirteen (15.1%), 4 (4.6%), 1(1.2%) and 1 (1.2%) Aeromonas isolates were resistant to ampicillin / tetracycline / cotrimoxazole, ampicillin / cotrimoxazole, ampicillin / tetracycline and ampicillin / chloramphenicol / tetracycline / cotrimoxazole respectively (table 5). None of the isolates were sensitive to all antibiotics tested.

Table 5. The frequency of antibiotic resistant types among the *Aeromonas* isolates

Resistant type	Frequency	% Frequency
AMP	67	77.9
AMP/TE/SXT	13	15.1
AMP/SXT	4	4.6
AMP/TE	1	1.2
AMP/CHLOR/TE/SXT	1	1.2

AMP - ampicillin, TE – tetracycline, SXT - co-trimoxazole, CHLOR - chloramphenical

3.4 Plasmids from Aeromonads

All the *Aeromonas* isolated were analysed for the presence of plasmids (Fig. 8). Small and large plasmids were identified with fragment size ranging from 6 to > 100 and 46 Mega dalton (MDa). Two common plasmid profiles were observed. The most frequent (9) plasmids had fragment sizes 51, 7.2 and >100 MDa. The common small plasmids had the fragment size of 7.2 MDa while the medium sized plasmids were 51, 7.2 and the largest common plasmid were > 100 MDa. Thirty seven (42.5 %) isolates had no plasmid detected as shown in Table 6.

Table 6: The frequency of different plasmid fragment sizes (Mega dalton) from *Aeromonas* isolates

Plasmids	Frequency
6	1
7.2	4
7.2, 6	3
46	2
51, 7.2	9
51, 42, 7.2	1
51, 44, 17	1
51, > 100	4
56	5
58, 47	1
> 100, 41, 6	6
> 100	9
> 100, 6	3
> 100, 46	1
No. Plasmid	37
Total	87

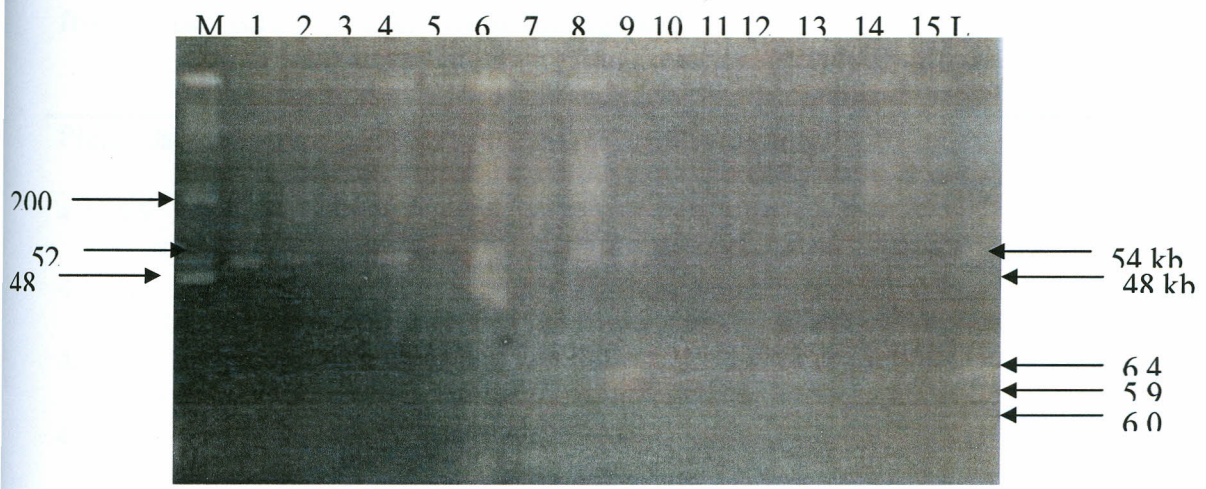


Figure 8: Ethidium bromide stained 1.5 % agarose gel illustrating different plasmids extracted from *Aeromonas* bacterium. Samples 4, 6, 8 and 9 have different plasmid of varying fragment sizes. M is *E. coli* 39R 861 while L is V517 *E. coli* used as markers

3.5 Plasmid sizes of transconjugant *E. coli* K12 F^- NA^r Lac^+

All the 87 *Aeromonas* isolates were subjected to conjugation using standard strain (*E. coli* K12 F^- NA^r Lac^+). Sixty six (75.9 %) *Aeromonas* isolates transferred resistance to E. *coli*. Plasmids of fragment sizes 4.5, 4.9 to 58 MDa were identified as shown in Figure 9. The smallest common plasmid were 4.5, 4.9 to 6 MDa in size with frequency of 1. The medium sized plasmid with frequency of 4 had fragment size of 47 MDa, while the largest common plasmid had 56 MDa fragment size with a frequency of 8. Ten isolates could not transfer any resistance to *E.Coli* K12 (Table 7).

Table 7: The frequency of different plasmid fragment sizes (Mega dalton) isolated from *E. coli* K12 F^- NA^r LA after conjugation

Plasmids	Frequency
4.5, 4.9	1
4.9, 5.3	1
46	1
47	4
47, 6	2
48	1
49	7
49, 6	1
6	1
51	16
51, 4.6	1
54	4
54, 6	3
56	8
56, 6	1
58	4
No. Conjugation	10
TOTAL	66

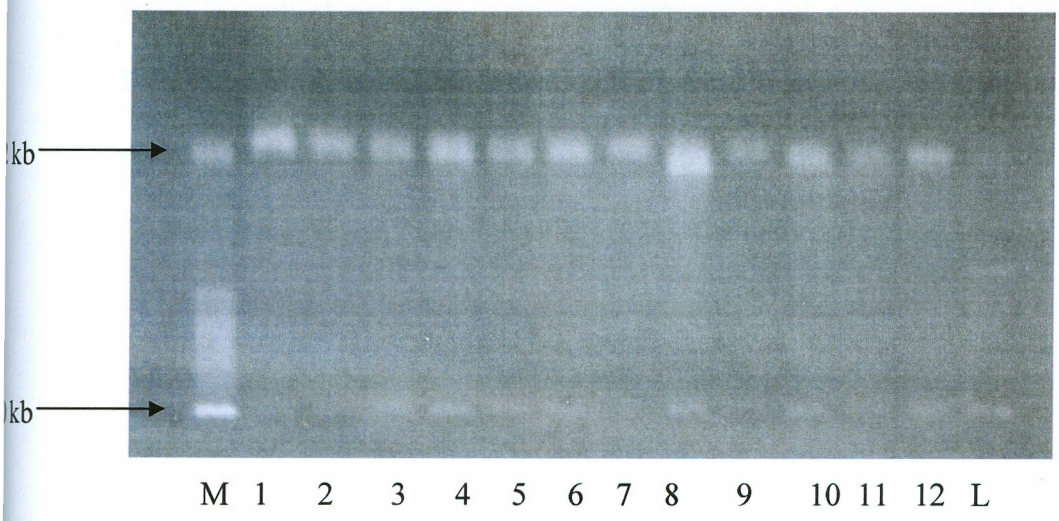


Figure 9: Ethidium bromide stained 1.5 % agarose gel illustrating different plasmids extracted from *Aeromonas* transconjugant bacterium. Samples 1 - 12 have different plasmids of varying fragment sizes. M is *E. coli* 39R 861 while L is V517 *E. coli* used as markers

3.6 Antimicrobial sensitivity profile of transconjugants

3.6.1 Antimicrobial sensitivity profile for *E.coli* transconjugants for *A.hydrophila*

All the 18 (100 %), 8 (100 %) and 17 (100 %) *E.coli* transconjugants for A.hydrophila for isolates from Ongata Rongai, Lamu and Wajir respectively, were sensitive to cefotaxime, ceftazidime and ciprofloxacin. Sixteen (94.1 %) transconjugants for isolates from Wajir were sensitive to chloramphenical. The sensitivity profile was significantly different for isolates from the three locations ($t = 4.583$; $df = 2$; $P = 0.044$). However, 17 (94.4 %), 7

(87.5 %) and 16 (94.4 %) *E.coli* transconjugants for *A.hydrophila* from Ongata Rongai, Lamu and Wajir respectively were sensitive to tetracycline. The sensitivity patterns were not significantly different with the location of isolation ($t = 4.193$; $df = 2$; $P = 0.052$). Sixteen (88.9 %), 7 (87.5 %) and 14 (77.8 %) from Ongata Rongai, Lamu and Wajir respectively, were sensitive to co-trimoxazole and the patterns were significantly different among the three locations of isolation ($t = 4.520$; $df = 2$; $P = 0.046$).

All the *E.coli* transconjugants for *A.hydrophila* were resistant to ampicillin and nalidixic acid. One (14.3 %) transcojugant for isolate from Wajir was resistant to chloramphenical and the patterns were not significantly different among the three locations ($t = 1$; $df = 2$; $P = 0.423$). Similarly, 2 (11.1 %), 1 (12.5) and 3 (17.6 %) *A. hydrophila* isolates from Ongata Rongai, Lamu and Wajir respectively were resistant to co-trimoxazole. However, the resistant profile was not significantly different ($t = 3.464$; $df = 2$; $P = 0.074$) (Fig. 10).

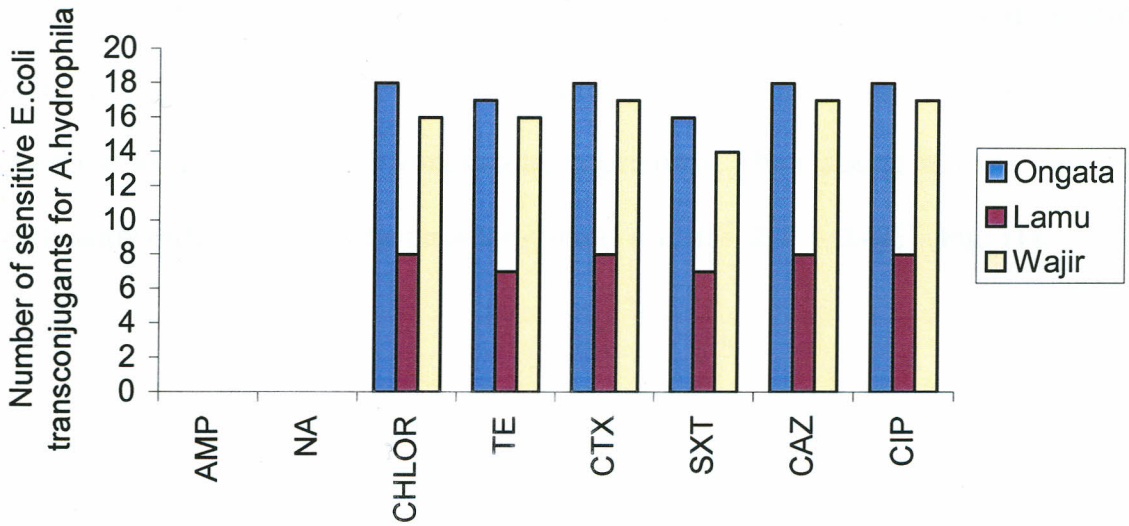


Figure 10: Sensitivity profile of *Ecoli* transconjugants *A.hydrophila* isolated from Ongata Rongai, Lamu and Wajir.

3.6.2 Antimicrobial sensitivity profile for *E.coli* transconjugants for *A.caviae*

All the 3 (100 %), 2 (100 %) and 8 (100 %) *E.coli* transconjugants for A.caviae isolated from different water wells and boreholes in Ongata Rongai, Lamu and Wajir respectively, were sensitive to chloramphenical, cefotaxime, ceftazidime and ciprofloxacin. Similarly, 3 (100 %), 2 (100 %) and 7 (87.5 %) *E.coli* transcojugants for *A. caviae* isolate from Ongata Rongai, Lamu and Wajir were sensitive to tetracycline. However, the sensitivity patterns were not significantly different regardless of the location of isolation ($t = 2.619$; $df = 2$; $P = 0.12$). While 3 (100 %), 2 (100 %) and 6 (75 %) *A. caviae* isolates from Ongata Rongai, Lamu and Wajir respectively were sensitive to co-trimoxazole. The sensitivity patterns were not significantly different ($t = 3.051$; $df = 2$; $P = 0.093$).

All the E.coli transcojugants for *A. caviae* were resistant to ampicillin and nalidixic acid. However, 1 (12.5 %) and 2 (25 %) E.coli transcojugants for *A. caviae* from Wajir were resistant to tetracycline and co-trimoxazole, respectively. The resistant patterns were not significantly different within the three locations (t = 1; df = 2; P = 0.423) (Fig. 11).

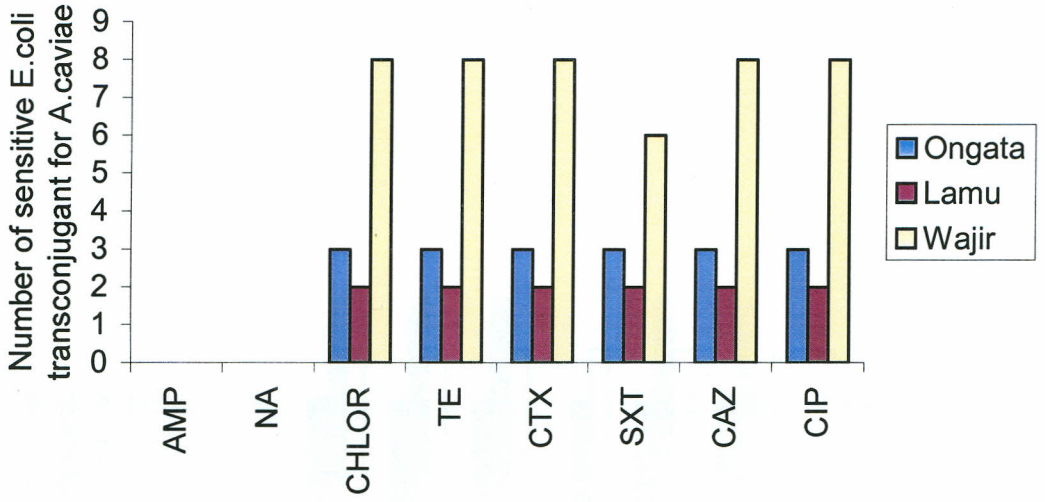


Figure 11: Sensitivity profile of *E.coli* transcojugants for *A. caviae* isolated from Ongata Rongai, Lamu and Wajir

3.6.3 Antimicrobial sensitivity profile for *E.coli* transcojugants for *A. sobria*

All the 3 (100 %), 5 (100 %) and 4 (100 %) *E.coli* transcojugant for *A. sobria* isolated from Ongata Rongai, Lamu and Wajir respectively were sensitive to chloramphenical, cefotaxime, ceftazidime and ciprofloxacin. The sensitivity patterns were significantly

different within the three locations (t = 6.928; df = 2; P = 0.02). Similarly, 1 (33.3 %), 3 (60 %) and 4 (100 %) E.coli transconjugant from Ongata Rongai, Lamu and Wajir respectively, were sensitive to tetracycline and co-trimoxazole. The sensitivity patterns were not significantly different within the three locations (t = 2.774; df = 2; P = 0.109) and (t = 3.024; df = 2; P = 0.094), respectively. All the E.coli transcojugant for *A. sobria* from the three locations were resistant to ampicillin and nalidixic acid and differed significantly within the three locations of isolation (t = 6.928; df = 2; P = 0.02) (Fig 12)

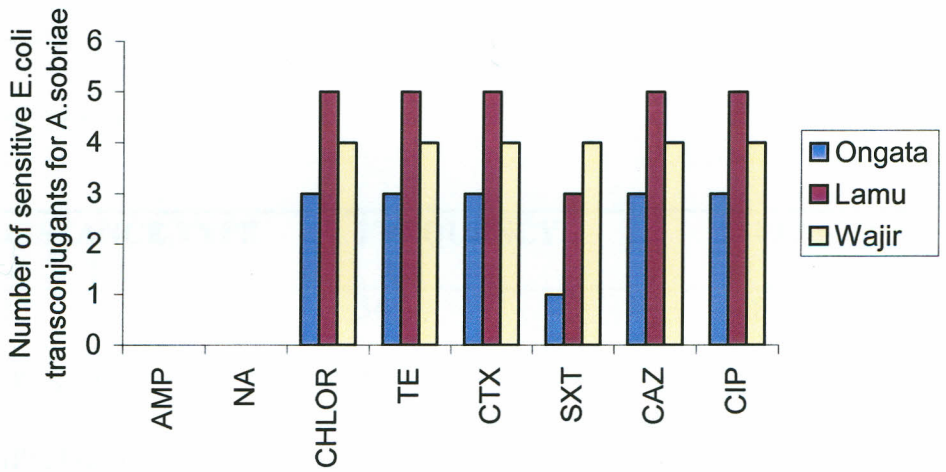


Figure 12: Sensitivity profile of *E.coli* transcojugant for *A. sobria* isolated from Ongata Rongai, Lamu and Wajir

3.7 Antibiotic resistant types of the Aeromonas transconjugants

Four different resistant types were observed among the Aeromonas transconjugants.Fifty four (81.8%), 6 (9.1%), 5 (7.6%) and 1 (1.5%) Aeromonass transconjugants were resistant to ampicillin, ampicillin/tetracycline/cotrimoxazole, ampicillin/cotrimoxazole and ampicillin/chloramphenical/tetracycline/cotrimoxazole respectively (Table 8).None of the isolates were sensitive to all the antibiotics tested.The most common resistant type was ampicillin.

Table 8: The frequency of resistant types of the *E. coli* K12 F^- NA^r Lac^+ after conjugation

RESISTANCE TYPE	FREQUENCY	PERCENT
AMP	54	81.8
AMP/ SXT	5	7.6
AMP/CHLOR/TE/SXT	1	1.5
AMP/TE/SXT	6	9.1

AMP - ampicillin, TE – tetracycline, SXT - co-trimoxazole, CHLOR - chloramphenical

3.8 Toxin assays

3.8.1 Haemolytic patterns of the *Aeromonas* isolates

All the 87 *Aeromonas* isolates were tested for the production of haemolysin on 4 different types of blood (sheep, horse, rabbit and human) (Fig. 13). Seventy seven (88 %) of the isolates exhibited beta (β) haemolysis. The remaining 10 (12 %) had no haemolytic activity (Table 9).

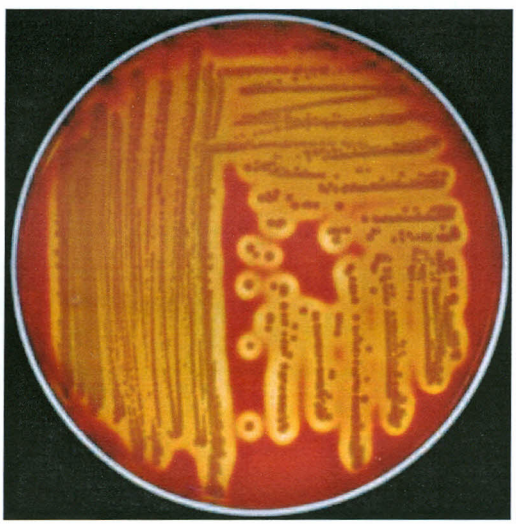


Figure 13: A sheep blood agar plate showing the beta (β) haemolytic activity of an *Aeromonas* isolate

Table 9: The levels of haemolytic activity of *Aeromonas* isolates from the three study locations.

Isolates	Location	β- Haemolytic	Non haemolytic
A. hydrophila	Ongata Rongai	20 (39.2 %)	0
A. sobria		3 (20 %)	0
A. caviae		4 (19 %)	0
A. hydrophila	Lamu	9 (17.6 %)	1 (2 %)
A. sobria		5 (33 %)	2 (13.3)
A. caviae		3 (14.3 %)	0
A. hydrophila	Wajir	20 (39.2 %)	1 (2 %)
A. sobria		4 (26.6 %)	1 (6.6 %)
A. caviae		9 (42.8 %)	5 (23.8 %)

3.8.2 Enterotoxin assays

All the 87 *Aeromonas* isolates were assessed for the presence of toxins. Ten (11.5 %) *Aeromonas* isolates had the enterotoxins whose distributions within the three locations were 2 % *A. hydrophila*, 13.3 % *A. sobria* from Lamu, 2 % *A. hydrophila*, 6.6 % *A. sobria* and 23.8 % *A. caviae* from Wajir (Table 10). Two types of toxins were identified;

these were 6 heat labile enterotoxin (LT) and 4 heat stable enterotoxin (3 STH and 1 STP) (Fig. 14).

Table 10: The distribution of the heat labile enterotoxin (LT) and heat stable enterotoxin (STH and STP) within the three study locations

Location	LT	STH	STP
Wajir	3	2	1
Lamu	2	1	0
Ongata Rongai	1	0	0

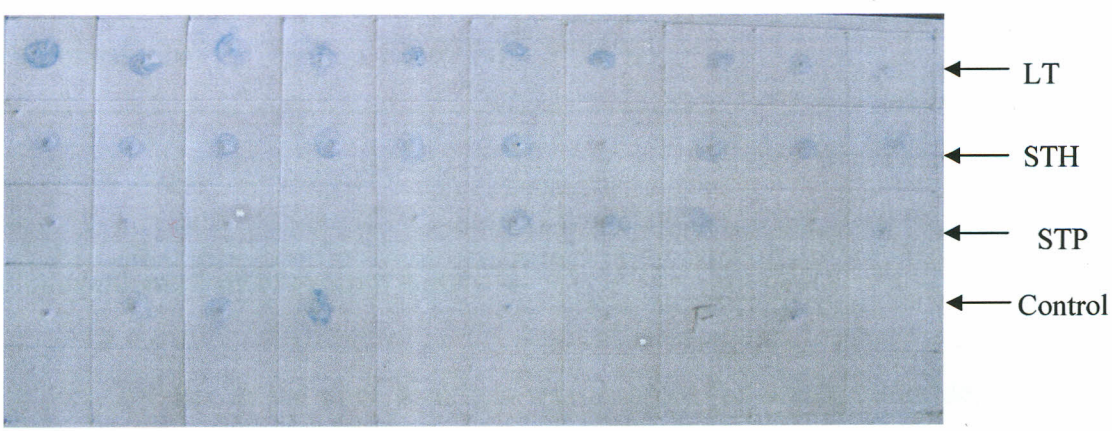


Figure 14: Nitrocellulose membrane colony blot hybridization assay illustrating heat labile enterotoxin (LT) and heat stable enterotoxin (STH and STP) toxins.

3.9 Bacterial pollution of the ground waters in the three study locations

All the ground water in the three locations was assessed for the prevalence of coliforms and faecal *E. coli*. All the 20 (100 %) wells in Wajir and Lamu and 12 (60 %) in Ongata Rongai had coliforms. Similarly, 17 (85 %), 16 (80 %) and 5 (25 %) ground waters from Wajir, Lamu and Ongata Rongai respectively had faecal *E. coli* present (Table 11).

Table 11: Number of ground water samples positive for coliforms and Faecal *E. coli* from the three study locations

Location	No. Ground water samples	Coliforms	Faecal *E. coli*
Wajir	20	20 (100 %)	17 (85 %)
Lamu	20	20 (100 %)	16 (80 %)
Ongata Rongai	20	12 (60 %)	5 (25 %)

3.10 Chemical analysis of ground water

All the ground water in the three locations was assessed for nitrate, chloride, sodium, fluoride, lead and total dissolved solids. Nineteen of the 20 wells in the Wajir and Lamu had nitrate. Chloride was present in 19 and 4 wells found in Wajir and Lamu, respectively. Sixteen and 5 ground water from Wajir and Lamu respectively, had sodium, while 18 ground waters in Ongata Rongai had fluoride. Two ground waters in Lamu had

lead while 16 and 3 ground waters from Wajir and Lamu had total dissolved solid particles (Table 12).

Table 12: The distribution of different metals and solids in the ground water samples from the three study locations

Location	No. Ground water samples	Nitrate	Chloride	Sodium	Fluoride	Lead	TDS
Wajir	20	19	19	16	0	0	16
Lamu	20	19	4	5	0	2	3
Ongata Rongai	20	0	0	0	18	0	0

The normal ranges for the chemicals and the solids are nitrate 50 ppm, chloride 250 ppm, sodium 200 ppm, flouride 1.5 ppm; lead 0.01 ppm and total dissolved solid 1500 ppm.All chemicals assessed were exceeding the maximum permissible WHO guidelines limit for drinking water.(WHO, 2003).

CHAPTER FOUR

DISCUSSION, CONCLUSIONS, AND RECOMMENDATIONS

4.1 Discussion

Aeromonas species namely *Aeromonas hydrophila, A. caviae* and *A. sobria* were isolated from all the ground water sources examined in the three different towns in Kenya. Similar results were also observed by other investigators (Ghenghesh *et al.*, 2001; Massa *et al.*, 2001; Kuhn *et al.*, 1997). *A. hydrophila* (59 %) was numerically the dominant organism both in the boreholes and wells water in the three areas. This observation is similar to that noted by other investigators (Ghenghesh *et al.*, 2001; Kuhn *et al.*, 1997; Mark *et al.*, 2003), who found that *A. hydrophila* was the most common species representing 58.6 % of *Aeromonas* identified to the species level in ground water. *Aeromonas* species were isolated in the 60 (100%) water samples from the ground waters in the three study areas. In this study 45 (75 %) of all groundwater sampled contained pollution of faecal indicator bacteria. *A. hydrophila* was the most common in ground waters with faecal indicator bacteria. Legnani *et al*; (1998) also observed similar prevalence of faecal contaminant in ground waters. However, Araujo *et al;* (1991) observed *A. caviae* as the most common species in water with high faecal contamination levels and were found in 24 % of groundwater specimen sampled. In this study the proportion of *A. sobria* to other species in water with high faecal bacteria pollution was considerably higher, compared to less faecal bacteria polluted water as seen in Ongata Rongai. On the basis of these results it is reasonable to conclude that the *Aeromonas* isolated could be of faecal origin.

A. hydrophila, *A caviae* and *A. sobria* in the present study showed that they can tolerate and grow in natural mineral ground water. Thus, some components of mineral water could influence the growth of *Aeromonas* in drinking water. These observations agree with those of other investigators (Kersters *et al.*, 1996; Brandi *et al.*, 1999), that *Aeromonas* species were able to replicate in mineral ground water and that the extent of growth and survival were water type dependent (Kersters *et al.*, 1996). *Aeromonas* species is adaptable to physicochemical stress. Kuhn *et al;* (1997) observed that this adaptability could be due to some strain thriving for years in a single well. Due to the build up of nutrients in the unsaturated zone and possibly the aquifer, increases the survival time of the microbes. Ghenghesh *et al.*, (2001) found that no difference existed in the isolation rates of *Aeromonas* species in the water samples obtained from wells with different depths. This observation agrees with the present study whereby the isolation of *Aeromonas* was equivocal in all the 3 study areas regardless of the depth of the ground water. The study by Ghenghesh *et al;* showed that most of the sampled ground water had elevated levels of nutrients well above the WHO allowable maximum limits. About 80 % of rural drinking water wells of Lamu and Wajir contain nitrate above the recommended health advisory level by WHO (>250 ppm), which raises considerable public health concern. Kross *et al*; (1993) found a consistently similar proportion of ground water having nitrate content.

From the present study the proximity of latrines or septic tanks to water wells probably contributed to the high levels of nitrate in water due to seepage and contamination. Gbodi and Atawodi (1987) also observed that the proximity of latrine to wells contributed to

high nitrate content. In Lamu and Wajir there was heavy presence of *Aeromonas* species and faecal *E. coli* probably due to the high levels of nitrate in the water. Elevated levels of chlorides of 250 ppm above the maximum permissible WHO guideline were noted in Wajir and Lamu. These may be responsible for the brackish taste in water and is also an indicator of sewage pollution due to the chloride content of urine. Typically, this is indicative of anthropoic pollution, a fact that indicates the increasing risk of pollution of drinking water resources. The study shows that the well and borehole environment provides a suitable habitat for the survival and multiplication of *Aeromonas*. The widespread circulation of these species of organisms throughout the ground water and their considerable adaptability can be seen from the high percentage of recovery in all the ground water samples examined.

Antibiotic susceptibility of *Aeromonas* species in the aquatic environment was performed against 8 antimicrobials. The 87 isolates from the 3 different geographical areas in Kenya were similar in many aspects including being resistant to either two or more antimicrobial agents. The *Aeromonas* species isolated were all resistant to ampicillin. This observation is in agreements with the findings of (Mckeon *et al.*, 1995; Ghenghesh *et al.*, 2001) who reported that all of *Aeromonas* strains isolated from rural ground water supplies in Libya were resistant to amplicillin antibiotics. Thus, treatment of *Aeromonas* infection with antibiotic such as ampicillin to which these organisms are resistant may be predisposing factors to the development of chronic gastroenteritis (Holmberg *et al.*, 1986; Moyer, 1996). The emergence of ampicillin resistance in *Aeromonas* species makes it important and avoidance of use of ampicillin would be sensible. In this study the third generation

cephalosporin such as cefotaxime, ciprofloxacin, ceftazidime and nalidixic acid were all active against *Aeromonas* species. These agree with observations of Ghenghesh *et al*; (2001) who observed that these antibiotics are 100 % effective against *Aeromonas* species. The percentage of strains with resistance to tetracycline and co-trimoxazole in this study ranged between 17.5 % and 20 % which were similar to findings of (Ghenghesh *et al.*, 1998; Ghenghesh *et al*; 2001). These levels of resistance could be related to the extensive application of these antimicrobial agents in the 3 study areas on human and veterinary related needs. The low resistant rate of chloramphenicol in this study agrees with the rare resistance rates of this antibiotic to *Aeromonas* species reported by Jones *et al*; (1995) and Pathak *et al*; (1993). The low resistance rates could be explained by restricted use of chloramphenicol in Kenya.

Antibiotic resistant strains of *Aeromonas* have been isolated from aquatic environment and the resistance is principally plasmid mediated (Hedges *et al.*, 1985; Borrego *et al.*, 1991). Similarly, in the present study, some of the resistance was plasmid mediated as evidenced by the transfer of resistance to *E. coli* K12 via conjugation. Studies by Adam *et al*; (1998), Mckeon *et al*; (1995) and Chang *and Bolton* (1987) observed high incidence of plasmid conferring multidrug resistance in Aeromonads species and Enterobacteria. Common plasmid of medium and small molecular weight of 48 and less than 4.6 Mega daltons (MDa) were present in the transformant and the donor bacteria. These were transferred in the conjugation experiments thus, implicating them in multi-drug resistance. This study therefore shows the potential of *Aeromonas* (drug resistant) to transfer resistance to same spp or other bacterial spp.

Among the environmental *Aeromonas* species isolated in this study, 77 (88 %) were able to haemolyse the erythrocytes of sheep, human, horse and rabbit. The high prevalence of beta- haemolysin production among all *Aeromonas* strains isolated in ground water agrees with observations of other investigators (Majeed and McCrae, 1994; Singh and Sanyal, 1992; Inger *et al.*, 1997; Alavandi and Ananthan, 2003) who reported a higher prevalence of beta- haemolysin properties among environmental strains. In contrast Burk *et al*; (1984) reported no heamolytic activity in *A. caviae* from ground water sources. In this study, no significant association was noted between any particular *Aeromonas* species and haemolysis.

Erythrocytes from small laboratory animals (mouse, rabbit and guinea pig) have been reported to be more sensitive than human, horse and sheep erythrocytes in *Aeromonas* haemolysis assay (Handfield *et al.*, 1996). The finding of the present study and those of others (Monfort and Baleux, 1991; Ghenghesh *et al.*, 2001), however support the use of human, sheep or horse erythrocytes in the haemolysin test for the detection of enteropathogenic mesophilic *Aeromonas* strains in ground water. The predominant beta-haemolysin mesopholic *Aeromonas* species has been suggested to be a cytotoxin (Singh and Sanyal, 1991). These observations suggest that *A. hydrophila, A. caviae* and *A. sobria* posses enterotoxigenic disease causing properties.

Other studies have demonstrated that many mesophilic Aeromonads isolated from drinking water can exhibit toxigenic properties. Out of the 87 isolates from water samples

tested for enterotoxin like activities, 10 (12 %) showed the ability to produce the toxin. Millership *et al.,* (1986) found that enterotoxin production was demonstrated in 28 % of *Aeromonas* isolates while Holmes *et al.*, (1996) found 20 % of *Aeromonas* isolates exhibiting enteroxin activities. These findings, therefore, indicate that *Aeromonas* may represent a true human pathogen. Contrary to the present study where there was no difference in the toxin production among species, other studies showed that enterotoxin production was species specific and that only *A. hydrophila* and *A. sobria* produced enterotoxin (Turnbull *et al.*, 1984). Since *Aeromonas* species are suspected as causative agents of acute diarrhoeal disease in humans following water consumption, it could therefore be assumed that special virulence traits exist among the environmental isolates.

The ease of transfer of drug resistance plasmids in the environment is not only a reminder for good hygiene but it also illustrates the reduction of use of antimicrobials in order to forestall increase in resistance or emergence of new resistance. There is a general agreement that the pool of resistance genes in the environment is amplified by the use of antimicrobial agents in human, animals and agricultural produce. Keeping the use of antimicrobial agents to a minimum will lower the frequencies of resistant plasmids among bacteria and consequently reduce the risk of spread of such factors in the environment, therefore securing the continuous benefits of antimicrobial drugs.

4.2 Conclusions

1. Aeromonads are widely distributed in fresh, brackish and saline ground water in the study areas of Ongata Rongai, Lamu and Wajir
2. There was considerable bacteriological contamination and physicochemical pollution of the ground water.
3. The depth of the well is the best predictor of well –water contamination. High contamination was noted in shallow and unprotected wells were most likely to have been contaminated by surface run-off and direct human intervention.
4. There was high incidence of enteropathogenic *Aeromonas* strains in sampled ground water posing a public health hazard for the inhabitants of the study area as well as a challenge to their dependence on these water sources.
5. The antibiotic resistant strains act as reservoirs for resistance genes, which could subsequently be transferred to bacteria of public health significance. This study suggests that transfer of the R-plasmids between *Aeromonas* isolated could be extended through the presence of carrier or a mediator bacterial species such as normal gut flora *E. coli*. A cycle of resistance transfer could exist in the environment with transfer occurring between *Aeromonas* and a range of unidentified mediator bacterial species.

4.3 Recommendations

1. Since Aeromonads showed high resistance to Ampicillin, this increase warrants a restructuring of the chemotherapeutic regimen for enteric diseases as well as restricting its use.
2. Presence of Aeromonads in water supplies posses a public health hazard and needs public health appraisal.
3. Surveillance of environmental isolates antibiotic resistance to be undertaken.
4. It will be important to investigate the correlation between antibiotic resistance genotypes and heavy metal resistance patterns in ground water.
5. There is need to study the effect of physicochemical parameters on the health of the population in the 3 study areas.
6. To control the frequent outbreaks of waterborne diseases, wells should be protected and fitted with hand pumps.
7. Water for human consumption should be systematically examined for *Aeromonas* species and *Aeromonas* should be adopted as an index of hygienic quality.
8. Latrines and other point sources of potential faecal contamination should be located sufficiently far from ground water sources intended for human and animal consumption.

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APPENDICES

APPENDIX 1: BACTERIOLOGICAL EXAMINATION OF WATER

REPUBLIC OF KENYA

NATIONAL PUBLIC HEALTH LABORATORY SERVICES
P.O. BOX 20750
NAIROBI
Date .....

Lab. Ref. No..... Sample No .....
Time and date sample taken .....
Time and date sample examined .....
Taken by .....
Authority .....
Reason for sampling .....
(If water is suspected of causing ill health please say so).
Source of sample .....
(State if well, spring, stream or public supply).

Is it protected? .....
If so, how? .....
(Is it completely covered, or sides only?)
Is there a pump? .....
If so, how long has it been in use? .....
Has it been overhauled recently? .....
Exact site sample taken from .....
(i.e. tap in kitchen, through cistern or direct from mains?)
Are there any latrines or other sources of pollution? .....

If so, where? .....

Is it a chlorinated supply? .....

Report

.....

.....

.....

.....

.....

Copies to:
Date .....

APPENDIX 2. 20 NE READING TABLE

TESTS	SUBSTRATES	REACTIONS/ENYMES	RESULTS	
			NEGATIVE	POSITIVE
NO_3	Potassium nitrate	Reduction of nitrates to nitrites	NIT 1 + NIT 2 / 5MIN colorless	NIT 1 + NIT 2 / 5MIN pink - red
		Reduction of nitrates to nitrogen	**Zn / 5 min** pink	**Zn / 5 min** colorless
TRP	tryptophane	Indole production	**JAMES / immediate** colorless pale green / yellow	**JAMES / immediate** pink
GLU	Glucose	acidifiaction	Blue to green	yellow
ADH	arginine	Arginine dihydrolase	yellow	Orange / pink / red
URE	urea	Urease	yellow	Orange / pink / red
ESC	esculin	Hydrolysis (β-glucosidase)	yellow	Grey / brown / black
GEL	gelatine (with India ink)	Hydrolysis (protease)	No pigment diffusion	Diffusion of black pigment
PNPG	p-nitrophenyl-β-D-galactopyranoside	B-galactosidase	colorless	yellow
GLU	glucose	assimilation	transparent	opaque
ARA	arabinose	assimilation	transparent	opaque
MNE	mannose	assimilation	transparent	opaque
MAN	mannitol	assimilation	transparent	opaque
NAG	N-acetyl-glucosamine	assimilation	transparent	opaque
MAL	maltose	assimilation	transparent	opaque
GNT	gluconate	assimilation	transparent	opaque
CAP	caprate	assimilation	transparent	opaque
ADI	adipate	assimilation	transparent	opaque
MLT	malate	assimilation	transparent	opaque
CIT	citrate	assimilation	transparent	opaque
PAC	Phenyl-acetate	assimilation	transparent	opaque
OX	Tetramethyl-p-phenylene diamine	Cytochrome oxidase	**OX / 1 – 2 min** colorless	**OX / 1 – 2 min** violet

Appendix 3. Probability Table according to McCrady

QUANTITY OF WATER	50 ml	10ml	1ml	
No. of samples of each quantity tested	1	5	5	
Number giving positive reaction (acid and gas).	0	0	0	0
	0	0	1	1
	0	0	2	2
	0	1	0	1
	0	1	1	2
	0	1	2	3
	0	2	0	2
	0	2	1	3
	0	2	2	4
	0	3	0	3
	0	3	1	5
	0	4	0	5
	1	0	0	1
	1	0	1	3
	1	0	2	4
	1	0	3	6
	1	1	0	3
	1	1	1	5
	1	1	2	7
	1	1	3	9
	1	2	0	5
	1	2	1	7
	1	2	2	10
	1	2	3	12
	1	3	0	8
	1	3	1	11
	1	3	2	14
	1	3	3	18
	1	3	4	20
	1	4	0	13
	1	4	1	17
	1	4	2	20
	1	4	3	30
	1	4	4	35
	1	4	5	40
	1	5	0	25
	1	5	1	35
	1	5	2	50
	1	5	3	90
	1	5	4	160
	1	5	5	180

Probable number of coliform bacilli in 100 ml of water

APPENDIX 4. REAGENT PREPARATION FOR PLASMID EXTRACTION AND CHARACTERIZATION

1. LB broth

Bacto Tryptone	10 g
Bacto yeast extracts	5 g
Sodium Chloride	10 g
Distilled water	1000 ml

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

1. 10 M NaOH was made by dissolving 80g sodium hydroxide pellets in 200 ml distilled water.
2. 20% Sodium Dodecyl Sulphate (SDS) was prepared by dissolving 40g SDS in 160ml distilled water.
3. 0.5M EDTA, pH 8.0 was prepared by adding 37.22g $Na_2EDTA.2H_2O$ to 160 ml distilled water. pH was adjusted to 8.0 with 4 g sodium hydroxide pellets and made to 200 ml. This was sterilized by autoclaving at 121°C for 15 minutes.
4. 20 % Glucose was prepared by dissolving 40g anhydrous dextrose in 200 ml distilled water and which was then sterilized by filtration through a 0.22 µm disposable filter. This was then stored at 4°C until use.
5. 1 M Tris-HCl, pH 8.0 was prepared by dissolving 121.1g Tris base in 800 ml distilled water. pH was adjusted to 8.0 with HCl and made up to 1000ml with distilled water. This was sterilized by autoclaving.

6. 3 M Sodium acetate, pH 5.2 (B-III) was prepared by dissolving 204.12 anhydrous sodium acetate in 400 ml distilled water. pH was adjusted to 5.2 with 100 ml acetic acid and made to 500ml with distilled water and sterilized by autoclaving.
7. 7.5M Ammonium acetate was prepared by dissolving 289.05 ammonium acetate in 500ml distilled water and sterilised by autoclaving.
8. 20 X TE buffer (1X is 10mM Tris, 1mM EDTA) was made by dissolving 24.11 g Tris base and 7.45g $Na_2EDTA.2H_2O$ in 800 ml distilled water. The pH was adjusted to 8.0 with HCl and made to 1000 ml with distilled water
9. Stop mix (0.07% BPB, 7% SDS and 20% Ficoll) was prepared by dissolving 70mg bromophenol blue, 7g SDS and 20g Ficoll type 400 in 80 ml distilled water. This was warmed to dissolve the content, mixed and made to 100 ml with distilled water.
10. 5 X TBE electrophoresis buffer (used as 1:10 solution in the bath) (1X is 89 mM Tris, 89 mM boric acid and 2.8 mM EDTA) was prepared by dissolving 53.89 g Tris base, 27.51g boric acid and 5.21g $Na_2EDTA.2H_2O$ in 1000ml distilled water.
11. 10 mg/ml ethidium bromide was prepared by dissolving 0.5 g ethidium bromide in 50 ml distilled water in a pre-sterilized bottle. The mix was stored at 4°C until ready for use. Ethanol (80%) was prepared by mixing 160ml ethanol with 40 ml sterilized distilled water in a pre-sterilized bottle.

Solution I (B-I)		
Lysozyme	20 mg	2mg/ml
1M Tris-HCl, pH 8.0	0.25 ml	25 mM
0.5M EDTA, pH 8.0	0.20 ml	10 mM
20% Glucose	0.45 ml	50 mM
Distilled water	9.10 ml	
Total	10.00 ml	

Solution II (B-II)		
10 M NaOH	0.4 ml	0.1 N
20% SDS	1.0 ml	1.0%
Distilled water	18.6 ml	
Total	20.00 ml	

APPENDIX 5. REAGENT PREPARATION FOR COLONY HYBRIDISATION TEST

1. LB Broth

Bacto Tryptone	10 g
Bacto Yeast extracts	5 g
Sodium Chloride	10 g
Distilled Water	1000 ml

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

1.0.5 N NaOH was prepared by dissolving 10 g sodium hydroxide pallets and 5 g Sodium Dodecyl Sulphate (SDS) in 500 ml deionised water.

2. SSC was prepared by dissolving 175 g sodium chlorides and 88 g sodium citrate dehydrates in 1litre-deionised water.

3. 0.5% Triton X-100 was prepared by taking 0.5ml into 9.5ml of Triton X-100, fill up to 100ml with deionised water.

4. Tris HCL PH 7.4 was prepared by dissolving 121.1 g Tris base in 800 ml and at adjusting PH to 7.4 with HCL.

5. Hybridisation buffer was prepared by dissolving 2.5 g BSA 2.5 g polyvinyl pyrrocidone and 5 g SDS in 500ml distilled water.

6. Alkaline Phosphates buffer was prepared by dissolving 6.1 g Tris base 6.8 mg Zinc Chloride and 0.25 g Sodium azide in 400 ml distilled water and adjusting the PH to 8.5 with HCL.

7. Nitro blue tetrazolum solution was prepared by dissolving 100 mg NBT in 1.33ml 75% N-dimethyl formanide.

8. BCIP (5-brome-4-chloro-3-idolyl phosphate tohidine salt) solution was prepared by dissolving 100 mg BCIP in 2 ml N, N-dimethyl formanide.

9. Alkaline phosphate linked oligonucle-otide probe was prepared by adding oligonucleotide probe.

APPENDIX 6. PROTOCOLS FOR MEDIA PREPARATION

SULPHUR-INDOLE MOTILITY DECARBOXYLASE MEDIUM (SIM OXOID CM 435)

SIM Medium, Dehydrated	30 g
Distilled water	1000 ml

Boil to dissolve completely. Dispense in 4ml aliquots into 13 x 100 mm screw cap tubes.

Sterilize by autoclaving at 121^{0}C for 15 min and store at 4^{0}C until use.

TRIPLE SUGAR IRON (TSI) AGAR (OXOID CM 277)

TSI Agar, dehydrated	65 g
Distilled water	1000 ml

Boil to dissolve completely. Dispense into test tubes and autoclave at 121^{0}C for 15 min.

Slant tubes to form deep butts and moderate slants. Allow to harden completely and store at 4^{0}C until use.

CONTROLS

Escherichia coli	ATCC 25922
Proteus vulgaris	ATCC 13315
Salmonella enteritidis	ATCC 13076

TRYPTONE SOYA BROTH GLYCEROL MEDIUM (15%)

Tryptone soya broth base (OXOID CM 129)	30 g
Glycerol (BDH 284546F)	150 ml
Distilled water	850 ml

Dispense in 1ml amounts in cryovial and autoclave at 121^{0}C for 15mins. Cool and store at 4^{0}C until use.

UREA AGAR (OXOID CM 53)

Urea agar base, dehydrated	2.4 g
Distilled water	95 ml

Boil completely to dissolve, sterilize by autoclaving at 121^{0}C for 15 min. Cool to 50-55^{0}C. Aseptically add 5ml sterile 40% urea solution SR 20 (OXOID). Mix well and dispense into 2ml aliquots in sterile containers and allow setting in slope position. Store at 4^{0}C.

CONTROLS

Positive control- *Proteus vulgaris* ATCC 13315

Negative control- *Escherichia coli* ATCC 25922

BLOOD AGAR (OXOID CM 331)

Blood agar base, dehydrated 40 g

Distilled water 1000 ml

Sterile whole blood, (horse, sheep, human and rabbit)

Boil to dissolve completely. Sterilize by autoclaving at 121^{0}C for 15 minutes. Cool to 45-50^{0}C and add 7 % sterile blood. Mix in gentle rotation and pour onto sterile petri dishes.

CONTROLS

Positive control - *Staphylococcus aureus* ATCC 25923

Negative control - Uninoculated media.

MACCONKEY AGAR TWEEN 80 (OXOID CM 7)

MacConkey agar dehydrated 52 g

Distilled water ***1000 ml***

Boil carefully to dissolve completely. Autoclave at 121^{0}C for 15 minutes. Cool to 50^{0}C and Tween 80 aseptically, dispense into sterile petri dishes. Allow to solidify completely and store at 2-8^{0}C until use.

Positive contro***l-*** *Escherichia coli* ***ATCC 25922***

Negative control- *Enterococcus faecalis* ATCC 29212

MCFARLAND STANDARD NO 0.5

Add 0.5 ml of a 1.175% solution of barium chloride dihydrate ($BACL_2 2H_20$) to 99.5 ml of 0.36 N (1%) sulphuric acid. Dispense 5ml aliquots into screw cap bijou bottles and seal with cap. The turbidity standard can be stored in the dark room temperature for 6 months or more, provided the bottle is sealed to prevent evaporation. The standard must be thoroughly mixed just before use, preferably on a vortex mixer.

MRVP MEDIUM (OXOID CM43)

MRVP dehydrated	15 g
Distilled water	1000 ml

Suspend the medium in distilled water. Mix well and distribute into final containers and sterilize by autoclaving at 121^0C for 15 minutes. Store at 4^0C until required.

CONTROLS

MR POSITIVE	*Escherichia coli*	***ATCC 25922***
MR NEGATIVE	*Klebsiella pneumoniae*	***ATCC 13883***
VP POSITIVE	*Enterobacter cloacae*	ATCC 23355
VP NEGATIVE	*Escherichia coli*	***ATCC 25922***

MUELLER-HINTON AGAR (OXOID CM337)

Mueller-Hinton dehydrated medium	38 g
Distilled water	1000 ml

Suspend in distilled water. Boil to dissolve. Autoclave at 121^{0}C for 15 minutes and allow to cool up to 50^{0}C. Pour into sterile petridishes. Store at 4^{0}C until required.

CONTROL

POSITIVE *Escherichia coli* ***ATCC 25922***

POSITIVE *Staphylococcus aureus* ATCC 25923

NEGATIVE ***Uninoculated media***

SIMMONS CITRATE AGAR (OXOID CM 155)

Simmons Citrate agar	23 g
Distilled water	1000 ml

Boil carefully to dissolve completely. Aliquot 2 ml in to 13 mm x 100 mm tubes. Sterilize by autoclaving at 121^{0}C for 15 minutes, set to cool in a slanted position. Store at 4^{0}C.

Positive Control	*Citrobacter freundii*
Negative Control	*E. coli* ATCC 25922

ALKALINE PEPTONE WATER

Bactopeptone medium dehydrated	10 g
Distilled water	1000 ml

Suspend the medium in distilled water. Adjust pH with sodium hydroxide. Mix well, distribute into final containers and sterilize by autoclaving at 121^{0}C for 15 minutes. Store at 4 ^{0}C until required.

Positive Control	*Vibrio cholerae* ATCC 14035
Negative Control	*E. coli* ATCC 25922

THIOSULPHATE CITRATE BILE SALT SUCROSE (Biotec)

TCBS Agar, dehydrated	88 g
Distilled water	1000 ml

Suspend 88 g in 1000ml of distilled water and bring to the boil to dissolve the agar. Cool to 47^0C and pour into petri dishes. Keep at 4^0C until needed for use.

Positive Control	*Vibrio cholerae* ATCC 14035
Negative Control	*E. coli* ATCC 25922

Appendix 7. The green shaded regions show geographical location of Wajir, Lamu and Ongata Rongai