RELATIONSHIP BETWEEN MICROBIAL AND PHYSICO-CHEMICAL POLLUTANTS AND REISTANCE OF WATER-BORNE DIARRHOEA-RELATED BACTERIA AGAINST ANTIBIOTICS IN THIBA RIVER BASIN, KENYA

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DECLARATION

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

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This work is dedicated to my dear wife Anne Muturi and our three lovely sons, Kelvin Mwangangi, Vincent Kinyua and Fredrick Mugo, and to all residents of Thiba River basin.

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ACRONYMS AND ABBREVIATIONS

AAS	Atomic absorption spectrometry
amp	Ampicillin
API	Analytical Profile Index
ARBs	Antibiotic resistance-carrying bacteria
ARGs	Antibiotic resistant genes
ATP	Adenosine tri-phosphate
BG agar	Brilliant green agar
BS agar	Bismuth sulphite agar
cap	Chloramphenicol
cip	Ciprofloxacin
CDC	Centers for Disease Control and Prevention
CMR	Centre for Microbial Research
EAAS	Electrothermal atomic absorption spectrometry
EMB	Eosin Methylene Blue stain
ery	Erythromycin
EXPEC	Extra-intestinal Pathogenic *E. coli*
FAAS	Flame atomic absorption spectrometry
GISA	Glycopeptide – Intermediate Sensitive *Staphylococcus aureus*
GoK	Government of Kenya
Ha	Hectares
H.E.P	Hydroelectric power
HGT	Horizontal gene transfer
H_2S	Hydrogen sulphide gas
KEMRI	Kenya Medical Research Institute
lcm	Lincomycin
LZ	Lower zone
MDR	Multiple drug resistance
meth	Methicillin
MGEs	Mobile genetic elements
min	Minocycline
MIS	Mwea Irrigation Scheme

MRGs	Heavy metals resistant genes
MRSA	Methicillin Resistant *Staphylococcus aureus*
mtz	Metronidazole
MZ	Middle zone
NIB	National Irrigation Board
NTS	Non-typhoid Salmonella
RBC	Red blood cells
SS agar	Salmonella-Shigella agar
sxt	Cotrimoxazole/ trimethoprim/sulfamethoxazole
TN	Total nitrogen
TP	Total phosphorus
TSI	Triple- sugar- iron
TSS	Total suspended solids
USEPA	United States Environmental Protection Agency
UZ	Upper zone
VBNC	Viable But Non-Culturable State
VGT	Vertical gene transfer
VRE	Vancomycin Resistant Enterococcus
WHO	World Health Organisation

ABSTRACT

Bacteria-related water-borne diarrhoea is amongst major cause of illness and death to children under five years in developing countries. Reports by health workers in Thiba river basin indicate that this diarrhoea and levels of resistance of the bacteria causing it against the commonly used antibiotics to treat it are on increase. This study was to isolate and identify the bacteria responsible for water-borne diarrhoea in Thiba river basin and to determine the levels of their resistance against commonly used antibiotics. Also the levels of microbial and physico-chemical pollutants of Thiba river basin water and rain water; and their relationship with the antimicrobial resistance of the bacteria causing water-borne diarrhoea against the commonly used antibiotics in this region were to be determined. A total of 168 water samples were obtained, distributed uniformly in upper, middle and lower zones within Thiba river basin in September 2016 during dry season and in October 2016 during wet season. Rain water samples were also taken as control. Entero-pathogenic bacteria causing water-borne diarrhoea were isolated by use of Membrane Filter Technique (MFT) and selective media. Vitek 2 System: 07.01 test and other biochemical tests were used to identify the species, which were enumerated using the most probable number (MPN). Kirby-Bauer Disc Technique was used to determine antimicrobial susceptibility. The mean levels of physico-chemical pollutants were measured using different methods. Total suspended solids were measured using glass-fibre filters; total nitrogen and total phosphorus using persulfate in acid and heavy metal ions (Cd^{2+}, Cu^{2+}, Zn^{2+} and Cr^{3+}) using flame atomic absorption spectrometry. Microbial contaminants' levels were estimated using the most probable number (MPN) of coliforms and pathogenic *E. coli* bacteria. Of the total 168 samples analysed, 143 samples were detected with at least pathogenic bacteria. Those identified were *Salmonella paratyphi, Pseudomonas aeruginosa, Salmonella enteritidis, Shigella flexineri, Klebsiella pneumonia, Enterobacter liquefaciens, Proteus mirabilis* and *Escherichia coli*. *Salmonella* spp., *Pseudomonas aeruginosa, Shigella flexineri* and *E. coli* were resistant to erythromycin, with *Salmonella* showing the highest resistance; while *Klebsiella pneumoniae* and *Shigella flexineri* were resistant to methicillin. Of the bacteria tested, 75 % showed moderate sensitivity to minocycline, cotrimoxazole, chloramphenicol, ampicillin, ciprofloxacin, metronidazole and lincomycin antibiotics, with highest sensitivity during dry season. Student's T-test and Single-factor ANOVA statistical tests showed no significance difference on mean values of levels of these microbial and physico-chemical pollutants in the Thiba river basin water across the sampled zones and within the two seasons at $p=0.05$. However, the mean values of these pollutants were found to be significant as they were higher than those admissible by World Health Organisation, except for Cu^{2+}. Positive correlation was established between the increased levels of physico-chemical pollutants and increased resistance of the pathogenic bacteria causing water-borne diarrhoea against commonly used antibiotics in Thiba river basin using Pearson's Correlation Coefficient and Bivalent Regression ($R^2=0.58$) tests. Therefore, the study showed that the Thiba river basin river water had significant levels of water-borne bacteria pathogens, with 53 % of them showing some resistance against commonly used antibiotics for treatment of the diarrhoea they cause to the basin residents living there. It also found some modulating effect of the Thiba river water contaminants on bacteria antimicrobial resistance, as increase in pollutants increased bacteria resistance against the antibiotics. The national government and county governments of Embu and Kirinyaga should have constant surveillance of microbial resistance of bacteria causing water-borne diarrhoea for prompt treatment. In addition, there should be improvement of sanitation of Thiba river basin water and beyond.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Diarrhoea is a major cause of death of children below 5 years in Kenya, after HIV & AIDS and respiratory infections (CDC, 2017; Institute for Health Metrics and Evaluation, 2017; Kawakatsu *et al*., 2017), with prevalence of 16 % (Regidor *et al*., 2017). The dehydration and malnutrition causes about 525000 children deaths annually (GoK, 2015; Sokhina *et al*., 2017; WHO, 2017a; Mutama *et al*., 2019). Most malnourished people and those having other health conditions like HIV & AIDS are quite vulnerable to diarrhoea (Mutama *et al*., 2019). Pre-disposing factors like poverty, poor hygienic conditions, contaminated drinking water and poor disposal of human waste expose many people to water-borne diarrhoea (Nsagha *et al*., 2015; Abok *et al*., 2018; Mutama *et al*., 2019; WHO, 2019a). The residents of the study area, herein known as Thiba river basin, usually experience many cases of bacterial diarrhoeal diseases, like cholera and typhoid fever (CDC, 2017).

The most common bacteria-related water-borne diarrhoea diseases include cholera caused by *Vibrio cholerae* species; typhoid fever caused by *Salmonella enterica* serotype *typhi* and to a lesser extent, *S. enterica* serotypes *paratyphi* A, B, and C; and shigellosis or bacillary dysentery mostly caused by *Shigella dysenteriae* (Brusch, 2019). Water pollutants like suspended solids and heavy metals, are known to increase the susceptibility of pathogens, for instance *Salmonella* sp. and *Shigella* sp. are susceptible towards antibiotics used to treat water borne diarrhoea (Ganesan *et al*., 2017). Many human activities along the rivers of Thiba river basin have contributed much to the pollution of the water (Muriuki *et al*., 2015).

Heavy metals ions like those of cadmium, copper, zinc and chromium, which mainly come from agrochemicals used in the Thiba river basin, and some of the major pollutants. They either have some antimicrobial properties or together with other chemical pollutants, cause selective pressure in the aquatic environment. This brings about mutations in the bacteria responsible for causing water-borne diarrhoeal diseases, making them to develop some forms of resistance (Wilfred *et al*., 2015).

1.2 Statement of the problem

Outbreaks of acute water-borne bacterial diarrhoeal diseases have become a national concern. Reports by the Kenyan Government on outbreaks of diarrhoea in Embu where part of Thiba river basin is located, indicates that bacterial diarrhoeal diseases like typhoid fever and cholera are on increase (GoK, 2015; Muriuki *et al*., 2015). The Global Burden of Disease estimates that in 2016, Kenya had 97,762 typhoid cases, 62% among children aged less than 15 years; and 1,075 typhoid deaths, 66% among children aged less than 15 years (Simiyu *et al*., 2018; Wierzba, *et al*.; 2019).

Antimicrobial resistance, especially by the enteric bacteria that cause the water-borne diarrhoeal diseases, has been increasing in the Eastern Africa (Omulo *et al*., 2015). The sensitivity of enterobacteria in Kenya towards antibiotics like ciprofloxacin, ampicillin, erythromycin, trimethoprim/sulfamethoxazole, streptomycin, ampicillin/sulbactam and tetracycline has been found to be high (Shah *et al*., 2016). For instance, bacteria like diarrheagenic *Escherichia coli* has showed multidrug resistant (MDR) to trimethoprim/ sulfamethoxazole 97.6%, amoxicillin 97.6%, erythromycin 96.9%, ampicillin 96.6% and streptomycin 89%. This is related to indiscriminate use of the antibiotics, seasonal variations and pollution of the rivers. There has been uncotrolled release of physico-chemical pollutants in the Thiba river basin water that is linked to

the increase in antimicrobial resistance by bacteria causing water-borne diarrheal diseases against the commonly used antibiotics to treat the diseases. Accumulation of heavy metal ions, which originate from agrichemicals used in the area, is of much concern to the residences of the area.

1.3 Justification of the study

Despite the increase in incidence of bacterial diarrhoeal diseases in the Thiba river basin, no conclusive study has ever been reported on the extent of water pollutants from Mwea Irrigation Scheme and main urban centres like Embu and Mwea on the efficacy of the commonly used antibiotics in curing bacteria-related water-borne diarrhoea. The major source of pollution of the waters within the Mwea area is water drained from the rice paddies having high concentration of agro-chemicals and untreated human waste mostly from local urban centres and villages (Reserve Areas). This usually leads to water-borne bacteria diarrhoea outbreaks that make the residents of Thiba river basin to spend a lot of money and time trying to fight back the effects, hence jeopardizing their health and economic activities (Muriuki *et al*., 2015). This was a major concern to both residents and the Kenyan government. The study was therefore carried out to establish the correlation between the microbial and physico-chemical pollutants of river water and rain water on the resistance of bacteria causing water-borne diarrhoeal diseases against antibiotics commonly used by residents of Thiba river basin.

1.4 Research questions

(i) Are the water-borne bacteria species that cause diarrhoeal diseases in the Thiba River basin water and rain water significant during dry and wet seasons?

(ii) Is there a significant difference in the antibiotic resistance of the water-borne bacteria species that cause diarrhoeal diseases in the Thiba river basin and rain water against the commonly used antibiotics by residents to treat them?

(iii) Are there significant levels of microbial pollutants in the Thiba river basin water and rain water during dry and wet seasons?

(iv) Are there significant levels of physico-chemical pollutants in the Thiba river basin water and rain water during dry and wet seasons?

(v) Is there any relationship between the microbial and physico-chemical pollutants of the Thiba river basin water on the levels of resistance of diarrhoea-related bacteria species against commonly used antibiotics?

1.5 Hypotheses

(i) The bacteria species responsible for water-borne bacteria diarrhoeal diseases in the Thiba river basin water and rain water are not significantly different during dry and wet seasons.

(ii) There is no significant resistance of the water-borne bacteria species that cause diarrhoeal diseases in the Thiba river basin water and rain water against the commonly used antibiotics by residents to treat them.

(iii) There are no significant levels of microbial pollutants in the Thiba river basin water and rain water during dry and wet seasons.

(iv) There are no significant levels of physico-chemical pollutants in the Thiba river basin water and rain water during dry and wet seasons.

(v) There is no relationship between the microbial and physico-chemical pollutants of the Thiba river basin water and the levels of resistance of diarrhoea-related bacteria species against commonly used antibiotics.

1.6 Objectives

1.6.1 General objective

To investigate the relationship between microbial and physico-chemical pollutants of Thiba river water and the levels of resistance of diarrhoea-related bacteria species to antibiotics used against them in the Thiba river basin.

1.6.2 Specific objectives

(i) To isolate and characterize the bacteria species associated with water-borne bacteria diarrhoeal diseases in the Thiba river basin water and rain water during dry and wet seasons.

(ii) To determine the levels of resistance of the water-borne bacteria species that cause diarrhoeal diseases in the Thiba river basin water and rain water against the commonly used antibiotics by residents to treat them.

(iii) To determine the microbial pollutants found in the Thiba river basin water and rain water during dry and wet seasons.

(iv) To determine the physico-chemical pollutants found in the Thiba river basin water and rain water during dry and wet seasons.

(v) To determine the relationship between the microbial and physico-chemical pollutants of river water and the levels of resistance of diarrhoea-related bacteria species against commonly used antibiotics in the Thiba river basin.

1.7 Significance of the study

The study was meant to generate information on bacteria that causes water-borne diarrhoeal diseases in the Thiba river basin and their resistance against the antibiotics commonly used to treat the diseases. It was also to give more light on the relationship

between the microbial and physico-chemical pollutants that included the heavy metals, in the Thiba river basin water and the antimicrobial susceptibility of bacteria pathogens causing the water-borne diarrhoeal diseases. It was expected that such information was to greatly benefit the health workers and residents of the area and the country at large, in their endeavour to combat these diseases. In extension, the information was expected to contribute to the improvement of the health and economic status of Kenyans, especially the infants.

CHAPTER TWO

LITERATURE REVIEW

2.1 Water pollution

Many rivers globally are polluted mainly by raw sewage mostly from urban centres, industrial effluents such as heavy metals, agrochemicals, soaps and detergents (Halder *et al*., 2015). The raw sewage is the major source of microbial contaminants like coliforms (Al-Gheethi, 2018; Ashish *et al*. 2019). Coliforms are rod-shaped Gram-negative non-spore forming and motile or non-motile bacteria that can ferment lactose with the production of acid and gas when incubated at 35–37°C (Daoliang *et al*., 2019). They are commonly found in the environment and intestines of endotherms. They are usually more in contaminated water compared to the dangerous pathogenic bacteria. Though non-pathogenic, some may cause complications like diarrhoea to some people. There are many strains of coliform genera that include *Escherichia, Klebsiella, Citrobacter* and *Enterobacter* (Hervet *et al*., 2016). Faecal coliforms are members of total coliforms that grow and ferment lactose in 24 hours at higher temperature of 44.5°C with *Escherichia coli* being the most common (Malcolm *et al.,* 2017; Abok *et al*., 2018). Measurement of total coliforms provides the standard by which microbial contamination of water is measured.

2.2 Common Enterobacteria causing water-borne diarrhoeal diseases

2.2.1 *Vibrio*

Vibrio is a genus found in family Vibrionaceae consisting of more than 30 species that include major human foodborne pathogens causing cholera and other Vibrio illness (Kim *et al*., 2015). *Vibrio cholerae* is the most important species, which has three

serotypes; Ogawa, Inaba and Hikojima (WHO, 2017a). Vibrios are aquatic especially in marine water. They are Gram negative and motile rods. They are facultative anaerobes and oxidase positive. They form colonies 2-3 mm in diameter on blood agar, while on thiosulphate citrate bile salt sucrose (TCBS) agar, they are either yellow or green (WHO, 2017a).

2.2.2 *Salmonella* spp.

Salmonella is a genus within the family Enterobacteriaceae. Members are rod-shaped cells, which do not form spores. They stain negatively with Gram stain, oxidase negative, catalase positive, facultative anaerobes and reduce nitrate to nitrite. It comprises of *Salmonella enterica* and *S. bongori* species, each subdivided into several subspecies and serovars (Mikoleit, 2015; WHO, 2017a).

2.2.3 *Shigella* spp.

Shigella is a genus in the family Enterobacteriaceae. Members are rod-shaped cells that stain negatively with Gram stain and don't form spore. They are oxidase negative, catalase positive (except *S. dysenteriae* Type 1), facultative anaerobes and reduce nitrate to nitrite. The genus has four species: *Shigella dysenteriae* (subgroup A), *Shigella flexineri* (subgroup B), *Shigella boydii* (subgroup C) and *Shigella sonnei* (subgroup D). Each species may be further divided into serogroups, except *S. sonnei* (Mikoleit, 2015; WHO, 2017b).

2.2.4 *Escherichia coli*

Members of *Escherichia coli* live in the colon of healthy animals as normal flora. They are rod-shaped bacteria that stain negatively with Gram stain. They ferment lactose producing pink colonies in MacConkey agar and metallic sheen colour in EMB plate

(WHO, 2017a; Paramesh *et al*., 2018). Most strains are non-pathogenic. Those that cause diarrhoea include Enterotoxigenic *E. coli* (ETEC, O148), enteropathogenic *E. coli*, Shiga toxin-producing *E. coli* (STEC), enteroaggregative *E. coli*, Enterohaemorrhagic *E. coli* (EHEC, O157:H7) and Enteroinvasive *E. coli* (EIEC, O124) (Tejan *et al*., 2019).

2.2.5 *Enterobacter*

Enterobacter are motile short rods mostly found in the intestines of animals. They do not produce spores and stain negatively with Gram stain. They are catalase-negative, citrate positive, indole negative, Methyl-Red negative and facultative anaerobes. They synthesize ornithine decarboxylase enzyme. In humans, species which are opportunistic pathogens include *E. cloacae*, *E. aerogenes*, *E. gergoviae* and *E. agglomerans* (WHO, 2017b; Kara, 2019).

2.2.6 Enterococci

Enterococci are usually found as commensals of gastrointestinal tract (GI) in humans and other animals. They are Gram-positive bacteria that utilize a wide variety of carbohydrates for growth (WHO, 2017a).

2.3 Bacteriological water analysis

Water samples to be analysed for bacteria that cause water-borne diarrhoea that include *Shigella, Salmonella, Vibrio cholerae,* and *Escherichia coli* O157:H7, are transported in media such as Cary-Blair medium, ensuring bacteria flora remain unchanged until when streaking onto plating agar occurs (Tankeshwar, 2016). In the laboratory, the water samples are inoculated in enrichment broth media like Selenite broth or Tetrathionate broth. Selenite broth is used for the isolation

of *Salmonella* and *Shigella* from stool, urine, water and food products. The inoculated broths or media are incubated at appropriate temperature for 12-18 hours at 35°C-37°C. Isolated colonies are then sub-cultured in selective media like bismuth sulfite or desoxycholate citrate agar (DCA) to isolate and identify specific pathogens (Nisha, 2018). Differential media like Eosin Methylene Blue (EMB) agar, Mannitol Salt Agar (MSA) and Mac Conkey's agar are used to differentiate closely related organisms or groups of organisms. Bio-chemical and serological tests are carried out to differentiate between the different biotypes and serotypes respectively (Nisha, 2018). For quick identification of members of the Enterobacteriaceae, the Analytical profile index (API) is used, which has miniaturized clinical biochemical tests (Syria *et al*., 2015). Traditional tests for coliforms include plate count method and pour plate methods; the multiple-tube fermentation and membrane filtration techniques and the ATP test.

2.3.1 Plate count or the pour plate count method

In the plate count method, bacteria are grown on a nutrient medium to form colonies that are counted directly or indirectly. The original sample is diluted severally before culturing to obtain appropriate number of colonies of bacteria being investigated, usually between 25 and 250 colonies (Madhusudana *et al*., 2018). The number of viable (living) cells in a known dilution are known as colony-forming units (CFUs). Through simple proportions, the total number of CFUs in the original sample is calculated using the value obtained for each sample. To determine the number of CFUs per milliliter (ml) of sample, the number of colonies counted is multiplied by the number of times the original millimeters of bacteria was diluted (the dilution factor of the plate counted). The number obtained after the calculation is used to quantify the number of bacteria cells capable of multiplying (Stiefel *et al*., 2015).

The direct methods for counting colonies include use of microscope with special counting chambers like Haemocytometer and Most Probable Number (MPN).

2.3.2 Multiple-tube fermentation method

This is a statistical estimation technique based on the most probable number (MPN) that indicates that there is 95 % chance that the bacterial population falls within a certain range (Nisha, 2017). The method involves three steps; the first presumptive test, then the confirmed test and the last completed test. In all these steps, the media and apparatus used are sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. For presumptive test, samples of appropriate dilution are inoculated on moderately selective media like Lactose broth medium, Mac Conkey Broth or Lauryl tryptose (lactose) broth containing Durham's tube. The tubes with growth of coliforms produce gas and are noted as positive. The number of positive tubes is compared to a standard chart on MPN values to estimate the number of bacteria present in them, which is then recorded (Nisha, 2017).

In the confirmed test, samples from the positive tubes are inoculated into media like Brilliant Green Lactose Broth (BGLB) or Eosin Methylene Blue (EMB) broth which are more selective. This is meant to eliminate all organisms that produce acid and gas from lactose fermentation except true coliforms. The inoculated tubes and agar slants are incubated at 37°C for 24 hours. Preparations made from the slants are Gram-stained and examined under a microscope. Production of gas in lactose broth indicates presence of Gram negative short bacilli, non-spore-forming coliform group while absence of gas indicates absence of coliforms in the tested sample (Nisha, 2017).

In the completed test, a sample from positive BGLB tube with the highest dilution is inoculated onto selective medium like EMB agar or Endo's medium and incubated for 24 hours at 37°C and another at 44.5°C. In EMB medium, metallic green colonies indicates positive. Presences of typical colonies at 44.5 °C indicate presence of thermotolerant *E. coli* (Nisha, 2017).

2.3.3 Membrane Filter Technique (MFT)

Different samples of water are passed through separate membrane filters with pore sizes of 0.45 µm. The membranes are then placed on agar plates having standard coliform media or media containing the enzyme ß-glucuronidase, like mEndo agar LES for total coliforms and mFC agar for faecal coliforms. Bacterial cells trapped on the membranes grow into colonies (Nisha, 2019). On mEndo agar, coliforms form red colonies with a metallic sheen and on mFC agar, faecal coliforms form dark blue colonies. The colonies are then counted, and a bacterial density of the water samples can be calculated (Nisha, 2019).

2.3.4 Enzymatic methods

Some strains of bacteria like *Escherichia*, *Citrobacter*, *Enterobacter* and *Klebsiella* produce β-D-galactosidase enzyme that hydrolysis lactose at 37°C into glucose and galactose that are detected by use of coloured or fluorescent markers. This activity is not found in strains of *Proteus*, *Salmonella* and *Edwardsiella* as they don't produce the enzyme (Andrėe *et al*., 2015). Likewise, some Gram-negative bacteria, mostly strains of *E. coli* and some strains of *Salmonella* and *Shigella*, produce another enzyme called β-D-glucuronidase whose activity at 44.5°C is also detected by use of coloured or fluorescent markers such as XGLUC (5-bromo-4-chloro-3-indoxyl-β-D-glucuronide)

and MUGLU (4-methylumbelliferyl-β-D-glucuronide) respectively (Andrée *et al*., 2019). *E. coli* colonies can be distinguished from other coliforms on membrane filters and plates of violet red bile agar if MUG (4-methylumbelliferyl-beta-D-glucuronide) is incorporated into the culture media (Andrée *et al*., 2015). Some bacteria like *Enterobacter, Klebsiella*, *Proteus, Vibrio*, and most *Salmonella* strains do not display β-glucuronidase activity (Andrée *et al*., 2015).

2.3.5 Other microbial parameters in water

Other microbial parameters in water that may be investigated include presence or absence of enteric viruses of humans and other animals, parasitic protozoa and Microsporidia, the spore-forming unicellular fungal parasites. The viruses of interest could be *Norovirus,* Human Adenovirus, *Rotavirus and Enterovirus*. The protozoa include *Entamoeba histolytica*, *Giardia lamblia*, *Cryptosporidium parvum*, *Toxoplasma gondii* and *Balantidium coli* (Nicholas, 2015; Christesson *et al*. 2018). The detection of the pathogenic microbes involves sedimenting the cells using centrifugation; filtration; flotation; chemical precipitation and assay and characterization procedures (like PCR, hybridization, RFLP analysis, and nucleotide sequencing). Protozoan population can be enumerated by the Most Probable Number (MPN) method and represented as cells/ml of the sample (Nicholas, 2015; Christesson *et al*. 2018; Mark, 2019).

2.4 Antimicrobial used to treat bacteria-related water-borne diarrhoea

Antibiotics used in the treatment of bacterial diarrhoeal diseases include penicillin, streptomycin, chloramphenicol (chlornitromycin), tetracycline, amoxicillin, azithromycin, ceftriaxone metronidazole, co-trimoxazole, rifaximin, vancomycin and

ciprofloxacin (Eugenia *et al*., 2018). The effect of these antibiotics on bacteria may be cidal (killing) or static (inhibitory) (Garima *et al*., 2017). The most preferred antibiotic for treating severe bacteria-related water-borne diarrhoea is azithromycin. Levofloxacin and ciprofloxacin are also preferred, especially against *Shigella*, but are becoming less effective due to increasing resistance by bacteria (Tribble, 2017). Others include metronidazole, erythromycin and methicillin. Levofloxacin and ciprofloxacin are quinolones that act by preventing the unwinding and duplication of bacteria DNA. Erythromycin and azithromycin are macrolides that function by preventing protein synthesis of bacteria when they bind to the bacterial 50S ribosomal subunit (Patel *et al*., 2021). Chloramphenicol also functions by blocking the synthesis of bacterial proteins by binding to the 23S rRNA on the 50S subunit of bacterial ribosome and inhibits the action of peptidyl transferase enzyme (Kumar, 2017). Metronidazole interferes with protein synthesis by causing a loss of helical DNA structure and strand breakage (Weir *et al*., 2021). Methicillin is in category of penicillins that block the synthesis of bacterial cell-wall (DoctorAlerts, 2017).

2.4.1 Antimicrobial resistance

Antimicrobial resistance (AMR) has become a widespread health problem globally. When the microorganisms are exposed to antimicrobial drugs for some times, they develop resistance making the drugs ineffective. The resistance can be intrinsic or acquired (WHO, 2020).

2.4.2 Antibiotics resistance

Some bacteria usually develop resistance to those commonly used antibiotics upon frequent exposure. Examples include resistance of *Klebsiella pneumoniae*, *E.*

coli, methicillin-resistant *Staphylococcus aureus* (MRSA) and penicillin-resistant *Streptococcus pneumoniae* towards carbapenem and fluoroquinolone antibiotics. *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* show multi-drug resistance. *Mycobacterium tuberculosis* has been reported to develop multiple drug resistant tuberculosis (MDR-TB), showing resistance to at least four of the core anti-TB (Francesca *et al*., 2015).

Antibiotics exert selective pressure against bacteria. The susceptible bacteria are killed or inhibited from carrying out their life processes, while those with intrinsic or acquired antibiotic-resistance survive and multiply. Misuse of antibiotics is a major contributor of increased resistance by the bacteria (Francesca *et al*., 2015; Bingyun *et al*., 2017; Huang *et al*. 2018).

2.4.3 Different mechanisms of antibiotics resistance by bacteria

Bacteria antibiotics resistance involves several mechanisms. One mechanism involves decrease in number of porin channels located in outer membrane of Gram-negative bacteria. This blocks the entry of β-lactams, quinolones and the glycopeptides antibiotics into the cell to inhibit cell wall synthesis. The resistance of *Pseudomonas aeruginosa* towards imipenem, which is a beta-lactam antibiotic, is a good example (Bingyun *et al*., 2017; Garima *et al*., 2017).

Another mechanism involves increasing the efflux mechanism of the antibiotics from the bacterial cells by efflux pumps before they reach their target. This maintains their low-intracellular concentrations. The resistance of *E. coli* and other Enterobacteriaceae towards tetracyclines; Enterobacteriaceae towards chloramphenicol; *Staphylococci* towards macrolides and streptogramins; and *Staphylococcus aureus* and *Streptococcus*

pneumoniae towards fluoroquinolones occurs in this manner (Bingyun *et al*., 2017; Garima *et al*., 2017).

Modification of ribosomes and penicillin-binding protein (PBPs) due to spontaneous gene mutation on the bacterial chromosome leads to another mechanism of bacterial resistance shown by *Enterococcus faecium* towards ampicillin; *Streptococcus pneumoniae* towards penicillin and *Staphylococcus aureus* towards methicillin and oxacillin (Bingyun *et al*., 2017; Garima *et al*., 2017).

Another mechanism involves some pathogens producing enzymes that inactivate or modify antibiotics making them impossible to function. Such enzymes include β-lactamases, aminoglycoside-modifying enzymes and chloramphenicol acetyl-transferases. β-lactamases hydrolyze β-lactam ring in penicillins and cephalosporins, interfering with their binding ability onto penicillin binding proteins (PBPs). *Staphylococci, Enterococci, Mycobacterium spp*. and *Haemophilus influenzae* strains exhibits this mechanism towards methicillin, Vancomycin, streptomycin and chloramphenicol respectively (Bingyun *et al*., 2017; Garima *et al*., 2017).

2.4.4 Detecting antimicrobial resistance

Several antibiotic sensitivity tests are used that include dilution growth inhibition assays and disc-diffusion susceptibility (Kirby-Bauer) tests (Weinstein *et al*., 2018); E-test; automated methods; mechanism-specific tests and genotypic methods (WHO, 2019b; Zeeshan *et al*., 2019). The test to be used is determined by several factors that include availability of resources, degree of accuracy, convenience and cost (Tim, 2016).

When using dilution growth inhibition assays and disc-diffusion susceptibility tests, minimal inhibitory concentration (MIC) is determined, usually in accordance with the Clinical and Laboratory Standards Institute (Weinstein *et al*., 2018). In this study, standardized disk diffusion method was used. Filter paper disks impregnated with standardized concentrations of different antibiotics are placed on the surface of appropriate medium like Müller-Hinton (MH) agar in a petri dish on to which a standardized isolate suspension has been swabbed (Weinstein *et al*., 2018). After incubation at appropriate temperature and time, the size of zones of growth inhibition around each of the discs are related to the MIC by use of reference tables. The results are recorded as whether the organism is susceptible (S), moderately susceptible (MS) or resistant (R) to that antibiotic (Tim, 2016; Christesson *et al*., 2018; Zeeshan *et al*., 2019).

Automated methods include the BD Phoenix Automated Microbiology System (BD Diagnostics), automated systems, such as the Vitek®2 System (bioMérieux) and the Sensititre ARIS 2X (Trek Diagnostic Systems) (Zhou *et al*., 2018). These diagnostic methods are more effective than other methods (Zeeshan *et al*. 2019). In this study, the Vitek®2 System (bioMérieux) was used as it is more reliable and accurately detects methicillin resistant and vancomycin resistant enterococci (VRE) among coagulase-negative staphylococci (CoNS) isolates in the clinical laboratory (Susmitha *et al*., 2016; Zhou *et al*., 2018; Zeeshan *et al*., 2019). It is highly automated and uses very compact plastic reagent cards (credit card size) that contain microliter quantities of antibiotics and test media in a 64-well format. There are four reagents cards for identification of different organism classes, which are: GN: Gram-negative fermenting and non-

fermenting bacilli; Gram-Positive cocci and non-spore forming; YST: Yeast and yeast-like organisms and BCL: Gram-positive spore-forming bacilli (Susmitha *et al*., 2016).

Epsilometer test (E-test) (AB Biodisk, Solna, Sweden), uses multiple plastic test strips coated with pre-defined antibiotic concentrations to determine the MIC (Zeeshan *et al*., 2019). Genotypic methods involve test for the specific genes that confer antibiotic resistance. They include polymerase chain reaction (PCR) which involves detection of target DNA sequence of interest, amplifying and analysing it; DNA hybridization and modifications of PCR and DNA hybridization (Zeeshan *et al*., 2019).

2.5 Physico-chemical pollutants in river water

Most physico-chemical pollutants in rivers like raw sewage, wastewater, chemical substances, pharmaceutical residues (mostly antibiotics), antibiotic resistant genes (ARGs), suspended particles and sediments are due to human activities. Chemicals are either inorganic of organic compounds. Inorganic compounds are mostly heavy metals like lead, mercury, cadmium, arsenic, chromium, zinc, nickel and copper. Others include total suspended solids -TSS, plant nutrients like nitrates and phosphates. Organic compounds are like oil, plastics, biocides, pesticides, persistent organic pollutants, cleaning solvents and detergents (Mominul *et al*., 2018). Most of the chemical substances are toxic as they persist in the environment bringing about various health problems in human populations and other animals (Masayuki *et al*., 2018; Melissa, 2018).

Nitrogen forms various inorganic and organic compounds in water. Inorganic ones include nitrite, nitrate, ammonia or ammonium compounds, organic compounds are like amino acids, proteins, humic acids and urea (Rohrer *et al*., 2017; WHO, 2017c; WHO,

2018). Phosphorus forms inorganic compounds like orthophosphate and polyphosphate, and organic phosphate. Particulate phosphorus may also be found in suspension, sediment, minerals or adsorbed on particles. Total nitrogen (TN) is a measure of all forms of nitrogen found in water while total phosphorus (TP) is a measure of all forms of phosphorus found in water (WHO, 2017c; WHO, 2018).

Persulfate in an acid or alkaline is used to measure total phosphorus (TP) and total nitrogen (TN) in water samples. A mixture of river water sample and persulfate salt and sodium hydroxide gives a pH >12. Under these alkaline conditions, nitrogen in the sample is oxidized to nitrate. Boiling the mixture at high temperature of 120°C, more digestion leads to decomposition of persulfate to form bisulfate ions which are neutralized and then acidified by the reaction mixture in the chemical reaction (Maximilian *et al*., 2015):

$$S_2O_8^{2-} + H_2O \xrightarrow{\Delta} 2\,HSO_4^{-} + \tfrac{1}{2}\,O_2$$

The low pH obtained of about 2 favours hydrolyses of all dissolved phosphorus in the sample into orthophosphate respectively (De Borba *et al*., 2016). The concentrations of nitrogen and phosphorus in these compounds are then determined by use of colorimetry (e.g. for phosphorus, the digest mixture is reacted with an ammonium molybdate/antimony potassium tartrate solution along with ascorbic acid. This forms molybdenum blue, the intensity of which is proportional to the amount of phosphorous in solution). Alternatively, ion chromatography can be used (Maximilian *et al*., 2015).

The levels of heavy metals in river water are determined using Atomic Absorption Spectrometry (AAS) (Harvey, 2019). The analyte is first converted into free gaseous atoms after being heated in an atomizer using such flames like air-acetylene flame

(2300 °C) for most elements. Depending on the type of the atomizer, the two main analytical techniques are flame AAS (FAAS) and electrothermal AAS (ETAAS). The free gaseous atoms absorb electromagnetic radiation at a specific wavelength to produce a measurable signal. The absorption signal is proportional to the concentration of those free absorbing atoms in the optical path. Typical atomic absorption lines are observed as dark lines in the solar spectrum, usually narrower than 0.002 nm (Fernández, *et al*., 2019).

2.5.1 River water pollution and antibiotics resistance

There are many antibiotics residues and other pollutants that are released into the rivers of most countries that contribute to antimicrobial resistance (Andrew *et al*., 2016; Bengtsson *et al*., 2018). The high concentration of pollutants and antibiotics residues in such river causes selective pressure bringing about mutations that lead to formation of antibiotic resistant genes (ARGs) responsible for antibiotic resistance (Macharia, 2015; Wales *et al*., 2015; Webber *et al*., 2015; Zhang *et al*., 2017; Zhang *et al*., 2018). The ARGs may bring about resistance of bacteria to other chemicals like biocides, a phenomenon known as cross-resistance, or may appear together with resistant genes to other chemicals substances on mobile genetic elements (MGEs) such as conjugative plasmids, transposons and integrons, hence co-resistance (Pal *et al*., 2015; Wilfred *et al*., 2015). These MGEs increase bacteria adhesion on suspended particles of the river pollutants in waters, forming biofilms that enriches the river water with plasmids that carry ARGs (Zhai *et al*., 2016; Bhatty *et al*., 2017; Dang *et al*., 2017; Magali *et al*., 2018). This explains why high number antibiotic resistant bacteria are found in polluted water.

2.5.2 Antimicrobial effects of heavy metals

Heavy metals and their metalloids co-select for the development of antibiotics resistance by their co-existence with antibiotics in the environment (Songcan *et al*., 2015). They bring about resistance of bacteria towards antibiotics by modulation of the expression of antibiotic resistance genes, making them to have chemical reactions with the antibiotics thus decreasing bioavailability of each other (Songcan *et al*., 2015; Tong *et al*., 2015; Zhou *et al*., 2018; Kunihiko *et al*., 2019). The heavy metals also bring about resistance through co-resistance or cross-resistance. This involves transfer of antibiotic resistance properties in bacteria through vertical gene transfer (VGT) or horizontal gene transfer (HGT) by conjugation, transformation, or transduction (Pal *et al*., 2015; Wilfred *et al*., 2015).

Studies have shown that the combined presence of heavy metal ions like of zinc, lead, copper, cadmium and mercury in the rivers, leads to development of antibiotic resistant genes (ARGs) that get transferred amongst members of same species (horizontal gene transfer) between species (vertical gene transfer).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The study was carried out at Thiba river basin within Kirinyaga and Embu Counties along the Eastern side of Mt. Kenya region of Kenya (Figure 3.1). This constitutes a vast area drained by Thiba (Thitha) river and its tributaries.

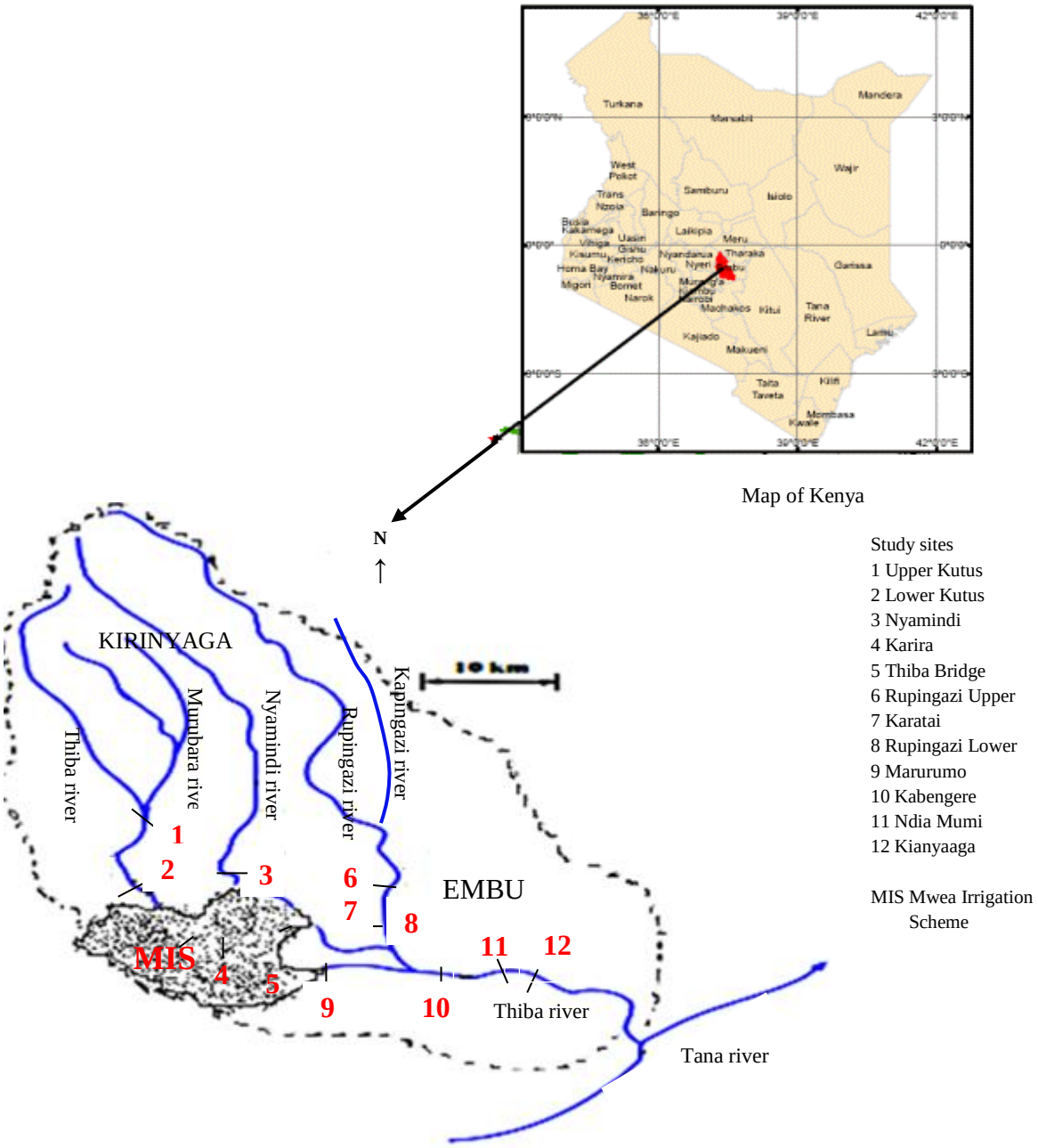


Figure 3.1: Thiba river basin showing position of the study sites (Courtesy Google map).

Embu County is located approximately between latitude 0°31'58.80" N and longitude 37°27'0.00" E, while the Mwea region that transects both Embu and Kirinyaga counties is located approximately between latitude 00°40'54" and 1°42'00" S and 37°30'00" E. The rivers originate from Mt. Kenya and drain through Kirinyaga and Embu Counties before joining the main Tana (Thagana) river at Kamburu Hydroelectric Power Station downstream. The main tributaries are Murubara, Nyamindi and Rupingazi rivers. Kapingazi river is a tributary of Rupingazi river. Thiba, Murubara and Nyamindi rivers are the main source of water for Mwea Irrigation Scheme (MIS) in Kenya. The predominant crop grown in Mwea Irrigation Scheme (MIS) is rice. A total of 16,000 acres are under paddy production. The residents of the basin use the river water for domestic purpose and subsistence as well as commercial farming. Therefore, the rivers are exposed to various forms of pollutants like agrochemicals and sewage, which favour multiplication of bacteria responsible for water-borne related diarrhoea.

3.2 Sample size determination

To determine the minimum river water and rain water sample size needed at 95 % confidence level and desired absolute precision of 5 %, a formula used was according to Taherdoost (2017):

$$n = \frac{Z^2}{(d/2)^2}\ pq$$

where n= the required sample size; p= the expected prevalence rate 16 % (which is average for diarrhoeal diseases among children under 5 years of all cases reported in Kenya (Regidor *et al*., 2017); q=1-p; Z= the value of the required confidence interval (1.96) and d= desired absolute precision or marginal error (5 %).

Substituting the values $n= \frac{(1.96)^2}{\left(\frac{5}{2}\right)^2} 16X(1-16)=167.186$, or approximately 168 samples.

3.3 Sampling design

The Thiba river basin was divided into three sampling regions, the upper, the middle and the lower zones. The upper zone was the area upstream before the Mwea irrigation scheme (MIS), the middle zone comprised of area within MIS, while the lower zone comprised of area downstream after the MIS. Each zone had four sampling sites (Table 3.1):

Table 3.1: Distribution of sampling sites per zone within Thiba river basin

Zone	Sampling sites
Upper zone	Upper Kutus
	Lower Kutus
	Nyamindi
	Rupingazi upper
Middle zone	Karira
	Thiba bridge
	Karatai
	Rupingazi lower
Lower zone	Marurumo
	Kabengere
	Ndia Mumi
	Kianyaaga

The sampling sites were uniformly distributed with a distance of approximate one kilometre from each another. The sampling of water was done during both wet and dry seasons to take care of seasonal variability. During dry season in the month of September 2016, twenty four (24) river water samples were collected from each of the three zones, giving a total of seventy two (72) water samples. From each sampling site, six (6) river water samples, each containing 2 litres, were collected for eleven days. Each time the water samples were placed in separate glass bottles that had been

sterilized in an autoclave (at 121^{o}C for 1 hour) and transported in a cool box to Microbiology and Analytical Chemistry laboratories at Kenyatta University for analyses of microbial and physico-chemical parameters. The same procedure was repeated in October 2016 during wet season to get river water sample from each sampling site for eleven days, giving a total of seventy two (72) river water samples. Two (2) rain water samples, each containing two litres, were also collected from each sampling site during rainy season, a total of twenty four (24) rain water samples. The total number of water samples (both river and rain water) collected was therefore one hundred and sixty eight (168) water samples. Rain water was sampled by placing opened sterilized bottles in open field when it was raining.

3.4 Secondary data on resistance of bacteria causing water-borne diarrhoea towards commonly used antibiotics from hospitals within the Thiba river basin

To obtain the status of common bacteria-related diarrhoea in residents of Thiba river basin, a structured questionnaire (Appendix II) was administered to sixty health workers within the region, in the main local health facilities; Embu Level 5 Hospital, Kerugoya Level 4 Hospital, Kimbimbi Sub-County Hospital and Our Lady of Lourdes Mwea Mission Hospital (Karira). The obtained data was on incidences of bacteria-related water-borne diarrhoeal diseases in the year 2016, the antibiotics commonly used to treat the diseases and the reported cases of bacteria resistance against these antibiotics. This was obtained from records in the registries.

3.5 Isolation, identification and enumeration of bacteria causing water-borne diarrhoea

3.5.1 Isolation of bacteria cultures using Membrane Filter Technique - MFT

Serial dilution of 10 ml of each water sample was carried out aseptically to get 0.1 % concentration. This involved pipetting 10 ml of each properly mixed sample into a dilution bottle containing 90 ml of sterile distilled water (Anupama, 2020). The whole of this content was poured through a sterile 0.45±0.02 µm pore size and 47 mm diameter nitrocellulose filter membrane placed into the funnel of the assembly of membrane filter technique apparatus. Aseptically, the membrane filter with trapped bacteria was then placed onto sterile Mac Conkey-sorbitol Agar and incubated for the 24 hours at 37°C. Bacterial culture characteristics like shape, size, colour, surface appearance and texture of colonies were used for further identification of the bacteria (Madhusudana et al., 2018)

3.5.2 Microbial load in the Thiba river basin water and rain water

Multiple tube fermentation technique was used to enumerate the total number of colonies of bacteria. Each water sample was diluted to 0.1 ml, 1 ml and 10 ml concentrations and placed in separate test tubes. Each concentration was aseptically inoculated into Lactose broth medium containing Durham tube and sealed. They were incubated at temperature of 37^{o}C for 24 hours. The Durham tubes which had a gas inside were recorded as positive for lactose fermentation. For each sample, MPN Index per 100 ml was calculated using the MPN Index table to give approximate total number of colonies in 100 ml of the sample. From each positive tube, 0.1 ml of the culture was dispensed aseptically into Eosin Methylene Blue (EMB) broth and incubated for 24 hours at 37^{o}C (Madhusudana *et al*., 2018). Positive for coliforms was indicated by dark

purple colonies and dark centres with greenish metallic sheens. Lastly, the colonies with 0.1 ml dilution tube, which was positive from step 2, were inoculated onto mEndo agar LES and mFC agar and streaked on Nutrient agar slant. The plates with inoculated mEndo agar LES and Nutrient agar slant were incubated at 37°C for 24 hours, while inoculated mFC agar plates were incubated for 24 hours at 44.4ºC. The colonies obtained were evaluated for typical coliforms by conducting Gram stain test (Madhusudana *et al*., 2018).

3.5.3 Identification of bacteria in Thiba river basin water and rain water that causes water-borne diarrhoea

Positively identified cultures from Membrane Filter Technique were sub-cultured onto Deoxycholate citrate (DCA) agar, Salmonella - Shigella (SS) Agar and Thiosulfate-Citrate-Bile salts-Sucrose (TCBS) Agar for further isolation and identification of the entero-pathogenic bacteria. Identification of the species and serotypes was carried out using standard conventional biochemical tests that included urease, hydrogen sulphide-TSI, motility, indole, simmon citrate, methyl red, Vp (Voges-Proskauer) and TSI (triple sugar iron) tests.

Some isolates of entero-pathogenic bacteria associated with diarrhoea from the river water and rain water samples were taken to Centre for Microbial Research (CMR) laboratories at Kenya Medical Research Institute (KEMRI) for further identification of bacteria species using VITEK 2 Systems: 07.01 tests (WHO, 2019b). To use the VITEK 2 Systems, the inoculum was placed into the VITEK®2 Cassette. The Vitek®2 Gram negative identification card (GN) with established biochemical methods 1, 2, 4, 8, 9, 10, 11, 12, 17, 18, 20, 21, 24, 25 and 27; carbon source utilization and enzymatic

activities was used to identify the Gram Negative bacilli. The incubation and reading of each reagent card occurred automatically (Susmitha *et al*., 2016).

3.6 Antimicrobial susceptibility testing

Inoculum of isolated *Pseudomonas aeruginosa* bacterium from dry season was prepared according to McFarland 0.5 standards and spread onto the Muller-Hinton agar plate (Shah *et al*., 2016). Same procedure was repeated on another plate using the bacterium isolated during wet season. Two sets of disks were impregnated with antibiotics that were commonly used in the Thiba river basin to treat bacteria-related water-borne diarrhoea. The antibiotics used in the test were those that were commonly used to treat diarrhoel diseases in Thiba river basin and represented variety mode of antimicrobial action. These were Minocycline (min 30 mg), cotrimoxazole (sxt 25 mg), methicillin (meth 5 mg), erythromycin (ery 15 mg), metronidazole (mtz 16 mg), ampicillin (amp 10 mg), chloramphenicol (cap 30 mg), ciprofloxacin (cip 5 mg) and lincomycin (lcm 2 mg) antibiotics. One set of impregnated discs was placed onto one Muller-Hinton agar plate inoculated with the bacterium isolated during dry season and the other set placed onto the agar plate with the bacterium isolated during wet season. The procedure was repeated for the other seven isolated bacteria, giving a total of sixteen (16) inoculated plates. Two plates were not inoculated to act as control. All the inoculated Muller-Hinton plates with bacteria and having impregnated discs and those not inoculated were incubated at 37°C for 24 hours (LibreTexts, 2019b). The diameters of zones of inhibition were then measured to the nearest whole millimetre using a sliding ruler and were compared to the Clinical and Laboratory Standards Institute (CLSI) interpretative charts to determine if the strains were resistant, moderate susceptible, or susceptible to the antibiotics tested (Hervet *et al*., 2016).

3.7 Measurement of physico-chemical pollutants in Thiba river basin water and rain water

To measure Total suspended solids (TSS), Glass-fiber filter (Whatman 934-AH, Cat No 1827-047, of pore size 1.5 µm and a diameter 47 mm) was washed with three successive 10 ml volume of reagent grade water, dried at 103-105^{o}C in an oven, cooled in a desiccator and weighed. One litre of each water sample was poured through the pre-weighed glass-fibre filter with vacuum pump. The filter was then removed and dried to constant mass to ensure that all the water was removed, and then it was reweighed. The total weight of TSS in milligrams per litre (mg/L) of the material was calculated using the formula (Arvind *et al*., 2016).

$$\text{TSS in mg/L} = \frac{(\text{Fresh filter weight in gms} - \text{Dry filter weight in gms})}{\text{Volume of sample in L}} \text{X1000mg/L}$$

Total phosphorus (TP) and total nitrogen (TN) were measured using persulfate in an alkaline using Standard Methods (De Borba *et al*., 2016). To 100 ml of each river water sample, sulfuric acid was added and the mixture was placed in a 250 mL HDPE container and then boiled gently in a HotBlock. Into a digestion vessel, 50 mL of the sample was placed and 1 mL 11 N sulfuric acid solution and 0.5 g potassium persulfate were added. The mixture was boiled gently for 30 minutes, ensuring it did not boil down past 10 mL. The samples were then removed from the HotBlock, cooled, pH adjusted using buffer solution (mixture of boric acid and low nitrogen sodium hydroxide diluted to two litres using reagent water) and the final volume of each sample was brought up to 50 mL using reagent water. The concentrations of nitrogen and phosphorus were then determined using colorimetric analysis (De Borba *et al*., 2016).

The levels of heavy metals in Thiba river basin water, namely cadmium, chromium, zinc and copper, were determined using Flame Atomic Absorption Spectrometer (F-AAS) principle (Harvey, 2019). For each water sample, 100 ml was measured and placed in culture test tube, then 10 ml of aqua regia (HNO_3 67 %: HCl 37 % = 3:1) and 1 ml of perchloric acid were added and the mixture was incubated at 80°C in a water bath. After total digestion and subsequent cooling, the solution was diluted to 50 ml and analyzed for Zinc, Lead, Cadmium, and Chromium ions in a closed system by atomic absorption spectrophotometry (Cristiana *et al*., 2014).

3.8 Data analysis

Statistical analysis was performed using MS excel considering P-values ≤ 0.05 as significant. One-factor ANOVA and Student's t-test were used to determine whether there was any significant difference in the microbial and physico-chemical pollutants in the three study regions within the Thiba river basin at 5 % significance confidence level during dry and wet seasons. Pearson Correlation Coefficient (r) and Bivalent Regression Analysis tests were done to determine whether there was relationship between the levels of microbial and physico-chemical pollutants in the Thiba river basin waters and bacteria resistance against the antibiotics commonly used to treat the bacterial-related water-borne diarrhoea. The Regression model equation used was given by:

$y=b+ax_1^2+ax_2+c$

Where

y= dependent variable; x= independent variable; a= y-intercept; b= slope of regression line and c= random error.

The Fisher's least significant difference (LSD) tool was used to separate means that were found to be significantly different.

CHAPTER FOUR

RESULTS

4.1 Secondary data on diarrhoea incidences in the Thiba river basin obtained from local hospitals and levels of river water pollution

Secondary data on incidences of water-borne diarrhoea obtained from the daily records from the major hospitals within the Thiba river basin, namely Embu Level 5 Hospital, Kerugoya Level Hospital, Kimbimbi Sub-County Hospital and Mwea Mission Hospital - Karira, showed the highest incidence of diarrhoeal diseases was recorded at 15 % to 19 % of total diarrhoea cases (Appendix III). For all the cases of diarrhoeal diseases affecting the residents of Thiba river basin, bacteria-related diarrhoea was reported to be frequent in these children compared to those suffering from protozoa-related diarrhoea. During the time of this study, only eight (8) cases of cholera, twenty one (21) cases of shigellosis and sixteen (16) cases of typhoid fever were reported in the four main hospitals sampled. The Kimbimbi Sub-County Hospital led in 2016 with ninety seven (97) cases of patients treated for bacteria-related diarrhoea, followed by Embu Level 5 Hospital with ninety four (94) cases (Appendix III).

According to the respondents, the antibiotics commonly used for treatment of these diseases in the hospitals within the Thiba River basin were cotrimoxazole, metronidazole, ciprofloxacin and doxycycline, since the pathogens causing bacteria-related diarrhoea showed no resistance against them. However, these bacteria showed some resistance against streptomycin, chloramphenicol, penicillin, amoxicillin and ampicillin, hence rarely used for treatment of bacterial diarrhoea (Appendix III).

In the process of sampling river water from the rivers draining Thiba river basin, it was found that the water, especially in the Middle Zone during dry season, was having many

physico-chemical pollutants that included suspended solids, nutrients, agrochemicals and and raw sewage. The sewage was mostly generated from many urban centres with high population of people. The region lacked adequate sanitation facilities in rural areas and urban centres such as Ngurubani (Mwea) town; as well as in the rice paddies. This led to the raw sewage being discharged into the rivers. The workers in the rice paddies were seen defecating and bathing in the rivers openly. Raw sewage is known to have high concentration of pathogens and physico-chemical contaminats. The various farming activities produced a lot of agrochemicals usually emptied in to the rivers. The commonly observed chemical pollutants in the rivers were agrochemicals, mostly fertilisers and detergents. The medical personnels from the various health centres in the region associated the diarrhoea diseases affecting the residents with the contamination of river water by microbial and physico-chemical pollutants (Appendix III).

4.2 Isolation and identification of water-borne diarrhea associated bacteria

In a total of one hundred and sixty eight (168) water samples analysed, one hundred and forty three (143) or 85 % of them were contaminated with coliforms and pathogenic bacteria. Out of these, ninety six (96) or 68 % were from dry season and forty seven (47) or 32 % were from wet season. The trapped bacteria on the membrane filter formed colonies on Mac Conkey-sorbitol Agar. The bacterial culture characteristics like shape, size, colour, surface appearance and texture of colonies were used for further identification of the bacteria (Madhusudana et al., 2018). The conventional biochemical and the VITEK 2 Systems: 07.01 tests identified S*almonella paratyphi; Salmonella enterica*; *Klebsiella pneumoniae*; *Shigella flexineri*; *Enterobacter liquefaciens; Proteus mirabilis*; *Pseudomonas aeruginosa* and *Escherichia coli* (Table 4.1). More samples were contaminated with *Salmonella paratyphi*, *Shigella flexineri* and *E. coli*.

Salmonella paratyphi had the highest value in both dry and wet seasons for both upper and lower zones at 6.2 % and 26.7 % in dry season and 3.3 % and 23.8 % in wet season respectively. *Escherichia coli* mean values were second highest for both seasons in Middle and Lower zones at 8.8 % and 15.8 % in dry season and 7.5 % and 8.3 % in wet season respectively. *Proteus mirabilis* and *Enterobacter liquefaciens* were quite rare in both seasons, with highest for *Proteus mirabilis* being 0.8 % in the Middle zone during dry season. The highest mean value for *Enterobacter liquefaciens* was during wet season in the lower zone at 0.6 %. The Middle zone had the highest total mean values of most of the identified bacteria in both seasons.

Table 4.1: Identified pathogenic bacteria in water samples and rain water during dry and wet seasons

Zone	Sampling site	Bacteria identified (%)															
		SP		KP		SF		SE		EL		EC		PM		PA	
		D	W	D	W	D	W	D	W	D	W	D	W	D	W	D	W
UZ	Upper Kutus	4.3	4.2	0.0	0.0	0.0	0.0	0.0	0.0	1.6	0.0	0.0	0.0	0.0	0.0	3.1	2.8
	Lower Kutus	7.4	2.0	0.0	1.5	0.0	0.0	0.0	0.0	2.4	0.8	8.0	5.1	0.0	0.0	0.0	0.0
	Nyamindi	6.0	2.3	2.4	3.6	6.8	0.0	0.0	0.0	0.0	1.5	3.0	6.9	0.0	2.1	0.0	0.0
	Rupingazi Upper	7.0	4.6	4.1	4.4	3.0	0.0	3.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Mean value	6.2	3.3	1.6	2.4	2.5	0.0	0.9	0.0	1.0	0.6	2.8	3.0	0.0	0.5	0.8	0.7
MZ	Karira	0.0	23.6	5.6	0.0	3.0	0.0	0.0	0.0	3.0	0.0	0.0	13.4	0.0	0.0	0.0	23.0
	Thiba Bridge	0.0	0.0	0.0	0.0	3.0	6.5	0.0	0.0	0.0	2.6	0.0	0.0	0.0	0.0	0.0	35.0
	Rupingazi Lower	35.1	9.5	0.0	13.7	3.0	0.0	8.0	5.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Karatai	47.5	38.0	29.0	12.0	3.0	0.0	0.0	0.0	0.0	0.0	35.0	16.5	0.0	0.0	17.4	0.0
	Mean value	20.7	17.8	8.7	6.4	3.0	1.6	2.0	1.5	0.8	0.7	8.8	7.5	0.0	0.0	4.4	14.5
LZ	Marurumo	25.7	34.0	0.0	0.0	3.0	0.0	0.0	0.0	0.0	0.0	12.4	12.0	0.0	2.5	0.0	13.0
	Kabengere	16.1	23.0	22.0	0.0	3.0	3.0	0.0	0.0	0.0	0.0	17.9	5.0	0.0	0.0	0.0	8.0
	Ndia-Mumi	38.0	26.0	15.5	0.0	3.0	3.0	0.0	0.0	1.5	0.9	8.7	6.0	0.0	0.0	0.0	0.0
	Kianyaaga	27.0	12.2	21.0	13.0	3.0	0.0	12.1	0.0	0.0	0.0	24.0	10.3	0.0	0.0	11.7	0.0
	Mean value	26.7	23.8	14.6	3.3	3.0	1.5	3.0	0.0	0.4	0.2	15.8	8.3	0.0	0.6	2.9	5.3
Rain water		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Key: SP, *Salmonella paratyphi*; KP, *Klebsiella pneumoniae*; SF, *Shigella flexineri*; SE, *Salmonella enterica*; EL, *Enterobacter liquefaciens*; EC, *Escherichia coli*; PM, *Proteus mirabilis*; PA, *Pseudomonas aeruginosa*; UZ, Upper zone; MZ, Middle zone; LZ, Lower zone.

There were no bacteria detected in the rain water.

4.3 Levels of microbial contaminants in Thiba River basin water

In DCA medium, the red colonies were for total coliforms while colourless colonies were for entero-pathogenic *Escherichia coli* (Plate 4.1 C). The mean values of total coliforms and pathogenic *E. coli* were higher in dry season than in wet season, a difference 87.39 CFU/100 ml for total coliforms and 43.01 CFU/100 ml for *E. coli*. The mean values of total coliforms were higher compared to those of the pathogenic *E. coli* in both seasons, difference of 46.51 CFU/100 ml in dry season and 2.13 CFU/100 ml in wet season. The Middle zone had highest mean value for total coliforms, at 185.37 CFU/100 ml, the only one beyond the admissible levels of 100 CFU/100 ml by WHO.

In the rain water count, there were no total coliforms and pathogenic *E. coli* found, which was in agreement with the admissible level by WHO of 100 CFU/ 100 ml (table 4.4).

Table 4.2: Mean values of enumerated colonies of coliforms in Thiba river basin water during dry and wet seasons

Sampling Zone	TC (CFU/100 ml)		*E. coli* (CFU/100 ml)		WHO admissible levels of coliforms in river water CFU/100 ml (2017c)
	Dry	Wet	Dry	Wet	
Lower zone	91.83[a]	25.03[a]	9.33[a]	28.24[a]	100.00
Middle zone	185.37	39.67[a]	6.25[a]	37.60[a]	100.00
Upper zone	87.60[a]	26.72[a]	29.70[a]	33.08[a]	100.00
Mean	121.60	34.21	75.09	32.08	100.00
Rain water	0.00	0.00	0.00	0.00	0.00

Key: TC, total coliforms; EC, *Escherichia coli*; CFU, coliform forming unit; WHO, World Health Organisation.

Comparison of mean values of total coliforms enumerated during both dry and wet seasons, a value t=2.28 was obtained, where ts<tc at p=0.05, showing no significant

difference. However, comparison of mean values of *E. coli* in both seasons, gave t=6.90 where ts>tc at p=0.05, showing significant difference. The ANOVA test of mean values for total coliforms and *E. coli* for all zones in both seasons showed significant difference (Fs>Fc at p=0.05: 7.492145). Thus the hypothesis was rejected at p=0.05.

The calculation of the least significant difference (LSD) at 95 % confidence gave a value of 7.51. All the means showed no significance difference, except the mean for total coliforms in the middle zone during dry season (Table 4.4).

Colonies of *Salmonella* spp. in SS-Agar media appeared colourless with black centres while those of *Shigella flexineri* were colourless on same medium (Plate 4.1 D).

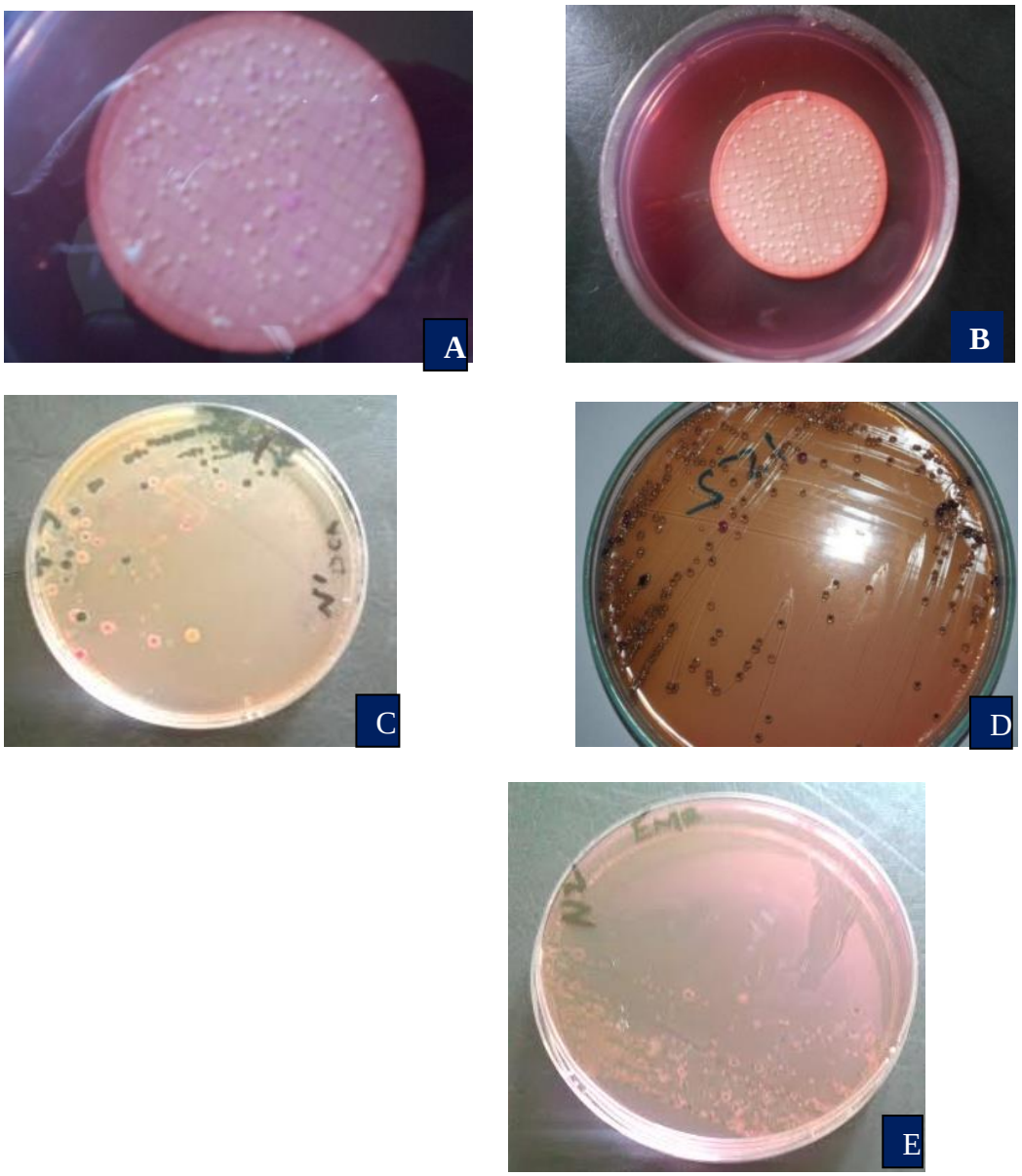


Plate 4.1: Pathogenic bacteria in different media

A&**B**, filter membranes with trapped bacteria from Thiba river basin water; **C**, pathogenic bacteria sub-cultured in DCA medium; **D**, *Salmonella* spp. in SS agar medium; **E**, pathogenic bacteria sub-cultured in EMB medium.

4.4 Antibiotics susceptibility test

The susceptibility of each microorganism to nine commonly used antibiotics in Thiba river basin in both seasons was interpreted as S for susceptible, MS for moderately sensitive and R for resistant (Table 4.2). The two plates that were not inoculated lacked any zone of inhibition and the bacteria colonies there grew normally.

Table 4.3: Sensitivity of identified pathogenic bacteria from Thiba river basin water against commonly used antibiotics

Test antibiotic	Antiobitic concentration (µg/l)	Average zone of inhibition (mm) against susceptibility of the pathogenic bacteria (S, MS or R)							
		PA		KP		SF		EL	
		D	W	D	W	D	W	D	W
Min	30	17(S)	18(S)	19(S)	20(S)	25(S)	30(S)	24(S)	26(S)
Sxt	25	14(MS)	19(S)	22(S)	20(S)	21(S)	28(S)	16(S)	19(S)
Cip	5	24(S)	27(S)	16(S)	18(S)	18(S)	16(S)	19(S)	16(S)
Mtz	16	16(S)	20(S)	17(S)	19(S)	26(S)	25(S)	20(S)	26(S)
Cap	30	17(S)	21(S)	13(MS)	9(R)	25(S)	21(S)	26(S)	30(S)
Meth	5	16(S)	12(MS)	9(R)	10(MS)	9(R)	10(MS)	15(MS)	18(S)
Amp	10	20(S)	17(S)	10(MS)	17(S)	16(S)	16(S)	22(S)	24(S)
Ery	15	12(MS)	15(S)	15(MS)	23(S)	9(R)	8(R)	11(MS)	14(MS)
Lcm	2	20(S)	18(S)	17(S)	21(S)	17(S)	19(S)	18(S)	21(S)

Test antibiotic	Antiobitic concentration (µg/l)	Average zone of inhibition (mm) against sensitivity of the pathogenic bacteria (S, MS or R)							
		EC		SE		PM		SP	
		D	W	D	W	D	W	D	W
Min	30	24(S)	25(S)	22(S)	25(S)	18(S)	13(MS)	22(S)	19(S)
Sxt	25	28(S)	30(S)	26(S)	29(S)	20(S)	18(S)	24(S)	21(S)
Cip	5	26(S)	22(S)	17(S)	19(S)	21(S)	24(S)	15(MS)	17(S)
Mtz	16	21(S)	24(S)	20(S)	18(S)	16(S)	17(S)	11(MS)	17(S)
Cap	30	28(S)	26(S)	22(S)	21(S)	18(S)	17(S)	23(S)	26(S)
Meth	5	12(MS)	16(S)	10(MS)	13(MS)	23(S)	13(MS)	11(MS)	10(S)
Amp	10	11(MS)	14(MS)	10(MS)	8(S)	10(MS)	12(MS)	20(S)	14(S)
Ery	15	9(R)	12(MS)	19(S)	17(S)	11(S)	10(MS)	9(R)	7(R)
Lcm	2	21(S)	23(S)	22(S)	18(S)	10(S)	12(MS)	11(MS)	17(S)

Key: PA, *Pseudomonas aeruginosa;* KP, *Klebsiella pneumoniae*; SF, *Shigella flexineri*; EL, *Enterobacter liquefaciens*; EC, *Escherichia coli*; SE, *Salmonella enteritidis*; PM, *Proteus mirabilis*; SP, *Salmonella paratyphi*; Min, minocycline; Sxt, cotrimoxazole; Cip, ciprofloxacin; Mtz, metronidazole; Cap, chloramphenicol; Meth, methicillin; Amp, ampicillin; Ery, erythromycin; Lcm, lincomycin; S= Sensitive (≥ 16 mm diameter); MS= Moderately sensitive (10-15 mm diameter); R= Resistant (<9 mm diameter).

Table 4.4: Percentage resistant bacteria against the antibiotics

Bacteria	Isolates	% Antibiotic resistance																	
		Min		Sxt		Cip		Mtz		Cap		Meth		Amp		Ery		Lcm	
		D	W	D	W	D	W	D	W	D	W	D	W	D	W	D	W	D	W
PA	16	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
KP	16	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	23.5	0.0	32.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SF	16	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	46.4	0.0	0.0	0.0	82.7	0.0	66.4	0.0
EL	16	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	35.5	0.0	0.0	0.0	54.6	0.0	0.0	0.0
EC	16	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	74.2	0.0	0.0	0.0
SE	16	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	38.7	0.0	0.0	0.0	0.0
PM	16	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SP	16	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	84.2	78.0	0.0	0.0

Key: PA, *Pseudomonas aeruginosa;* KP, *Klebsiella pneumoniae*; SF, *Shigella flexineri*; EL, *Enterobacter liquefaciens*; EC, *Escherichia coli*; SE, *Salmonella enteritidis*; PM, *Proteus mirabilis*; SP, *Salmonella paratyphi*; Min, minocycline; Sxt, cotrimoxazole; Cip, ciprofloxacin; Mtz, metronidazole; Cap, chloramphenicol; Meth, methicillin; Amp, ampicillin; Ery, erythromycin; Lcm, lincomycin.

Pseudomonas aeruginosa did not show resistance against any tested antibiotic. It however showed moderate susceptibility towards cotrimoxazole and erythromycin during dry season and methicillin during wet season. It was susceptible to other antibiotics tested in both seasons (Table 4.2). *Klebsiella pneumoniae* showed resistance against methicillin (32.6 %) during dry season and chloramphenicol (23.5 %) during wet season (Table 4.3). It was moderately susceptible towards chloramphenicol, ampicillin and erythromycin during dry season and methicillin during wet season. It was susceptible to all other antibiotics tested in both seasons (Table 4.2). *Shigella flexineri* was resistant against methicillin (46.4 %) and erythromycin (82.7 %) during dry season and erythromycin (66.4 %) during wet season (Table 4.3). It only showed moderate susceptibility towards methicillin during wet season and susceptibility to all other antibiotics tested in both seasons (Table 4.2).

Escherichia coli showed resistance against erythromycin (74.2 %) only during dry season (Table 4.3). It was moderately susceptible towards methicillin and ampicillin

during both seasons and lincomycin during wet season. It was susceptible towards all other antibiotics tested in both seasons. *Enterobacter liquefaciens* was resistant against methicillin (35.0 %) and erythromycin (54.6 %) during dry season (Table 4.3) and moderately susceptible towards methicillin and erythromycin during dry season and erythromycin during wet season. It was susceptible to all other antibiotics tested in both season (Table 4.2).

Salmonella enteritidis showed resistance against ampicillin during wet season (38.7 %) and was moderately susceptible towards methicillin and ampicillin during dry season and towards methicillin during wet season. It showed susceptibility towards the other antibiotics tested in both seasons (Table 4.2).

Proteus mirabilis was not resistant against any antibiotic tested. However, it was moderately susceptible towards ampicillin, erythromycin and lincomycin in both seasons, while it showed susceptibility towards all the other antibiotics tested. It was susceptible towards all other antibiotics tested in both seasons. *Salmonella paratyphi* showed resistance against erythromycin during dry season (84.2 %) and during wet season (78.0 %) (Table 4.2), moderately susceptible towards ciprofloxacin, metronidazole, methicillin and lincomycin during dry season and methicillin and ampicillin during wet season. It was susceptible towards the other antibiotics tested in both seasons (Table 4.2).

The ANOVA analysis of the results gave F value of 2.310 at p=0.05, which is greater than the critical value of F=1.718. This indicates that the difference of resistance of the pathogenic bacteria identified against the antibiotics tested in the dry and wet

seasons were significant. This implied that the resistance of the bacteria against the antibiotics tested was affected by seasonal variations.

4.5 Physico-chemical analysis of river and rain water

4.5.1 Physical pollutants

The mean levels of total suspended solids (TSS) were quite high (7125.00 mg/l in dry season and 5566.67 mg/l during wet season) compared to admissible levels of 1500 mg/l by World Health Organisation, especially during dry season. The middle zone had the highest values at 10500 mg/l of river water (Table 4.5).

4.5.2 Chemical pollutants

Table 4.5: Mean values of physico-chemical parameters in Thiba river basin water during dry and wet seasons and rain water

Zone	Season	TP (mg/l)	TN (mg/l)	TSS (mg/l)	Cd^{+2} (mg/l)	Cu^{+2} (mg/l)	Zn^{+2} (mg/l)	Cr^{+3} (mg/l)
Lower	Dry	58.30	213.75	9500.00	1189.03	0.14	62.16	4.39
	Wet	379.70	207.61	1200.00	108.00	0.28	61.08	7.05
Middle	Dry	610.00	319.92	10500.00	17.87	0.95	73.06	5.33
	Wet	434.00	219.50	8000.00	60.03	0.51	192.51	4.59
Upper	Dry	133.40	53.50	1375.00	30.94	0.35	0.41	1.31
	Wet	473.10	218.42	7500.00	47.93	0.39	22.91	4.44
Mean	Dry	267.23	195.72	7125.00	412.61	0.48	45.21	3.68
	Wet	428.94	215.09	5566.67	71.99	0.39	92.17	5.36
WHO (mg/l) (2017c)		200.00	50.00	1500.00	0.01	1.00	5.00	0.05
Rain water (mg/l)		88.75	39.13	1250.00	2.20	0.01	41.83	1.17
Rain water values admissible levels by WHO (mg/l) (2017c)		0.00	0.00	0.00	0.00	0.00	0.00	0.00

Key: TN, total nitrogen; TSS, total suspended solids; TP, total phosphorus; Cd^{2+}, cadmium ions; Cu^{2+}, copper ions; Zn^{2+}, zinc ions; Cr^{3+}, chromium ions.

Generally, the mean values of all the physico-chemical parameters were higher in all zones during dry season than wet season, except for total phosphorus in the Lower and Upper zones, total nitrogen in Upper zone, cadmium and zinc in Middle zone and zinc

and chromium in Upper zone. The mean values for all parameters for both seasons were higher than admissible levels by World health Organisation (Table 4.5)

The mean levels of total of total nitrogen and total phosphorus were quite high for both seasons (134.12 mg/l in dry season and 215.09.67 mg/l during wet season for total nitrogen; and 267.23.00 mg/l in dry season and 428.94 mg/l during wet season for total phosphorus) compared to the admissible levels by World Health Organisation of 50 mg/l and 200.00 mg/l respectively. The Middle Zone showed the highest mean values for total nitrogen in both seasons, while the levels of total phosphorus were highest during dry season, with small difference of 476.60 mg/l between highest and lowest value (Table 4.5).

The mean levels of cadmium ions were quite high for both seasons, at 55.61 mg/l in dry season and 71.99 mg/l during wet season, compared to admissible levels in river water by World Health Organisation (WHO) at 0.01 mg/l. The wet season value was even higher as compared to that of the dry season, a difference of 16.38 mg/l (Table 4.5).

The highest mean level for copper ions was at 0.95 mg/l in the middle zone during dry season, with very low difference of 0.81 mg/l, between the highest and the lowest values. The mean values for the copper ions for both seasons, 0.48 mg/l in dry season and 0.39 mg/l during wet season, were lower compared to the World Health Organisation (WHO) admissible value of 1.00 mg/l (Table 4.5).

The wet season had higher mean levels for zinc ions compared to the mean value of dry seasons, a difference of 46.96 mg/l. The mean levels of the zinc ions for both seasons,

at 45.21 mg/l in dry season and 92.17 mg/l during wet season, were higher compared to the admissible levels by World Health Organisation (WHO) of 5.00 mg/l (Table 4.5).

The mean levels for chromium ions were higher, at 3.68 mg/l in dry season and 5.36 mg/l during wet season, compared to the World Health Organisation admissible mean values of 0.05 mg/l. The mean value in wet season was higher compared to that of dry season, with a difference of 1.68 mg/l (Table 4.5).

The rain water from the three zones of Thiba river basin sampled during rainy season had no physico-chemical pollutants. This was in agreement with the admissible levels by World Health Organisation of 0.00 mg/l (Table 4.4).

4.5.3 Comparison of mean values of physico-chemical parameters between dry and wet seasons per zone

Table 4.6: T-values on comparison of mean values of physico-chemical parameters between the dry and wet seasons per zone at p=0.05 in Thiba river basin water

Parameter	Season	LZ vs MZ		LZ vs UZ		MZ vs UZ	
TSS	D	0.71	NSD	1.26	NSD	3.46	NSD
	W	1.55	NSD	3.50	SD	0.28	NSD
TN	D	-1.15	NSD	-0.01	NSD	2.73	SD
	W	-0.66	NSD	-0.83	NSD	0.13	NSD
TP	D	-0.61	NSD	1.04	NSD	1.21	NSD
	W	-0.41	NSD	-1.39	NSD	-1.67	NSD
Cd^{2+}	D	4.68	SD	1.41	NSD	-0.56	NSD
	W	1.87	NSD	-0.41	NSD	1.79	NSD
Cu^{2+}	D	-1.19	NSD	-1.75	NSD	-0.71	NSD
	W	-1.01	NSD	-0.54	NSD	0.61	NSD
Zn^{2+}	D	-0.92	NSD	0.97	NSD	1.62	NSD
	W	-1.20	NSD	1.00	NSD	1.75	NSD
Cr^{3+}	D	2.13	NSD	-1.31	NSD	-0.24	NSD
	W	-0.13	NSD	-0.06	NSD	0.37	NSD

Key: NSD, no significant difference between the means; SD, significant difference between the means; TSS, total suspended solids; TN, total nitrogen; TP, total phosphorus; Cd^{2+}, Cadmium ions; Cu^{2+}, Copper ions; Zn^{2+}, Zinc ions; Cr^{3+}, Chromium ions; LZ, Lower zone; MZ, Middle zone; UZ, Upper zone; D, dry season; W, wet season.

On physical pollutants, the comparison of total suspended solids mean values between the three zones showed no significant difference for all in both seasons except for the one between Lower Zone vs Upper Zone during wet season that showed significant difference (Table 4.6).

The chemical pollutants also showed almost similar trends. Comparison of total nitrogen and total phosphorus mean values between the three zones in both seasons, all values showed no significant difference. Comparison of cadmium ions mean values between the three zones, all values showed no significant difference except for the one between Lower Zone vs Middle Zone during dry season (Table 4.6). Comparison of copper, zinc and chromium ions mean values between the three zones, showed no significant difference in both seasons (Table 4.6).

Table 4.7: Comparison of mean values of physico-chemical parameters between dry and wet seasons per zone in Thiba river basin water

Parameter	TSS (mg/l)	TN (mg/l)	TP (mg/l)	Cd^{2+} (mg/l)	Cu^{2+} (mg/l)	Zn^{2+} (mg/l)	Cr^{3+} (mg/l)
Significance	NSD for all	NSD for all	NSD for all	NSD for LZ and UZ. SD for MZ	NSD for all	NSD for LZ and MZ. SD for UZ	NSD for LZ and MZ. SD for UZ

NSD, no significant difference between the means; SD, significant difference between the means; TSS, total suspended solids; TN, total nitrogen; TP, total phosphorus; Cd^{2+}, Cadmium ions; Cu^{2+}, Copper ions; Zn^{2+}, Zinc ions; Cr^{3+}, Chromium ions; LZ, Lower zone; MZ, Middle zone; UZ, Upper zone;

Comparison of mean values of total suspended solids between the seasons per zone; none showed significant difference (Table 4.7).

On the other hand, comparison of mean values of total nitrogen and total phosphorus between the dry and the wet seasons per zone, none showed significant difference (Table 4.7). Comparison of mean values of cadmium ions between the dry and the wet

seasons per zone, Lower and Upper zones showed no significant difference while the Middle Zone showed significant difference (Table 4.7).

Comparison of mean values of copper and zinc ions between the dry and the wet seasons per zone, showed no significant difference for all. However, the comparison of mean values of zinc ions between the dry and the wet seasons per zone, Lower and Middle zones showed no significant difference, while Upper Zone showed significant difference. On the other hand, the comparison of mean values of chromium ions between dry and wet seasons per zone, Lower and Middle zones showed no significant difference, while Upper Zone showed significant difference (Table 4.7).

4.5.4 Comparison of mean values of physico-chemical parameters across the three zones during dry and wet seasons

After the physico-chemical parameters across the three zones during dry and wet seasons in Thiba river basin water were compared, some showed significance difference (Table 4.8).

Table 4.8: F-values for physico-chemical parameters across the three zones during dry and wet seasons in Thiba river basin water

Season	TSS (mg/l)		TN (mg/l)		TP (mg/l)		Cd^{2+} (mg/l)		Cu^{2+} (mg/l)		Zn^{2+} (mg/l)		Cr^{3+} (mg/l)	
Dry	1.26	NSD	186.82	SD	1.04	NSD	4.80	SD	1.52	NSD	0.76	NSD	0.65	NSD
Wet	5.09	SD	0.16	NSD	0.80	NSD	2.06	NSD	0.10	NSD	2.08	NSD	0.01	NSD

NSD, no significant difference between the means; SD, significant difference between the means; TSS, total suspended solids; TN, total nitrogen; TP, total phosphorus; Cd^{2+}, Cadmium ions; Cu^{2+}, Copper ions; Zn^{2+}, Zinc ions; Cr^{3+}, Chromium ions.

During dry season, only total nitrogen, and cadmium ions had significance mean levels across the three zones. However, during the wet season, only the mean value of total suspended solids was significant across the three seasons (Table 4.8).

4.6 Relationship between microbial and physico-chemical pollutants in Thiba river basin water and resistance of bacteria causing water-borne diarrhoea across the seasons

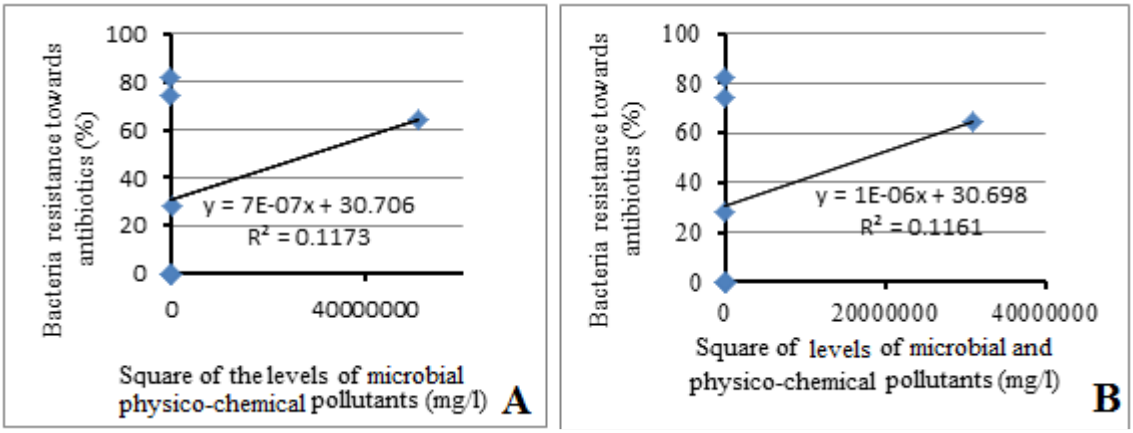
Comparison between the mean differences of resistance of the pathogenic bacteria causing water-borne diarrhoea against antibiotics used to treat them and mean values of Thiba river basin pollutants for both dry and wet seasons at p=0.05, some relationship was realised (Table 4.9).

Table 4.9: Statistical values on relationship between microbial and physico-chemical pollutants of Thiba river basin and bacteria resistance against antibiotics

Value	Season	
	Dry	Wet
r	0.323648	0.302009
T	0.815207	0.810325
R^2	0.117319	0.116608
Significant F	0.452022	0.454576

The coefficient of bacteria resistance against antibiotics and the mean values of water physico-chemical pollutants in Thiba river basin during dry season gave r value of 0.323648, while during wet season r=0.302009 (Table 4.9). The two showed weak positive correlation, which implied that the relationship between resistance of bacteria causing water-borne diarrhoea against commonly used antibiotics and levels of microbial and physico-chemical pollutants in Thiba river basin water was significant, as both values are greater than p=0.05. This was an indicator that as the level of pollutants increased in the river water, the resistance of the bacteria against antibiotics also increased.

The scatter diagrams for both seasons were non-linear; hence data conversion was done to obtain quadratic expressions that gave linear curves (Figure 4.1).



A, During dry season; **B**, During wet season.

Figure 4.1 Relationship between Thiba river basin physico-chemical pollutants and bacteria resistance against antibiotics during dry season.

R^2 values for both seasons indicate that it is only about 12 % of resistance of bacteria against antibiotics that could be explained by changes in the values of physico-chemical pollutants in Thiba river basin water, hence not a good fit. On the other hand, the values of significant F for both seasons (Table 4.9) were far much greater than p=0.05. This indicates that the tests were less statistically significant, meaning they are not much reliable. The shapes of the curves showed some positive modulating influence of the Thiba river water contaminants on the resistance of pathogenic bacteria causing water-borne diarrhoea towards commonly used antibiotics. Increasing the levels of microbial and physico-chemical pollutants in Thiba river basin water increased the levels of resistance of the bacteria causing water-borne diarrhoea against the antibiotics commonly used to tread them. Negative influence was found when there were low microbial and physico-chemical contaminants of Thiba river basin water.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Microbial contaminants of Thiba river basin water

The microbial contaminants, the total coliforms and pathogenic *E. coli*, in Thiba river basin waters were found to be higher compared to the international admissible values by World Health Organization (Table 4.4). The higher coliforms concentration was an indicator of higher contamination by pathogenic bacteria, some of which were isolated and identified in the Thiba river basin water (Table 4.1)

The membrane filters used with pore sizes of 0.45 µm, are tiny enough to block passage of coliforms and pathogenic bacteria. When the contaminated water sample is passed through the filters using filter funnels and vacuum system, the microorganisms are trapped and get concentrated on surface of the membrane. The filters with trapped bacteria are then placed on special pads saturated with appropriate media. Passing nutrients through the filters and incubating at appropriate temperature, the trapped bacteria grow into colonies that are identified through conventional biochemical tests or by use of Vitek 2 system. For instance, coliforms form red colonies with a metallic sheen on mEndo agar, while faecal coliforms form dark blue colonies on mFC agar (Nisha, 2019; Acharya, 2021).

Reports have shown that many residents of the Thiba river basin are prone to diarrhoeal diseases caused by entero-pathogenic bacteria isolated in the river water (GoK, 2015). This is clearly collaborated by the secondary data obtained from the hospitals within the basin on the category of the mostly affected age-groups by water-borne diarrhoea.

The bacteria identified in this study that cause water-borne diarrhoea diseases were *Salmonella paratyphi* and *S. enterica* that cause typhoid fever, *Shigella flexineri* that cause shigellosis, *Enterobacter liquefaciens* and *Escherichia coli* (Table 4.1). The recorded incidence of all diarrhoea in children below five (5) years in the two weeks preceding the Kenya Demographic and Health Services - KDHS survey was 11.5 % and 12.0 % of total diarrhoea cases in the households surveyed in Embu and Kirinyaga counties (KDHS Report, 2016).

According to a report by the Ministry of Health to World Health Organisation, Embu and Kirinyaga are amongst the seven counties that continue to have active water-borne bacteria-related diarrhoea outbreaks, with others being Garissa, Mombasa, Nairobi, Turkana and Wajir (WHO, 2017b). The people living around these regions are highly exposed to raw sewage from the urban centres, which have high concentration of these pathogens. High incidence of these diseases in the Thiba river basin is a clear indicator of significant presence of the pathogenic bacteria causing water-borne diarrhoea (GoK, 2015). Therefore, the hypothesis that the bacteria species responsible for water-borne bacteria diarrhoeal diseases in the Thiba river basin water and rain water are not significantly different during dry and wet seasons was rejected at p=0.05 and 95 % confidence level.

The source of these pathogens includes human faeces, animal droppings and poor domestic and personal hygiene (Ngari, 2015). Some raw sewage from sewerage treatment plant and water run-off from Embu Town and its environs are emptied into the nearby Rupingazi River. This raises major concern especially to the residents of Old Stadium and Emma villages, and others living downstream within Mwea and Mbeere

South sub-counties, who use the river water for domestic purposes, livestock and farming. This may explain the high levels of microbial and physico-chemical contaminants in the nearby Karatai sampling site, found just across Rupingazi River on Kirinyaga (Table 4.1).

5.1.2 Physico-chemical pollutants of Thiba river basin water and rain water

The levels of physico-chemical pollutants tested, except for copper ions (Cu^{2+}), were higher compared to the international admissible levels by World Health Organization (Table 4.5). Therefore, the hypotheses that there are no significant levels of microbial pollutants in the Thiba river basin water and rain water during dry and wet seasons; and there are no significant levels of physico-chemical pollutants in the Thiba river basin water and rain water during dry and wet seasons were rejected at p=0.05 and 95 % confidence level. The rain water sampled was found to have no significant levels of microbial and physico-chemical pollutants, a fact which is in agreement with the admissible levels by World Health Organisation. In the Thiba river basin, the major source of the heavy metal ions is agrochemicals and biocides used there. Macharia (2015) found that the commonly used agrochemicals in Kirinyaga for horticultural farming included dimethoate, labda, cyhalothrin, cymxanil, cypermethrin, cyfluthrin, mancozeb and deltamethrin.

The over-use of these agrochemicals with time may have brought about this high level of the heavy metal ions. Limited use of many agrochemicals by farmers that are copper based and the possibility of copper ions being eliminated faster from the water, may have contributed to the lower levels of copper ions compared to the admissible levels by World Health Organisation (Macharia, 2015).

Statistical analysis indicated no significant difference in the mean values of river water microbial and physico-chemical contaminants between dry and wet seasons. However, higher resistance by bacteria against antibiotics was witnessed during dry season than wet season. The cause of this could have been the carrying away of pathogenic bacteria having ARGs downstream when the rivers flood after heavy rains. During dry season, the shortage of water, high temperature and dusty conditions favour rapid proliferation of pathogenic bacteria and their resistance to antibiotics. During dry season, the river flows slowly and some water stagnates in pools. The water gets more concentrated with physico-chemical pollutants which may exert a lot of selective pressure on the bacteria causing water-borne diarrhoea diseases. This may lead to their mutations making them to be resistant against the antibiotics used (Shah *et al*., 2016).

According to previous studies, the significant presence of these contaminants in the water of Thiba river basin could be attributed to sources like raw sewage, detergents, fertilizers, herbicides and pesticides (Javier *et al*., 2018; UNICEF, 2019). In the Thiba river basin, rice growing and extensive horticultural farming are practiced by the small scale and commercial horticultural farms. These farms use different types of manure and fertilisers that release nutrients rich in phosphorus and nitrogen compounds. The soil erosion and surface water run-offs carry a lot of sediments in water that contribute to total suspended solids (TSS).

5.1.3 Microbial resistance towards commonly used antibiotics in Thiba river basin

The study showed that some bacteria causing water-borne diarrhoeal diseases are resistant against some commonly used antibiotics in Thiba river basin. The resistance

is mostly due to high concentration of contaminants in Thiba river basin water that exert environmental pressure on the bacteria inducing horizontal genes transfer and mutations. Those bacteria that undergo mutations develop intrinsic or acquired antibiotic resistance enabling them to survive and multiply (Oritogun *et al*., 2018). Misuse and overuse of antibiotics such as erythromycin and methicillin has contributed much to the increased resistance by the bacteria (Francesca *et al*., 2015; Bingyun *et al*., 2017; Zhang *et al*., 2017; Huang *et al*. 2018). Secondary data obtained from the health facilities within the basin showed that only metronidazole and doxycycline antibiotics were not resisted by bacteria (Appendix III), hence the drugs of choice. Therefore, the hypothesis that there is no significant resistance of the water-borne bacteria species that cause diarrhoeal diseases in the Thiba river basin water and rain water against the commonly used antibiotics by residents to treat them, was rejected at p=0.05 and 95 % confidence level.

5.1.4 Relationship between microbial and physico-chemical pollutants and resistance against antibiotics for treating water-borne bacteria diarrhoeal diseases in Thiba river basin

The study showed some positive correlation between levels of physico-chemical contaminants in the Thiba river basin and resistance of bacteria responsible for water-borne diarrhoea (Figure 4.1B). As the levels of the pollutants increases, the resistance of bacteria against antibiotics used increases. Increase in bacterial pollutants in water leads to increase in antibiotic resistance-carrying bacteria (ARBs) in different environments, making the resistance to spread rapidly (Kraemer *et al*. 2019). The higher values of significant F in the multiple regression test indicated that it was not very easy to predict bacteria resistance against commonly used antibiotics to treat bacteria-related

diarrhoeal diseases given the levels of microbial and physico-chemical contaminants of Thiba river basin water. Therefore, the hypothesis that there is no relationship between the microbial and physico-chemical pollutants of the Thiba river basin water and the levels of resistance of diarrhoea-related bacteria species against commonly used antibiotics was rejected at p=0.05 and 95 % confidence level.

The presence of heavy metals caused highest resistance (Appendix V). They contribute to the selection of antibiotic-resistant strains of bacteria. According to study by Nguyen *et al*. (2019), bacteria develop heavy metal resistance genes (MRGs) that enhance the action of antibiotic resistance genes (ARGs) in the same bacteria. the study also showed that zinc and cadmium were the metals mostly associated with resistance to antibiotics; while *Pseudomonas aeruginosa* and *Escherichia coli* showed highest co-occurrence of resistance to several heavy metals and antibiotic classes. Sabria *et al*., (2018), showed significant correlation between ARGs and the concentration of arsenic (As), zinc (Zn), lead (Pb) and mercury (Hg), suggesting that their combined presence may favour the propagation of ARGs. Antibiotic resistance due to the presence of cadmium, copper and zinc can take place via co-resistance or cross-resistance (Wilfred *et al*., 2015).

As observed earlier, the particulate matter like total suspended solids (TSS), sediments and nutrients offer surface for adhesion of these resistant bacteria forming reservoir for antimicrobial resistant genes (ARGs) (Huang *et al*., 2019). The ARGs are easily transferred or acquired rapidly among bacteria population, which could have been responsible for the resistance against erythromycin (Table 4.2). These genes may also result to multiple drug resistance (MDR) by pathogens (Francesca *et al*., 2015; Kraemer *et al*., 2019), especially in *Salmonella typhi,* lowering their susceptibility to

ciprofloxacin (Mutai *et al*., 2018). It is most likely that the MDR *Salmonella paratyphi* strains may have emerged in the Thiba river basin thus posing major public health problems.

5.2 Conclusions

(i) The study showed that the Thiba river basin river water had significant levels of coliform bacteria. It also identified the pathogens associated with water-borne bacteria diarrhoeal diseases, such as *Salmonella paratyphi*; *Klebsiella pneumoniae*; *Shigella flexineri*; *Salmonella enterica*; *Enterobacter liquefaciens* and *Escherichia coli*. This exposes residents to high risk of contracting the diseases.

(ii) The study found that some of these identified bacteria pathogens associated with water-borne diarrhoeal diseases showed resistance against some antibiotics tested except for minocycline, cotrimoxazole, ciprofloxacin and metronidazole. Many showed moderate susceptibility and susceptibility towards the antibiotics tested. The resistance of antibiotics may impact the residents of Thiba river basin negatively as they use antibiotics to treat the bacteria- related water-borne diarrhoeal diseases.

(iii) The higher levels of microbial contaminants in the Thiba river basin water and rain water during dry and wet seasons compared to the admissible levels by the World Health Organisation clearly indicates the danger the residents of the area are exposed to in contracting water-borne bacterial diarrhoeal diseases.

(iv) The Thiba river basin water had considerable amounts of physico-chemical pollutants above the admissible levels by World Health Organisation. The failure

to meet the standards set by this international body means that the water should be used with much caution.

(v) The study showed that the Thiba river basin water microbial and physical-chemical contaminants had some modulating effect on levels of resistance of the pathogenic bacteria against antibiotics commonly used by residents to treat diarrhoea caused by bacteria. Thus more effort should be put in place to ensure the cleanliness of the rivers.

5.3 Recommendations

(i) Proper treatment of river water in the Thiba river basin should be done before use to eradicate the pathogenic bacteria responsible for water-borne diarrhoeal diseases.

(ii) There is a need for constant surveillance by both the national government and county governments of Embu and Kirinyaga counties for antimicrobial resistance for prompt treatment.

(iii) Improvement of water sanitation should be practiced in the Thiba river basin to minimise microbial pollutants.

(iv) Similarly, improvement of water sanitation should be practiced in the Thiba river basin to minimise physico-chemical pollutants.

(v) The county assemblies of Embu and Kirinyaga should come up with strict legislation against emptying of pollutants, especially heavy metals, which have direct bearing on bacteria resistance to antibiotics.

5.4 Recommendations for further studies

There is need for thorough screening of bacteria-related water-borne diarrhoea cases in residents of Thiba river basin and in the whole country for the purpose of controlling its spread.

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APPENDICES

Appendix I: Authorization letters from the counties

MINISTRY OF HEALTH
OFFICE OF EMBU COUNTY DIRECTOR OF HEALTH

Telephone: 068-31883
Fax: 068- 31791

Email : cdhembu@gmail.com
When replying please quote our reference

COUNTY DIRECTOR OF HEALTH
EMBU COUNTY
P.O. BOX 273
EMBU

Ref: A.9/VOL. VI/50

Date: 7TH October, 2014

TO:
SCMOH – Mbeere South
Med Supt – Embu PGH

RE: RESEARCH AUTHORIZATION– MUTURI STEPHEN NJAGI

The above matter refers.

The above named officer is a post graduate student and is carrying out a research for a MSC Proposal entitled "Relationship between microbial and physico-chemical pollutants and resistance of water-borne diarrhoea-related bacteria against antibiotics in Thiba river basin - Embu and Kirinyaga counties, Kenya."

This office hereby, gives permission to collect data in your facilities.

Kindly accord him necessary support.

COUNTY DIRECTOR OF HEALTH
EMBU COUNTY
P O Box 273, EMBU
Fax: 068 - 319791
Tel: 068 - 31883 / 31081
Email: cdh embu@gmail.com

DR. PHILLIP MASAULO
COUNTY DIRECTOR OF HEALTH
EMBU COUNTY

KIRINYAGA COUNTY GOVERNMENT

OFFICE OF THE GOVERNOR
COUNTY DEPARTMENT OF HEALTH

Telegrams: "MEDICAL", KERUGOYA
Telephone: (060) 21564, 21058
Fax (060) 21564
E- mail: dmohkirinyaga@yahoo.com

COUNTY DIRECTOR OF HEALTH
KIRINYAGA,
P. O. BOX 24,
KERUGOYA

When replying please quote

23RD DECEMBER 2014.

REF; CDH/RES/VOL.I/65

TO WHOM IT MAY CONCERN

RE: APPROVAL TO UNDERTAKE MEDICAL RESEARCH IN KIRINYAGA COUNTY – MUTURI STEPHEN NJAGI

The above named student is currently pursuing a Master's of Science in Medical Microbiology at Kenyatta University.

For his Thesis, he intends to investigate on the relationship between microbial and physico-chemical pollutants and resistance of water-borne diarrhoea-related bacteria against antibiotics of Thiba river basin.

Attached is a concept note for his research project.

Kindly accord him the necessary assistance.

COUNTY DIRECTOR OF HEALTH
P. O. BOX 24, KERUGOYA

DR. ESBON GAKUO
COUNTY DIRECTOR OF HEALTH
KIRINYAGA.

CC:

- **Chief Officer - Health**

Appendix II: Questionnaire on status of diarrhoeal diseases in Thiba basin

I am a MSc Microbiology student at Kenyatta University, Department of Biochemistry, Microbiology and Biotechnology. I am conducting research to investigate the relationship between the levels of water pollutants and incidences of water-borne bacteria-related diarrhoea within the region drained by Thiba river and its tributaries within Kirinyaga and Embu counties. Also to be investigated are the levels of resistance of the bacteria causing bacteria-related diarrhoea against the commonly used antibiotics.

The purpose of this questionnaire is to enable me to acquire information on prevalence of the bacteria-related diarrhoea and the antibiotics commonly used to treat the disease. I will appreciate for your assistance by responding to the questions below. Please note that the data will only be for research.

A: Location

Date:……Health Centre:…… Status…… Sub County ……County……..

B: Water-borne diarrhoea incidence (Please use a tick (√) for answer in the appropriate box)

(i) How do you rate the incidence of water-borne diarrhoea in your area?

Very low ☐ Low ☐ Average ☐ High ☐ Very high ☐

(ii) Which category of people is mostly affected by this water-borne diarrhoea in your area?

Below 5 yrs ☐ 5-12 yrs ☐ 12-18yrs ☐ 18-24yrs ☐
25 yrs and above ☐

(iii) Indicate the status on occurrence of the diarrhoea in the following categories. The percentage should be out of the total reported cases of diarrhea, in the whole of 2016.

Bacteria-related
Below 10 % ☐ 10-14 % ☐ 15-19 % ☐ 20-24 % ☐
25 % and above ☐

Viruses-related
Below 10 % ☐ 10-14 % ☐ 15-19 % ☐ 20-24 % ☐
25 % and above ☐

Protozoans-related:
Below 10% ☐ 10-14 % ☐ 15-19% ☐ 20-24% ☐
25% and above ☐

C: Bacteria-related diarrhoea

(i) How many patients have been treated for each of the stated bacteria-related water-borne diarrhoea in your health centre since the start of this year? (use 1 to 9 as defined below)

1: 0 to 4	4: 15 and 19	7: 30 to 34
2: 5 to 9	5: 20 and 24	8: 35 to 39
3: 10 to 14	6: 25 to 29	9: Above 40

Bacteria-related water-borne diarrhoea	2016							
	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug
Cholera								
Shigellosis								
Typhoid fever								
Salmonellosis								

(ii) Indicate how often the antibacterial drugs listed below are used in treatment of these bacteria-related water-borne diarrhoea in your health centre? (Please tick where appropriate)

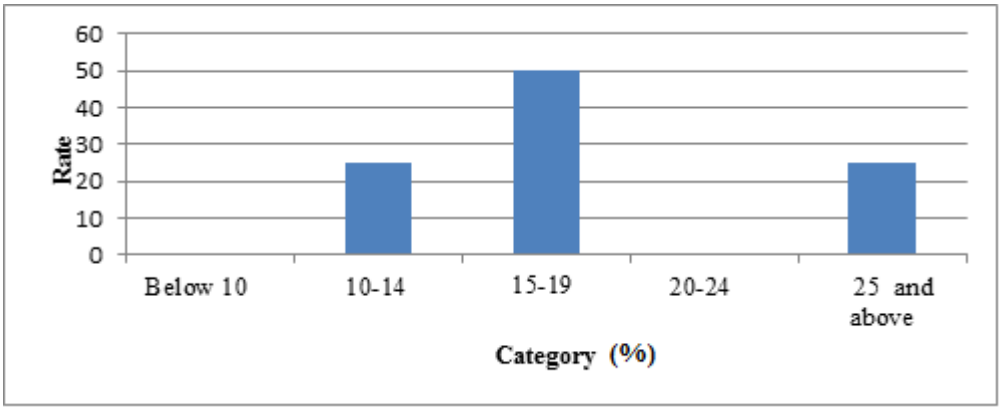
Antibacterial drug	Commonly used	Rarely used	Not used
tetracycline			
cotrimoxazole/ trimethoprim/ sulfamethoxazole			
streptomycin			
gentamicin			
chloramphenicol			
penicillin			
amoxicillin			
ampicillin			
ciprofloxacin			
metronidazole			
doxycycline			

(iii)Indicate whether there have been reported cases of resistance of bacteria against the following antimicrobial drugs commonly used in the treatment of this bacteria-related water-borne diarrhoea in the past five (5) years, and if so, indicate the trend.

Antibacterial drug	None	The trend of resistance			
		Decreasing	Constant	Increasing slowly	Increasing rapidly
tetracycline					
cotrimoxazole/ trimethoprim/ sulfamethoxazole					
streptomycin					
gentamicin					
chloramphenicol					
penicillin					
amoxicillin					
ampicillin					
ciprofloxacin					
metronidazole					
doxycycline					

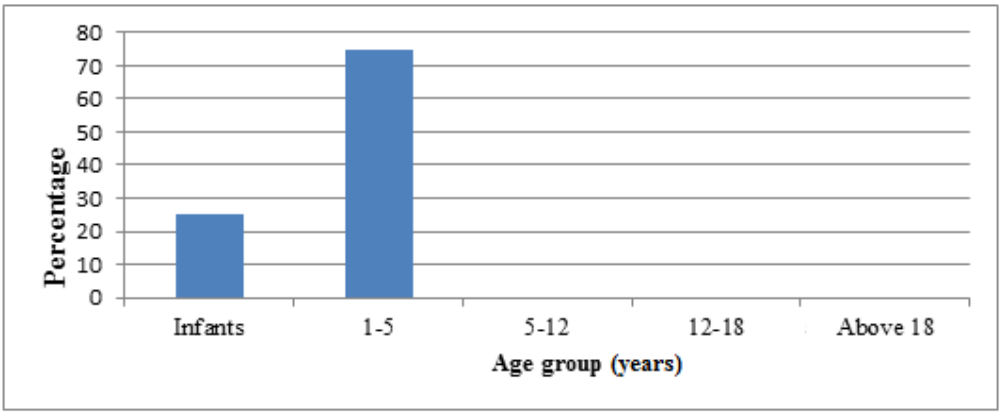
Appendix III: Analysis of Secondary data on responses from structured questionnaire

1.



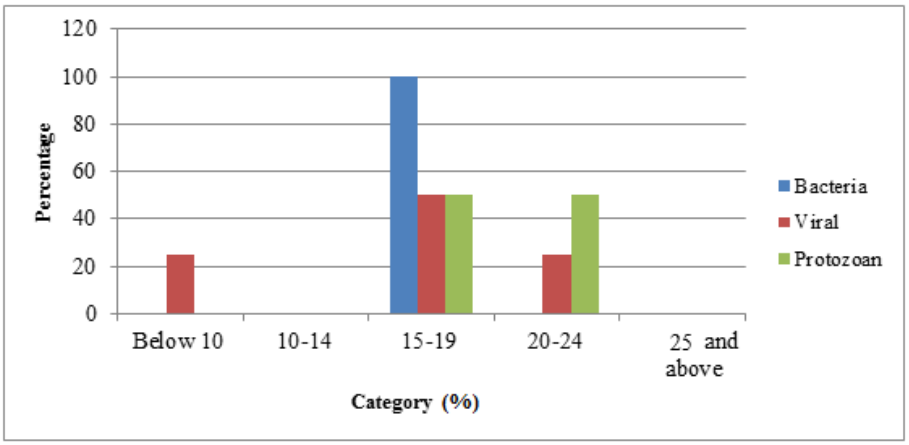
Incidence of water-borne diarrhoea affecting residents who visited the main hospitals within Thiba river basin in the year 2016.

2.



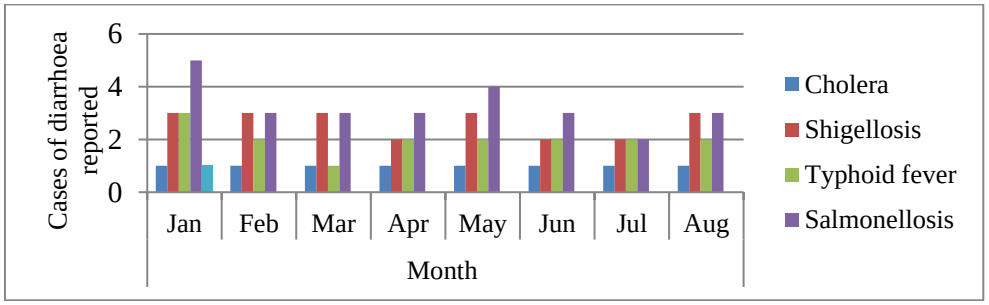
Categories of mostly affected age group by water-borne diarrhoea who visited the main hospitals within Thiba river basin as percentage of the total reported cases of diarrhoea in the year 2016.

3.



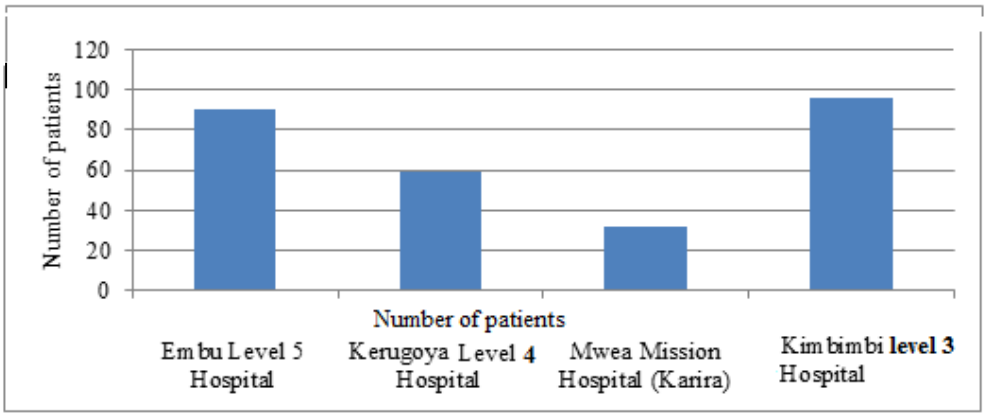
Categories of diarrhoea affecting residents who visited the main hospitals within the Thiba river basin in the year 2016.

4.



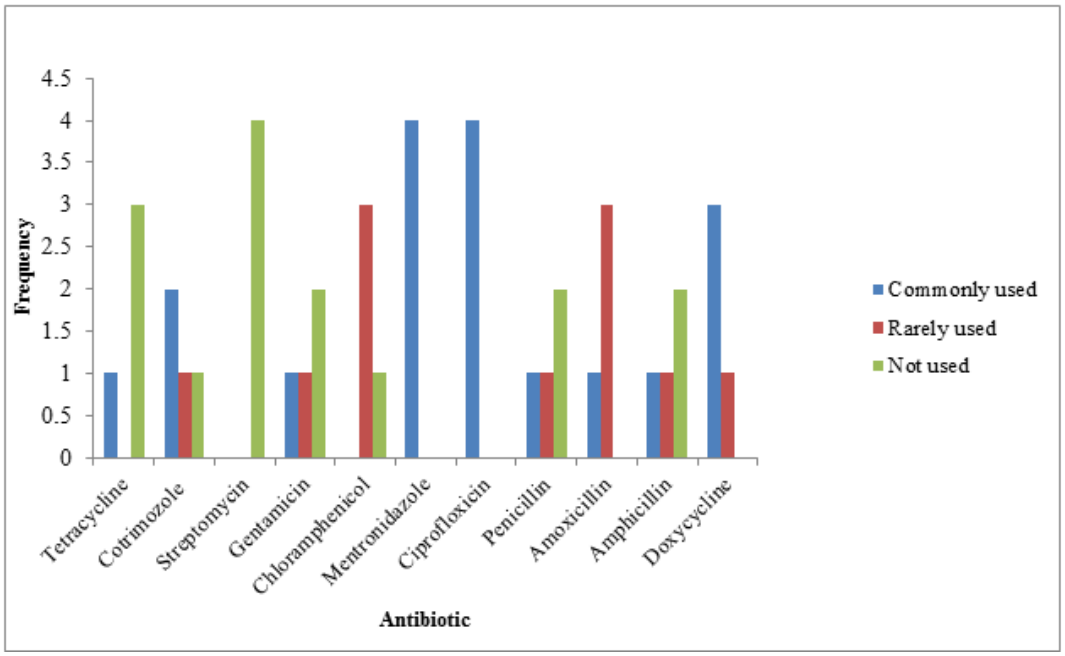
Cases of bacteria-related water-borne diarrhoea in residents who visited the main hospitals within the Thiba river basin from January to August 2016.

5.



Number of patients treated for bacterial-related water-borne diarrhoea in the main hospitals within Thiba Basin main health facilities in the year 2016.

6.



Antibiotics used in treating bacteria-related water-borne diarrhea to patients who visited the main hospitals within Thiba River basin in the year 2016.

Appendix IV: International standards of maximum admissible limits of some physico-chemical pollutants in river water (Gebrekidan, 2005)

	WHO (2008)	USEPA (2008)	EU (1998)	AU (1996)	IRAN (1997)	INDIA (2005)	NZ (2008)
TP (mg/l)	200						
TN (mg/l)	50						
TSS(mg/l)	0.01						
Cu^{+2} (µg/l)	1000	1300	2000	2000	1000	1500	2000
Cr^{+3} (µg/l)	50	100	50	50[c]	50[c]	50[c]	50
Cd^{+2} (µg/l)	1	5	5	2	10	10	4
Zn^{+2}(µg/l)	5000	5000	NM	300[b]	NM	5000	1500
Total coliform	10 per 100ml						
E. coli (µS/cm)	250	NM	2500	NM	NM	NGL	NM

[b]Based on quality (aesthetic) not safety (health risk). [c]Chromium as Cr^{+6} not Cr^{+3}.

NM, not mentioned; NGL**, no guideline because it is not a health concern at that concentration but may affect at concentration above 300 µg/l at which toxic effects may occur; WHO, World Health Organisation; USEPA, United States Environmental and Protection Agency; EU, European Union; AU, African Union; NZ; New Zealand.

Appendix V: Relationship between water pollutants and antibiotics resistance in Thiba river basin

Table 6.6 Comparison between mean values of physico-chemical pollutants of Thiba river basin and resistance of bacteria against commonly used antibiotics during dry and wet season

Pollutant	Dry season (mg/l)	Wet season (mg/l)	Antibiotic	Bacteria resistance towards the antibiotic (%)
TP	267.23	428.94	PA	0.00
TN	134.12	215.09	KP	28.05
TSS	7125	5566.67	SF	64.55
Cd^{2+}	55.61	71.99	EL	0.00
Cu^{2+}	0.48	0.39	EC	74.20
Zn^{2+}	45.21	92.17	SE	0.00
Cr^{3+}	3.68	5.36	SP	82.00

Total nitrogen (TN); TSS, total suspended solids; TN, total nitrogen; TP, total phosphorus; Cd^{2+}, cadmium ions; Cu^{2+}, copper ions; Zn^{2+}, zinc ions; Cr^{3+}, chromium ions; PA, *Pseudomonas aeruginosa;* KP, *Klebsiella pneumoniae*; SF, *Shigella flexineri*; EL, *Enterobacter liquefaciens*; EC, *Escherichia coli*; SE, *Salmonella enteritidis*; PM, *Proteus mirabilis*; SP, *Salmonella paratyphi*; Min, minocycline; Sxt, cotrimoxazole; Cip, ciprofloxacin; Mtz, metronidazole; CaP, chloramphenicol; Meth, methicillin; Amp, ampicillin; Ery, erythromycin; Lcm, lincomycin.

Appendix VI: Statistical tests for mean values

Table 6.11 Summary of hypotheses testing

Hypotheses	Estimate	p-value of T statistic	Conclusion
H_{01}: There is no significant presence of entero-pathogenic bacteria responsible for diarrhoeal diseases in the rivers of the Thiba river basin water and rain water.	15.67	0.00	Reject H_{01}
H_{02}: There is no relationship between levels of Thiba river basin water and rain water pollutants and increased resistance of entero-pathogenic bacteria to antibiotics commonly used by residents to treat these diseases.	-0.25	0.045	Reject H_{02}
H_{03}: The levels of pollution of rivers of Thiba river basin has no relationship with levels of resistance of pathogenic bacteria to antibiotics commonly used by residents to treat these diseases.	0.71	0.03	Reject H_{03}