PLASMID-MEDIATED ANTIBIOTIC RESISTANCE IN GRAM-NEGATIVE PATHOGENIC BACTERIA ISOLATED FROM RIVER SOSIANI AND WASTE WATER SYSTEMS IN UASIN GISHU COUNTY, KENYA

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2025

DECLARATION

Declaration by the student

This thesis is my original work and has not been submitted for any academic award in any institution; and shall not be reproduced in part or full, or in any format without prior written permission from the author and/or University of Eldoret.

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DEDICATION

This thesis is dedicated to my lovely husband, Mr. Elias Odhiambo Oriama, for the continual financial support that facilitated the success achieved. I also dedicate to my pretty daughter, Myled Vishaka Oriama, my mother Mss. Gladys Mildred Oyuga, my siblings and the rest of my family for their unwavering support, advice and infinite inspiration throughout my academic journey. May Almighty God bless you abundantly.

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ABSTRACT

The widespread of bacteria resistance to antibiotics poses an immense threat to the public health. Aquatic ecosystems receive waste water which invariably contain different bacteria, chemicals and antibiotic residues that induces bacterial resistance to antibiotics, resulting into several treatments failures and deaths. Plasmid-mediated antibiotic resistance of bacteria through horizontal gene transfer has been reported to significantly cause resistance in susceptible bacteria. The current study identified antibiotic resistance genes present in gram-negative bacterial plasmids, isolated from River Sosiani and wastewater systems in Uasin Gishu County, Kenya. The sampling sites included Outspan-Nairobi bridge, Pioneer bridge, Moi Teaching and Referral Hospital, Eldoret prison, Kipkaren bridge, Huruma quarry influent, Huruma quarry effluent, Kipkenyo boundary influent and Kipkenyo boundary effluent. The study entailed bacteria isolation, identification, antibiotic susceptibility testing, plasmid DNA extraction and polymerase chain reaction of the antibiotic-resistant genes. Mean and standard deviations were analyzed using Microsoft Excel while Chi square was used to determine significant relationship existing between the phenotypic and genotypic resistance in bacteria. Most of the bacteria coliforms obtained were too numerous to count. A total of 10 gram-negative pathogenic bacteria species were detected, including *Escherichia coli, Enterobacter aerogenes, Citrobacter freundii, Klebsiella pneumoniae, Klebsiella oxytoca, Proteus vulgaris, Proteus mirabilis, Pseudomonas aeruginosa*, *Salmonella enteriditis* and *Yersinia enterocolitica.* All the 10 bacteria conferred resistance to penicillin, ampicillin, amoxicillin-clavulanic acid, ceftazidime, tetracycline, streptomycin and sulfamethoxazole-trimethoprim. Multi antibiotic resistance index was higher in *E. aerogenes* (0.83) but lower in *Y. enterocolitica* (0.58). Out of the nine antibiotic-resistant genes investigated, *qepA* and *qnrA* genes were present in the plasmids of all the 10 bacteria species, 70% of the total bacteria species had *dfrA5*, 70% had *aadA1*, 50% had bla_{KPC}, 40% had *mphA*, 30% of the bacteria species had bla_{SHV} and *sul 1* genes ($P \geq 1$) while none of the bacterial plasmid DNA had *AmpC* gene. The study findings suggested that River Sosiani and the sampled wastewater systems contained different pathogenic bacteria and their plasmid-mediated antibiotic resistance genes which confer resistance to commonly used antibiotics. Therefore, effective waste disposal, continuous monitoring and proper pretreatments of water from River Sosiani and the sampled wastewater systems may reduce bacteria contamination and antibiotic resistance inducers, addressing challenges associated with antibiotic resistance in bacteria.

TABLE OF CONTENTS

DECLARATION ii
DEDICATION iii
ACKNOWLEDGEMENT iv
ABSTRACT v
TABLE OF CONTENTS vi
LIST OF TABLES ix
LIST OF FIGURES x
LIST OF APPENDICES xi
LIST OF ABBREVIATIONS AND ACRONYMS xii
CHAPTER ONE 1
INTRODUCTION 1
  1.1 Background of the Study 1
  1.2 Statement of the problem 3
  1.3 Justification of the study 4
  1.4 Study objectives 6
    1.4.1 General objective 6
    1.4.2 Specific objectives 6
  1.5 Null Hypothesis 6
CHAPTER TWO 7
LITERATURE REVIEW 7
  2.1 Prevalence of Bacteria in Water systems 7
  2.2 Common bacterial waterborne diseases 8
  2.3 Antibiotic susceptibility test 9
  2.4 Mechanisms of plasmid-mediated antibiotic resistance in Bacteria 10
    2.4.1 *Escherichia coli* 11
    2.4.2 *Salmonella species* 12
  2.5 Contamination of water bodies with antibiotics 13

CHAPTER THREE 16

MATERIALS AND METHODS 16

3.1 Study area..16

3.2 Study area map...17

3.3 Study design and sampling frame 18

3.4 Study population 18

3.5 Sample size determination 18

3.6 Sample collection and frequency 19

3.7 Chemicals and reagents...19

3.8 Laboratory analysis 19

3.8.1 Isolation and identification of bacteria from River Sosiani and wastewater systems 20

3.8.2 Antibiotic susceptibility test 23

3.8.3 Identification of genes in the plasmids of the selected bacteria for resistance against the antibiotics...24

3.9 Data quality control...28

3.10 Data analysis 29

3.11 Ethical approval 30

CHAPTER FOUR...31

RESULTS 31

4.1 Isolation and identification of Bacteria from River Sosiani and wastewater systems..31

4.1.1 Analysis of coliform bacteria in water...31

4.1.2 Bacteria sub culturing 33

4.1.3 Gram staining and biochemical characteristics.................................34

4.1.4 Distribution of Gram- negative pathogenic bacteria species in River Sosiani and wastewater systems 38

....40

4.2 Antibiotic susceptibility test 41

4.2.1 *Escherichia coli* 41

4.2.2 *Enterobacter aerogenes* 44

4.2.3 *Citrobacter freundii* 45
4.2.4 *Klebsiella pneumoniae* 45
4.2.5 *Klebsiella oxytoca* 46
4.2.6 *Proteus vulgaris* 47
4.2.7 *Proteus mirabilis* 48
4.2.8 *Pseudomonas aeruginosa* 49
4.2.9 *Salmonella enteriditis* 50
4.2.10 *Yersinia enterocolitica* 51
4.2.11 Determination of Multi antibiotic resistance index (MARI) in bacteria.........52
4.3 Antibiotic- resistant genes in plasmids of the isolated bacteria 54
4.3.1 Sub culturing of bacteria 54
4.3.2 Determination of purity in the plasmid DNA of Bacteria isolated from River Sosiani and wastewater systems 54
4.3.3 Determination of plasmid DNA concentrations of Bacteria isolated from River Sosiani and wastewater systems 55
4.3.4 Gel electrophoresis...56
4.3.5 PCR amplification of the antibiotic- resistant genes in the selected bacterial plasmid DNA 57
CHAPTER FIVE **63**
DISCUSSION **63**
5.1 Isolation and identification of Bacteria from River Sosiani and wastewater systems 63
5.2 Antibiotic susceptibility test 69
5.3 Antibiotic-resistant genes in plasmids of the isolated Bacteria 75
CHAPTER SIX **80**
CONCLUSIONS AND RECOMMENDATIONS...**80**
6.1 Conclusions...80
6.2 Recommendations..81
REFERENCES...**83**
APPENDICES **120**

LIST OF TABLES

Table 3. 1: Preparation of PCR mixtures 27
Table 4. 1: Analysis of coliform bacteria from River Sosiani and waste water systems...32
Table 4. 2: Bacteria species identified on biochemical tests 37
Table 4. 3:Antibiotic susceptibility profiles of *Escherichia coli* 42
Table 4. 4: Antibiotic susceptibility profiles of *Enterobacter aerogenes*..........................44
Table 4. 5: Antibiotic susceptibility profiles of *Citrobacter freundii*................................45
Table 4. 6: Antibiotic susceptibility profiles of *Klebsiella pneumoniae* 46
Table 4. 7: Antibiotic susceptibility profiles of *Klebsiella oxytoca*...................................47
Table 4. 8: Antibiotic susceptibility profiles of *Proteus vulgaris*......................................48
Table 4. 9: Antibiotic susceptibility profiles of *Proteus mirabilis* 49
Table 4. 10: Antibiotic susceptibility profiles of *Pseudomonas aeruginosa* 50
Table 4. 11: Antibiotic susceptibility profiles of *Salmonella enteriditis*...........................51
Table 4. 12: Antibiotic susceptibility profiles of *Yersinia enterocolitica*..........................52
Table 4. 13: Determination of purity and concentration of plasmid DNA in the Bacteria isolated from River Sosiani and wastewater systems 54
Table 4. 14: Determination of plasmid DNA concentrations of Bacteria isolated from River Sosiani and wastewater systems 55
Table 4. 15: The occurrence of genes for antibiotic-resistance in plasmids of the selected bacteria isolated from Sosiani River and waste water systems in Eldoret, Uasin Gishu, Kenya 58

LIST OF FIGURES

Figure 2. 1: Antibiotic resistance mechanisms in bacteria 13

Figure 3.1: Map of the study area showing locations of the nine sites sampled……………..17

Figure 4. 1: Isolation and identification of bacteria from River Sosiani and waste water systems.. 34

Figure 4. 2: Microscopic view of *E. coli* isolates from Huruma quarry influent site.. 35

Figure 4. 3: Different biochemical tests used in bacteria species identification................38

Figure 4. 4: Distribution of Gram- negative pathogenic bacteria species in River Sosiani and wastewater systems..40

Figure 4. 5: Antibiotic susceptibility tests of *Escherichia coli*...43

Figure 4.6: Multi antibiotic resistance indices (MARI) of different bacteria species. 53

Figure 4. 7: Gel quantification of bacterial plasmid DNA isolated from River Sosiani and wastewater systems.. 56

Figure 4. 8: *QnrA* gene detected in different bacteria species from River Sosiani and wastewater systems.. 59

Figure 4. 9: *AadA1* gene detected in different bacteria species from River Sosiani and wastewater systems.. 60

Figure 4. 10*: MphA* gene detected in different bacteria species from River Sosiani and wastewater systems.. 61

Figure 4. 11: *Bla*$_{KPC}$ gene detected in different bacteria species from River Sosiani and wastewater systems.. 62

LIST OF APPENDICES

Appendix I: Ethical approval letter 120
Appendix II: NACOSTI Research Permit 121
Appendix III: Authorized access letter from Eldoret Water and Sanitation Company ..122
Appendix IV: Laboratory results on general bacteriological culturing at the ELDOWAS, Kenya 123
Appendix V: A summary of break points/ cut off values of different bacteria species tested against specific antibiotics as per Clinical Laboratory Standard Institute guidelines (CLSI, 2023) 124
Appendix VI: Mean values and standard deviation of zones of inhibition for different antibiotics tested in various bacteria species 125
Appendix VII: Chi-square test analysis on the relationship between phenotypic resistance and genotypic resistance of the bacteria isolates from River Sosiani and wastewater systems 128
Appendix VIII: Sets of primers and PCR conditions used in the amplification of antibiotic resistance genes in the plasmid DNA of different bacteria species 129
Appendix IX: Similarity report 131

LIST OF ABBREVIATIONS AND ACRONYMS

AMC	Amoxicillin clavulanic acid
AMP	Ampicillin
AMR	Antimicrobial resistance
ANOVA	Analysis of variance
ARB	Antibiotic-resistant bacteria
ARGs	Antibiotic-resistant genes
AST	Antibiotic susceptibility test
AZT	Azithromycin
BP	Base pairs
BWDs	Bacteria waterborne diseases
CAZ	Ceftazidime
CFU	Colony forming units
CHL	Chloramphenicol
CI	Ciprofloxacin
CLSI	Clinical and Laboratory Standard Institute
DL	DNA ladder
DNA	Deoxyribonucleic acid
DOX	Doxycycline
ELDOWAS	Eldoret water and sanitation company
EMB	Eosin Methylene Blue
ESBL	Extended spectrum beta lactamases
GE	Gentamicin
GoK	Government of Kenya
IS	Intermediate susceptible
MAR	Multi antibiotic-resistant
MARI	Multi antibiotic resistance index
MDR	Multi drug resistant
MDRI	Multi drug resistant index
MHA	Mueller Hinton agar
MTRH	Moi Teaching and Referral Hospital

NACOSTI	National Commission for Science, Technology and Innovation
NAP	National Action Plan
NFW	Nuclease free water
PCR	Polymerase chain reaction
QGIS	Quantum geographic information system
R	Resistance
S	Susceptible
SC	Sample code
SUL	Sulfonamides
TBE	Tris borate EDTA
TET	Tetracycline
TNTC	Too numerous to count
TSI	Triple sugar iron
UTIs	Urinary tract infections
UV	Ultra violet
WHO	World Health organization
WWTS	Wastewater treatment systems
ZI	Zone of inhibition

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Gram-negative pathogenic bacteria are the key drivers in the rise and spread of various infectious bacteria diseases worldwide (Chahal *et al*., 2016; Numberger *et al*., 2019; Tiwari *et al*., 2022). Aquatic ecosystems and wastewater systems receive influxes from medical facilities, quarries, intensive agriculture, fish farms and hatcheries, logging and sites of deforestation, which contain varying species of bacteria (Fang *et al*., 2020; Khan *et al*., 2020; Shi *et al*., 2020). Bacteria spread severe infections to humans, animals and aquatic biota (Mancuso *et al*., 2021; Odonkor *et al*., 2022; WHO, 2023), including typhoid fever, cholera, pulmonary diseases, dysentery, skin diseases and gastro enteric infections, increasing the health cost implications (Deshmukh *et al*., 2016; Magana Arachchi & Wanigatunge, 2020). Some of these bacteria get discharged into the aquatic environments either through leakage or surface run off. In addition, aqueous environmental systems also accumulate a lot of antibiotic contaminants, which upon continual interaction with bacteria, they induce emergence of genes that foster resistance of bacteria towards many antibiotics (Hanna *et al*., 2020; Ngigi *et al*., 2020; Dawangpa *et al*., 2021). Antibiotic-resistant bacteria undergo proliferation and spread infections to people, animals and some aquatic biodiversity, whose treatments has been rendered ineffective (Kumar & Pal, 2018; Singh *et al*., 2019; Bhumbla *et al*., 2020).

Antibiotic resistance in bacteria is a major challenge to the to public health, as it contributed to 4.95 million deaths globally in 2019 (Murray *et al*., 2022; WHO, 2022; Salam *et al*., 2023). Bacterial infections which are resistant to antibiotics have presented a great burden to health care systems around the world (Asfaw *et al*., 2020), inclusive of increased human mortality rates especially in the tropical and subtropical areas. They also end up requiring alternative treatment approaches (Ho *et al*., 2024), which are expensive and incur prolonged hospital stays (Aslam *et al*., 2021; Pulingam *et al*., 2022). The widespread of bacteria resistance to enormous antibiotics has been driven by many factors (Kusi *et al*., 2022; Salam *et al*., 2023), including antibiotics residues that leak into aquatic ecosystems (Bielen *et al*., 2017; Ngigi *et al*., 2020; Mutuku *et al*., 2022). Bacteria end up acquiring various defense mechanisms to evade the activities of antibiotics, hence exhibiting resistance (Munita & Arias, 2016; Urban-Chmiel *et al*., 2022).

Plasmid-mediated antibiotic resistance in bacteria is triggered via horizontal gene transfer mechanisms such as transformation, conjugation and transduction (Malaka De Silva *et al*., 2022). A recent study showed a wide range of antibiotic resistance genes detected in plasmids of *E. coli* from the human samples against β lactams drugs (bla_{SHV}, bla_{TEM} and $bla_{CTX\text{-}M\text{-}14}$ genes), aminoglycosides (*aadA1, aadA5* and *aac(3) -IId* genes), tetracyclines (*tet A, tet B* and *tet D* genes), macrolides (*mphA* and *mphB* genes), quinolones (*qnrS* gene), phenicols (*cat* and *cmIA* genes), sulfonamides (*sul1, sul2, sul3*) and trimethoprim (*dfrA5* and *dfrA17*) (Stephens *et al*., 2020). Some of the mechanisms mediated by these antibiotic-resistant genes in plasmids of different bacteria include inactivation of the antibiotics, extrusion of antibiotic molecules outside the cell using efflux pumps, modification of antibiotics targets, reduction in permeability of the cell membrane that limits drug uptake, changing the genetic, enzymatic and metabolic activities (C Reygaert, 2018; Varela *et al*., 2021).

In line with the One Health approach to address the widespread global challenge of antibiotic resistance among patients at hospitals and the community level, as well as in agriculture, livestock and fisheries, the Government of Kenya developed the National Action Plan (NAP) on antimicrobial resistance (AMR) (GoK, 2017), in line with the Global Action Plan of 2015. Some of the objectives of the NAP are; to improve awareness and understanding of antimicrobial resistance through effective communication, education and training to strengthen the knowledge and evidence base through surveillance and research.

This study aimed at isolating gram-negative bacteria species in bridges along River Sosiani and waste water systems in Eldoret, Uasin Gishu county, carrying out antibiotic susceptibility test of the isolated bacteria and determining the presence of selected antibiotic-resistant genes in the extracted bacterial plasmids. The study therefore provides evidence-based data from aquatic and wastewater systems for the informed interventions, including the development of a suitable policy framework to address AMR in Kenya.

1.2 Statement of the problem

Aquatic ecosystems and wastewater systems accumulate intensive wastes from various sources (Bashir *et al*., 2020; Serwecińska, 2020) that harbor pathogens including different species of pathogenic microorganisms, pharmaceutical wastes, microplastics, heavy metals and other toxic substances (Sharma *et al*., 2022). This poses serious health risks to the people as well as deaths of marine plants and animals (Kolawole & Iyiola, 2023; Néstor & Mariana, 2017). Many people and animals in most parts of Kenya (Osiemo *et al*., 2019; Sila, 2019; Too *et al*., 2024) and sub-Saharan countries of Africa (Osunla & Okoh, 2017; Uzoka *et al*., 2021) often consume water directly from rivers without proper treatments. Thus, pathogenic bacteria present in such water might end up finding ways into their body systems and cause severe bacterial infections. Influx of antibiotic residues accumulating in aquatic systems from farms, wastewater treatment plants (Nnadozie *et al*., 2017) and medical facilities (Thai *et al*., 2018; Ngigi *et al*., 2020), triggers emergence of genes driving the resistance of bacteria to antibiotics (Akhter *et al*., 2024; Anand *et al*., 2021). Several antibiotic-resistant genes are mediated by plasmids from one bacterium to another through horizontal gene transfer (HGT) mechanisms (Stephens *et al*., 2020; Wang *et al*., 2024). Antibiotic-resistant infections have greatly affected the global and public health development, causing increased hospital stays for patients and caregivers, high mortality rates, need for expensive and intensive care including the chemotherapy and surgery due to recurrent episodes of treatments failures (Pulingam *et al*., 2022). This creates a significant research gap in antibiotic-resistant studies on bacteria isolated from aquatic ecosystems that have remained unexplored. Therefore, resulting into inadequate AMR data in Kenya, which is key in the management of diverse challenges associated with antimicrobial resistance.

1.3 Justification of the study

Although there is now substantial awareness on the diverse risks of pathogenic bacteria in the environment and associated resistance of bacteria to antibiotics, evidence-based data to support informed interventions and policy decisions is often lacking, especially on the African continent. Similarly, while pathogenic bacteria, antibiotic residues, toxic chemicals and micropollutants invariably find their way into rivers and wastewater systems (Thai *et al*., 2018; Haque, 2022), the aquatic ecosystems that have been studied vary greatly, based on regions, sizes, flow regimes, seasonality and anthropogenic impacts that affect different ecosystems. Also, various waste water systems receive high quantities and qualities of wastes, which are treated with different chemicals, frequency and efficiency. Thus, studies on bacteria species from such systems with varied resistance and susceptibility to common antibiotics have remain unexplored. Since 2017, Kenya has been implementing the National Action Plan (NAP) on AMR (Wesangula *et al*., 2020; WHO, 2022), in line with the global action (GAP) of 2015. One of the objectives was to strengthen the knowledge and evidence bases on AMR through surveillance and research, in order to boost the capacity of the country to address challenges of antimicrobial resistance (AMR). Isolation and identification of gram-negative pathogenic bacteria from River Sosiani and wastewater systems will provide an overview of bacteria species diversity, their profiles of susceptibility and resistance to common antibiotics and their actual patterns of resistance indices to multiple drugs. This will create awareness and concern among Kenyan citizens, public and environmental health personnel and authorities on the prevalence of increased fecal pollutions and bacterial contaminations from these sites. Thus, assisting in developing safety precautions measures and proper preventions at local and global level, in order to prevent the anthropogenic threats and risks from such environments. Identification of antibiotic resistance genes in different bacteria plasmids, will be a good indicator that River Sosiani and the sampled wastewater systems are accumulating enormous quantities of antibiotic residues and other chemicals which drive the emergence of genes, inducing bacteria resistance to antibiotics. Therefore, necessitating the need for effective search, modification and designing of new antibiotics to replace the ones bacteria have conferred resistance. This will also add efforts in combating the spread of bacterial resistance to antibiotics as well as protecting the general public health.

1.4 Study objectives

1.4.1 General objective

To identify gram-negative bacteria, antibiotic susceptibility and genes mediating antibiotic resistance in the plasmids of pathogenic bacteria isolated from River Sosiani and waste water systems in Uasin Gishu County, Kenya.

1.4.2 Specific objectives

1. To identify gram-negative bacteria species, present in waste water samples from River Sosiani and waste water systems in Uasin Gishu, Kenya.
2. To carry out antibiotic susceptibility tests of the isolated bacteria from the waste water samples.
3. To detect and characterized plasmid-mediated antibiotic resistance genes in the isolated bacteria.

1.5 Null Hypothesis

1. H_{01}: There are no gram-negative bacteria species in the River Sosiani and waste water samples.
2. H_{02}: There are no antibiotic-resistant bacteria in the River Sosiani and waste water samples.
3. $H_{03:}$ There are no plasmid-mediated antibiotic resistance genes present in bacteria isolates from River Sosiani and waste water samples.

CHAPTER TWO

LITERATURE REVIEW

2.1 Prevalence of Bacteria in Water systems

Bacteria are microscopic and unicellular prokaryotic organisms that have been considered as the driving tools in the rising and spread of various bacterial infections (Chahal *et al*., 2016; Tiwari *et al*., 2022). Several studies report that, most of the prominent causative agents of these diseases are gram-negative rod-shaped bacteria dominating rivers, lakes, seas and other wastewater systems (Mancuso *et al* 2021; Odonkor *et al*., 2022; WHO, 2023). Gram-negative pathogenic bacteria such as *Escherichia coli, Salmonella, Klebsiella, Proteus, Vibrio cholerae, Campylobacter* and *Shigella species* are well identified using biochemical tests such as catalase test, oxidase test, indole test and citrate test (Haile *et al*., 2022). Aquatic ecosystems provide water for domestic use, livestock watering points, shipping and transport, irrigation, industrial and recreational activities, which indirectly contribute antibiotic-resistant bacteria in the aquatic systems (Obayiuwana *et al*., 2018; Mahmood *et al*., 2019). Moreover, aquatic ecosystems and waste water treatment sites are dynamic systems, continuously receiving different materials, including bacteria species of varying quantities and concentrations, some of which accumulate in the system, while some are washed downstream. This naturally increases pathogenicity and toxicity in these water sources as well as wastewater systems, making the water unsafe to potential users (Serwecińska, 2020; Chauhan & Punia, 2023). Most aquatic and wastewater systems are known to contain many bacteria species resistant to several antibiotics (Levantesi *et al*., 2010; Vaz-Moreira *et al*., 2014) due to the presence of chemical determinants of antibiotic resistance in bacteria (Geta & Kibret, 2022; Samrot *et al*., 2023). Many studies report different confirmed cases of increased resistance to multiple antibiotics by *Escherichia coli*, *Enterobacter species, Pseudomonas species, Proteus species*, *Salmonella*, *Yersinia species* and other enteric bacteria isolates from water and hospital systems in Kenya (Odoyo *et al*., 2023), East Africa (Ampaire *et al*., 2016) and many other parts of the world (Lien *et al*., 2016; Asfaw *et al*., 2017). Increased rates of bacterial toxicity in these water sources as well as wastewater treatment systems (WWTS), highlight these sites with increased health risks of contacting bacterial waterborne diseases (Serwecińska, 2020).

2.2 Common bacterial waterborne diseases

Bacterial waterborne diseases (BWDs) have emerged as global health problems more so affecting largest group of population in the developing world (Deshmukh *et al*., 2016). Common bacterial waterborne infections reported by World Health Organization (WHO) include typhoid fever, pulmonary diseases, skin diseases, cholera, dysentery, and *E. coli* infections (Deshmukh *et al*., 2016; Magana Arachchi & Wanigatunge, 2020). There is a great challenge in treatments of these infections in most of the health care due to the emergence of bacteria resistance to antibiotics (Parmanik *et al*., 2022). Cholera is caused by *Vibrio cholerae* species, resulting into severe diarrhea (Chowdhury *et al*., 2022) and has been effectively managed in the past era till the evolution of its resistance towards the antibiotics (Ali *et al*., 2015; Montero *et al*., 2023). Typhoid fever is caused by *Salmonella typhi* while Salmonellosis is caused by *Salmonella enterica* (Deshmukh *et al*., 2016). The updated cases of *salmonella* species being resistant to many available drugs have been demonstrated and confirmed (Popa & Popa, 2021). Mostly, gastroenteritis is caused by

Campylobacter jejuni, Campylobacter coli and *Escherichia coli* (Poirel *et al*., 2018; Williams & Berkley, 2018; Metreveli *et al*., 2022). *Escherichia coli* infections have been severally demonstrated to be affecting both human and livestock at large (Baran *et al*., 2023). Additionally, *E. coli, E. aerogenes, K. pneumoniae, K. oxytoca, P. vulgaris, P. mirabilis, P. aeruginosa* *K. pneumoniae* and *P. mirabilis* also cause urinary tract infections (UTIs) in humans among other diseases, increasing health risks of pandemic outbreaks (Liu *et al*., 2018; Girlich *et al*., 2020; Zhou *et al*., 2023; Maghembe *et al*., 2024).

2.3 Antibiotic susceptibility test

Pathogenic bacteria are either susceptible, intermediate susceptible or resistant to the conventional antibiotics used in the treatment of various bacterial infections (Tankeshwar, 2013). Bacteria acquire various mechanisms of resistance, hence end up counteracting effects of various antibiotics (WHO, 2023; Brittanica, 2024). Recent researches demonstrate the possibility of some bacteria inheriting antibiotic resistance genes from the parental chromosomal DNA and transferring to their plasmid DNA, hence exhibiting intrinsic resistance (Sun *et al*., 2019). Also, exposing bacteria to the heavy metals, toxic chemicals, antibiotic residues (Ngigi *et al*., 2020), micropollutants and other environmental factors for prolonged period (Yu *et al*., 2017; Nguyen *et al*., 2019), induces plasmid mediated antibiotic resistance in bacteria (Samrot *et al*., 2023). Poor hygiene, wrong prescriptions due to misdiagnosis of bacterial infections, misuse and overuse of drugs have also contributed to the spread of antibiotic- resistant infections (Llor & Bjerrum, 2014; Mancuso *et al*., 2021; Kasse *et al*., 2024). However, some efforts emphasizing the need for continuous monitoring of aquatic ecosystems in order to safeguard general health are often overlooked (Asfaw *et al*., 2017; Samrot *et al*., 2023; Sravani *et al*., 2024).

2.4 Mechanisms of plasmid-mediated antibiotic resistance in Bacteria

Plasmids are mobile genetic extrachromosomal DNA elements which acquire and store antibiotic-resistant genes that mediate resistance of various bacteria dominating aquatic environments to different antibiotics (Wein & Dagan, 2020; Beltrán *et al*., 2021; Ajayi *et al*., 2024). The widespread occurrence of multi antibiotic resistance genes among bacteria occurs as a result of over exposure of bacteria to antibiotics (Shintani *et al*., 2015; Partridge *et al*., 2018; Darby *et al*., 2023). There has been rapid occurrence of plasmid-mediated antibiotic resistance genes in distinct bacteria (WHO, 2017; Benz & Hall, 2023; Kosterlitz *et al*., 2023) towards quinolone, cephalosporins and aminoglycosides observed in uropathogens (Elshamy *et al*., 2020) and other pathogenic bacteria (Husin & Razzaq, 2021). Khalifa *et al*., (2020) and others have also demonstrated that plasmids mediate resistance of *K. pneumoniae* to antibiotics, causing ineffective treatment of respiratory systemic illnesses (Guitor & Wright, 2018; Ahmed *et al*., 2021; Liu *et al*., 2023; Verburg *et al*., 2024). Plasmid-mediated ARGs were also detected in *Escherichia coli* (Suhartono *et al*., 2016), *Citrobacter freundii* and *Pseudomonas aeruginosa* (Stephens *et al*., 2020; Shi *et al*., 2022). Different strains of *E. coli* causing urinary tract infections (UTIs) have been also reported to confer resistance to the conventional antibiotics (Ranjan *et al*., 2021; Zhou *et al*., 2021; Harris *et al*., 2023). Besides, plasmid-mediated resistance in salmonella has been observed in the management of dysentery and typhoidal infections (Dela *et al*., 2022; Tawfick *et al*., 2022; Kessler *et al*., 2023). Plasmid-mediated ARGs acquired by the bacteria (San Millan, 2016; San Millan *et al*., 2018) via horizontal gene transfer (HGT) mechanisms (Li & Zhang, 2023), enabling bacteria to evade the effects of antibiotics

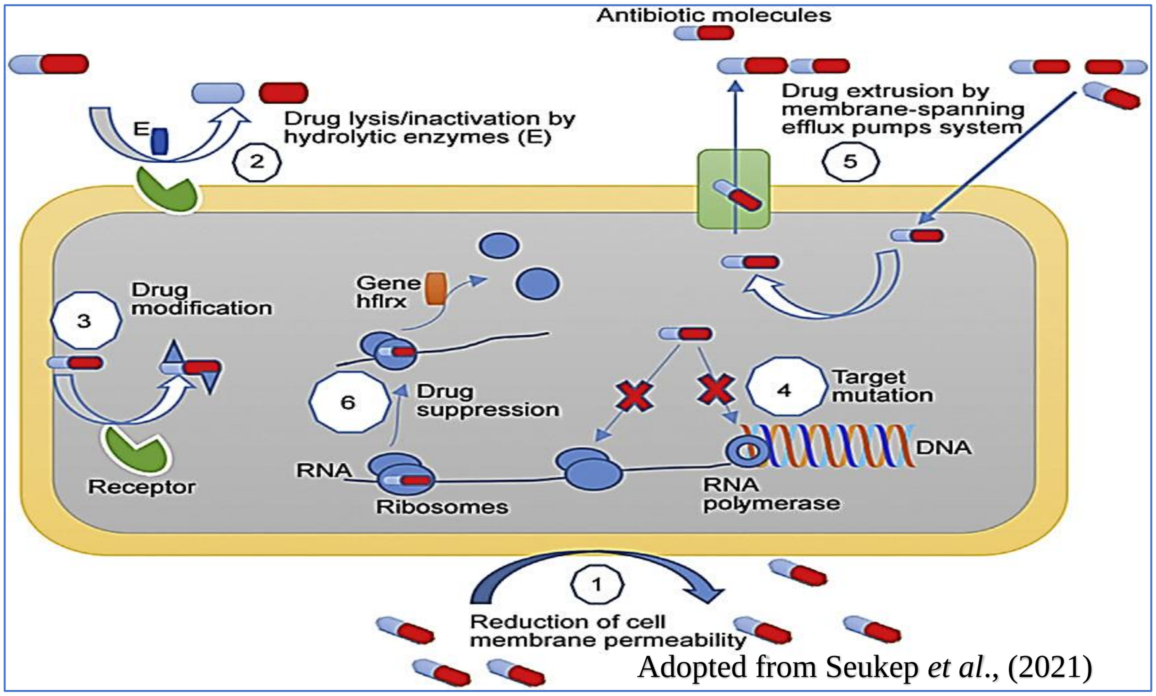
(Varela *et al.*, 2021). The HGT mechanisms include alteration in the target site of antibiotics, shifting in permeability of the cell membrane that limits drug uptake, changing the enzymatic and metabolic activities, inactivation of the antibiotics, extrusion of antibiotics using efflux pumps such as small multi drug resistance family (SMR), resistance nodulation division family (RND), ATP-binding cassette superfamily (ABC), multidrug and toxic compound extrusion family (MATE) and major facilitator superfamily (MFS) (C Reygaert, 2018; Varela *et al*., 2021). Mutational changes which limit drug uptake and over expression of resistance genes such as beta lactamases genes have greatly contributed to the resistance of *Pseudomonas aeruginosa* to antibiotics (Pang *et al*., 2019; Karruli *et al*., 2023).

2.4.1 *Escherichia coli*

Resistance mechanisms of *E. coli* to β-Lactams are through expression of extended spectrum β-lactamases encoding genes which produce enzymes capable of hydrolyzing penicillin and other drugs, hence antibiotic resistance (Poirel *et al*., 2018; Mahalleh *et al*., 2021). ARGs which induce *E. coli* resistance to fluoroquinolones include *GyrA* and *ParC* (Foudraine *et al*., 2021). A number of ARGs which extrude antibiotics in *E. coli* species via efflux pumps include *QepA, OqxAB, AcrAB-TolC, EmrAB, MdfA, TehA, EmrE, AcrE, and EmrD. Escherichia coli* resistance towards aminoglycosidic antibiotics is triggered by the presence of *Arman, RmtA, RmtB, RmtC, RmtD*, *RmtE, RmtF, RmtG,* and *RmtH* (Doi *et al*., 2016; Urban-Chmiel *et al*., 2022) while *tet(Y) tet (A), tet (B), tet (C*), *tet(D), tet(E), tet(G), tet(J)* and *tet(L)* trigger resistance of the species to the tetracyclines (Poirel *et al*., 2018). *Sul1, sul2 & sul3* genes mediates the resistance of *E. coli* to sulphonamides and trimethoprim (Sköld & Swedberg, 2017) while *cmlA*, gene *floR*, *cfr genes* and *catI* genes foster *E. coli* resistance to chloramphenicol (Urban-Chmiel *et al*., 2022).

2.4.2 *Salmonella species*

Salmonella confers intrinsic resistance through antibiotic hydrolysis hence inactivation of antibiotics such as aminoglycosides, chloramphenicol and β-lactams. β-lactam antibiotics such as cephalosporins and penicillin are hydrolyzed via β-lactamases while macrolides are inactivated by macrolide esterases (D'Costa & Wright, 2017; Teklu *et al*., 2019; Coipan *et al*., 2020). The *mphA, mphB* and *ereA* genes along with *Acr*A& *Acr*B have shown to confer azithromycin resistance in *Salmonella* while *floR* gene trigger resistance to phenicols (McDermott *et al*., 2018). *Salmonella* resistance genes to sulfonamides include *sul 1, sul2 & sul3* while for trimethoprim are *dfrA1, dfrA10, dfrA12* (Chaudhari *et al*., 2023). *Salmonella* also develop mechanisms of nucleotidylation, ribosylation, acetylation, phosphorylation, adenylation, thiol transfer and hydroxylation, showing resistance to tetracycline, aminoglycosides, chloramphenicol and macrolides (Chaudhari *et al*., 2023). *Salmonella species* have various drug efflux pumps which extrude different antibiotics, hence causing resistance (Amado *et al*., 2016; Greene *et al*., 2018; Yamagishi *et al*., 2020). Figure 2.1 shows antibiotic resistance mechanisms in bacteria.



Adopted from Seukep *et al*., (2021)

Figure 2. 1: Antibiotic resistance mechanisms in bacteria

Key: (1) Reduction of membrane permeability, (2) drug inactivation by hydrolytic enzymes, (3) modification of drug targets, (4) induction of target mutations, (5) drug extrusion by efflux pump systems and (6) Drug suppression by bacteria.

2.5 Contamination of water bodies with antibiotics

Antibiotics have different specified level of toxicity, upon their consumption, they undergo digestion, get absorbed into blood stream and become metabolized (Maghsodian *et al*., 2022). They release active molecules which either inhibit the growth and proliferation of pathogenic bacteria or cause prompt deaths of bacteria (Maghsodian *et al*., 2022). Based on the type of antibiotic, they are capable of eradicating many bacterial waterborne, food borne and other infectious diseases through inhibiting cell wall synthesis, blocking nucleic acid synthesis, damaging the cell membrane, hinders protein biosynthesis, interference with the normal transcription and translation processes, interrupting the metabolic and enzymic activities in bacterial cells (Kapoor *et al*., 2017; Eyler & Shvets, 2019; Hutchings *et al*., 2019). For instance, aminoglycosides, β lactams, glycopeptides, macrolides, oxazolidinones, polymyxins, quinolones, streptogramins, sulfonamides and tetracyclines (Samrot *et al*., 2023), have been widely targeted in the treatment of different bacterial infections (Carvalho & Santos, 2016). The continual release of antibiotics into aquatic systems have significantly resulted into serious potential risks to the environment and public health (Bengtsson-Palme *et al*., 2018; Kumar *et al*., 2019). Rivers and other waste water systems contain many pharmaceutical wastes inclusive of non- biodegradable antibiotic residues (Geta & Kibret, 2022). From the recent studies, antibiotics are considered among the emerging contaminants in water pollution and hotspots for the development of antibiotic resistance (Koch *et al*., 2021). Waste water is a habitat for many microbes including bacteria and supports the survival of other aquatic organisms (Cheng *et al*., 2020). Antibiotic contamination in both treated and untreated wastewater has been stewarded as a strong hotspot for the development and spread of antibiotic- resistant genes (Ben *et al*., 2019). Multiple bacterial infections which are resistant to antibiotics emerges due to the consistent association with antibiotics (Berendonk *et al*., 2015; Suzuki *et al*., 2017; Krzeminski *et al*., 2019). Nairobi River basin in Kenya (Ngumba *et al*., 2016) and other wastewater systems (Kairigo *et al*., 2020; K’oreje *et al*., 2020; Baralla *et al*., 2021) accumulates a lot of antibiotic residues, released from agricultural sites, waste water treatment effluents, aquaculture, livestock production (Kim *et al*., 2019; Fang *et al*., 2020; Shi *et al*., 2020; Kan *et al*., 2023) hospital, domestic wastes, municipal wastes and pharmaceutical industries (G *et al*., 2017; Müller *et al*., 2020). Raised concerns of antibiotic water pollution have been overlooked despite its severe threats to the public health (Godoy

& Sánchez, 2020; Chow *et al*., 2021; Roy *et al*., 2021). Accumulation of antibiotics in water systems fosters the occurrence of antibiotic-resistant genes in bacteria, interfering with natural immune systems in humans and animals (Kumar *et al*., 2020; Samrot *et al*., 2023), thus causing many treatment failures and increased mortality (Zhen *et al*., 2019; Teshome *et al*., 2020). Apart from detecting antibiotic residues in untreated wastes, other studies have also found them in the treated wastewater systems (Alonso *et al*., 2017; Riaz *et al*., 2018), supporting the existence of poor waste disposal and management practices in most of the aqueous environments (Asfaw *et al*., 2017; Papajová *et al*., 2022).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

The study was conducted along River Sosiani, which flows through Eldoret city in Uasin Gishu County, Kenya and the six wastewater systems serving the city. The sampling locations spanned from Kipkaren Bridge at 0°30'52''N, 35°15'22''E, to Pioneer Bridge at 0°30'46''N, 35°16'26''E, and the Outspan-Nairobi Bridge at 0°29'53''N, 35°18'09''E. The study also included influent and effluent points from the Huruma Quarry (influent: 0°31'21''N, 35°14'20''E; effluent: 0°31'52''N, 35°14'12''E) and Kipkenyo Boundary wastewater treatment systems (influent: 0°31'33''N, 35°12'44''E, effluent: 0°32'32''N, 35°12'49''E). The influent points represent the entry of untreated waste into the wastewater treatment systems, while the effluent points mark the discharge of treated wastes into the River Sosiani. Additional sites, including the waste discharge points from Moi Teaching and Referral Hospital (MTRH) (0°30′44″N, 35°16′50″E) and Eldoret Prison (0°31′34″N, 35°17′15″E), discharge pretreated waste into the municipal sewer lines, which are eventually directed to Huruma quarry and Kipkenyo boundary sites for further treatment.

The study area covering 42.2 km² within Eldoret, is heavily influenced by dense human settlements, agricultural activities, hotels and tourism, and small-scale industrial operations, all contributing to the discharge of wastewater and pollutants into River Sosiani. These activities create conditions which are conducive to the emergence of antibiotic-resistant bacteria in the river’s ecosystem, highlighting the challenges of management and treatment of wastes in the region.

3.2 Study area map

Quantum geographic information system (QGIS) version 3.34.3 (Pereira *et al*., 2022) , was used to design the study map area according to the specific geographical location coordinates of the sampling sites within Eldoret city, 0°30'54''N 35°16'19''E, as shown on Figure 3.1.

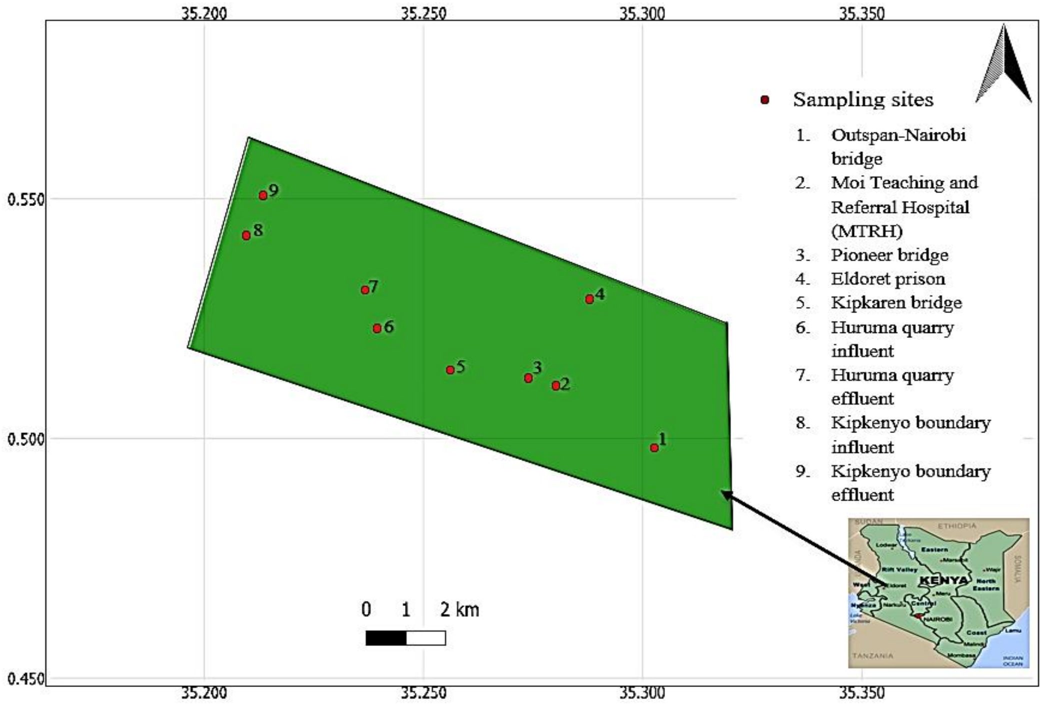


Figure 3. 1: Map of the study area showing locations of the nine sites sampled

Key: The nine sampling sites constituted those upstream, before the River Sosiani reaches city (Outspan-Nairobi bridge), those right inside city (Pioneer bridge and Moi Teaching and Referral Hospital), and those downstream, after the River Sosiani leaves the city (Eldoret prison, Kipkaren bridge, Huruma quarry influent, Huruma quarry effluent, Kipkenyo boundary influent and Kipkenyo boundary effluent).

3.3 Study design and sampling frame

The cross-sectional study was employed in systematic random collection of water samples (Fowler & Fleming, 2023), along bridges of River Sosiani that accumulates a lot of wastes from the heavy transport along the busy highways, nearby markets and hotels, carwashes, pharmaceutical wastes and other commercial centers. In addition, influents and effluents of wastewater treatment systems at Huruma quarry and Kipkenyo boundary as well as waste discharge screen sites of MTRH and Eldoret prison that releases their wastes into municipal sewers directed to Huruma quarry wastewater treatment plant were also sampled.

3.4 Study population

In this study, water samples from River Sosiani and wastewater treatment systems formed the sampling matter, where the gram-negative bacteria were isolated, their antibiotic susceptibility, intermediate susceptibility, resistance profiles and antibiotic resistance genes (ARGs) in the plasmids of the isolated bacteria were identified.

3.5 Sample size determination

The sample size was determined by the formula:

$N= z^2 P(1-p)/d^2$ adopted from (Abdulfatai *et al*., 2023).

N=sample size

Z= 1.96, confidence level at 95% CI

P= Assumed prevalence, 0.05

D= Absolute precision, 0.082209

N= 1.96^2x 0.05 (1-0.05) / ($0.082209^{2)}$ =27

Therefore, the sample size of this study was 27 i.e 9 sampling points x 3 (sampling in triplicates). This means that in each of the sampling site, 3 bottles filled with 100ml of the water samples were collected.

3.6 Sample collection and frequency

A total of 27 samples were collected in sterilized 100 ml plastic bottles from the nine selected sampling sites (sampling was done in triplicates). The samples were transported on ice to the central water testing and microbiology laboratory of the Eldoret Water and Sanitation company (ELDOWAS), Eldoret-Kenya, for general bacterial culturing. Samples were refrigerated at 4^oC till the processing time.

3.7 Chemicals and reagents

Laboratory chemicals and reagents including culture media and other materials used in microbiology assays as well as antibiotic disc cartridges were purchased from chemo-quip Himedia limited, Nairobi-Kenya. Plasmid bacterial extraction kit, nuclease free water, DNA ladder, loading dye, safe view, agarose powder, Tris Borate EDTA and primers were obtained from Inqaba Biotechnology, South Africa.

3.8 Laboratory analysis

Sub-culturing, biochemical tests and antimicrobial susceptibility tests were done at the Botany laboratory 1 of the Department of Biological Sciences, University of Eldoret. Plasmid DNA extraction, PCR and gel electrophoresis analysis were done in Fish Genetics and Genomics Research and Training laboratory at the University of Eldoret. Nanodrop analysis of bacterial plasmid DNA was processed at the Biotechnology laboratory, University of Eldoret.

3.8.1 Isolation and identification of bacteria from River Sosiani and wastewater systems

3.8.1.1 Total coliforms

Endo agar media was first prepared by dissolving 41.5 g of endo agar powder in 1,000 ml of distilled water, heated to near boiling point and sterilized in autoclave for 10 minutes (Himedia, origin) to destroy any microorganisms present, that might interfere with the study assays (Akita *et al.*, 2021). It was cooled for 3 minutes to 45^{o}C before being dispensed onto the 15 large sized petri dishes for solidification. Based on the concentration, different samples were prepared for the inoculation, whereby 0.5 mL of the highly concentrated sample was diluted with 100 mL of sterile distilled water while 1 mL of the less concentrated sample was diluted with 100 mL of distilled water. The samples were inoculated onto Endo agar media plates using pour plate technique (Yari *et al.*, 2018) and incubated at 37°C for 24 hours to allow the growth of the bacteria.

3.8.1.2 Fecal coliforms

Dilution of sample was done in physiological saline and filtered using the membrane filtration unit, following protocol from Akita *et al.*, (2021), with some modifications. Prior to the actual filtration, 70% of ethanol was spread evenly on the inner surface of the Buchner funnel and a flame lit for 3 minutes for sterilization. Using sterilized forceps, a sterile grid filter paper was placed over the porous plate of the apparatus with the grid side up and unit locked, after excess ethanol was drained off. The sample was then passed under partial vacuum until all the 100 ml sample was drained off into the volumetric flask and the grid unit was unlocked, from which the funnel was removed along with the soaked membrane filter using the sterilized forceps. The membrane filter containing bacteria was then placed onto the solidified endo agar plates in a rotational manner and incubated for 24 hours at 44^oC to allow the growth of fecal bacteria coliforms.

3.8.1.3 Bacterial count

Digital colony counter was used for the enumeration of freshly grown total coliforms and fecal coliforms and recorded in colony forming units per 100 ml (CFU/100ml). Total number of bacteria coliforms in the diluted samples from each of the sampling site was obtained by multiplying CFU value by the dilution factor (Journals & Songara, 2015). Bacteria coliforms from different sampling sites which recorded counts more than 10,000 CFU/100ml, were reported as too numerous to count (TNTC).

3.8.1.4 Bacteria sub-culturing

The total coliforms were further sub-cultured on Eosin Methylene Blue agar media (EMB) from the endo agar cultures (Himedia) (Jain *et al*., 2020). Sub-culturing was repeated four times to obtain pure colonies of each bacteria species. 50 g of EMB was suspended in 1000 ml distilled water, heated to boil until the media dissolved completely. It was cooled and poured onto the medium sized petri dishes to harden. Freshly grown bacteria coliforms were picked using a sterilized inoculating loop from the endo agar plates and streaked onto the EMB agar plates. Distinct identification of the isolated bacteria species colonies was based on the colony morphology and pigmentation on EMB plates, following the relevant researches (Antony *et al*., 2016; Patra *et al*., 2020).

3.8.1.5 Gram staining and biochemical characteristics

Gram-negative bacteria were identified based on their shape using gram staining technique and analyzed microscopically using a CXR2 LABOMED light microscope (Labo America, Inc. USA, origin), as per the protocol from Wu & Yang, (2020). The identified sub species of bacteria from EMB culture plates were further confirmed using biochemical tests (Patra *et al*., 2020; Masi *et al*., 2021). The selected five (5) biochemical tests were based on the colony morphological of bacteria isolates studied on the EMB culture plates. Catalase test identified the bacteria isolates capable of producing catalase enzyme, which hydrolyzes the breakdown of hydrogen peroxide into water and oxygen (Yuan *et al*., 2021). Small amount of freshly grown colony from EMB agar was collected using a sterile inoculating loop and placed onto the microscope slide mounted on the clean bench. 1 drop of 3% hydrogen peroxide was added onto the colonies on the microscope slide using a dropper. Bubbles were observed in some bacteria species, indicating positive reaction for catalase test. Citrate test aimed at identifying those bacteria isolates capable of utilizing citrate as an energy source. A freshly isolated colony of about 16- to 18-hours was picked using inoculating loop and lightly streaked on the surface of the agar slant containing citrate medium., followed by incubation at 28^{o}C (+/- 2^{o}C) for 18 to 48 hours. A change of color from green to blue slant was considered as positive for citrate test (Shoaib *et al*., 2020). Filter paper test method was performed for the oxidase test, to identify gram-negative bacteria having cytochrome c oxidase enzyme. A small piece of filter paper was soaked in 1% Kovács oxidase reagent and left to dry. 18 to 24 hours fresh culture colony from EMB plate was picked with a loop and rubbed onto Kovac’s treated filter paper. A positive reaction for the oxidase test was indicated by changing color from colorless to purple (Al-hmad & Roodsari, 2016). Indole test aimed at identifying those gram-negative bacteria which produce tryptophanase enzyme, catalyzing the breakdown of tryptophan into indole (Nieto & Lupton, 2021). Pure bacteria were inoculated into tryptone broth and later incubated at 28°C for about 48 hrs. After which, five (5) drops of Kovac's reagent were added for the indole production. A red ring formed on the surface of the solution indicated a positive reaction Nieto & Lupton, 2021). Triple sugar iron (TSI) test identified bacteria that ferment glucose, lactose, sucrose and produce hydrogen sulfide gas. Pure bacteria isolates were inoculated through streaking onto the surface of triple sugar iron agar slant that had been prepared. An overnight incubation at 32ºC followed with caps covered loosely to allow easy flow of ambient air. The change of color from red slant to yellow/ blue slant, black butt and presence of some bubbles or cracks were examined as positive reaction for the TSI test (Masi *et al*., 2021).

3.8.2 Antibiotic susceptibility test

Antibiotic susceptibility testing (AST) for different bacterial isolates was determined using the Kirby-Bauer disc diffusion method on Mueller Hinton Agar (MHA) (Wen *et al*., 2022; Regassa *et al*., 2023), following the guidelines from the Clinical and Laboratory Standards Institute (CLSI, 2014; Baseri *et al*., 2021). Pure bacterial isolates were suspended in normal saline and adjusted to 0.5 MacFarland turbidity standard (Ade Rizky *et al*., 2019), then spread evenly onto the prepared MHA plates. Using sterilized forceps, antibiotic discs were placed onto each of the MHA plates. The plates were incubated at 37°C for 24-48 hours to assess bacterial susceptibility and resistance to the tested antibiotics. All the bacteria species were tested against 12 standard antibiotic discs, which included Penicillin (P, 30 µg), Ampicillin (AMP, 10 µg), Streptomycin (STR, 10 µg), Sulfamethoxazole-trimethoprim (SXT, 20 µg), Doxycycline (DO, 30 µg), Amoxicillin-Clavulanic acid (AMC, 10/20 µg), Ceftazidime (CAZ, 30 µg), Tetracycline (TET, 30 µg), Azithromycin (AZT, 15 µg), Gentamicin (GE, 10 µg), Chloramphenicol (CH, 50 µg) and Ciprofloxacin (C, 30 µg). The AST test was done in triplicates whereby six Mueller Hinton Agar plates were prepared for each bacteria species and each of the plate was dispensed with 6 antibiotic discs. Those bacteria species with perfect growth characteristics were selected from different locations for AST test. The diameter of the standard antibiotic disc was measured to be 6mm. Therefore, the zones of inhibition for those bacteria species that indicated total resistance to particular antibiotics were recorded as 6mm, indicating the diameter of the standard antibiotic disc. The obtained mean values and standard deviations for the zone of inhibitions were also recorded.

3.8.3 Identification of genes in the plasmids of the selected bacteria for resistance against the antibiotics

3.8.3.1 Sub -culturing of bacteria

All the bacteria isolates were sub-cultured in nutrient liquid broth, following protocol from Bonnet *et al*., (2020), with some modifications. 48.5 g of nutrient agar powder (HiMedia) was weighed and suspended into 1000 ml of distilled water in conical flasks to make the liquid broth. The mixture was heated to boil in autoclave pressurized machine till complete dissolution and left to cool to 50^{o}C. It was divided into 50 ml sterilized bottle and antibiotic-resistant bacteria picked from EMB agar plates were then inoculated into liquid broth. Finally, the cultured bacteria were incubated overnight at 28^{o}C to allow them to grow.

3.8.3.2 Extraction of bacteria plasmid DNA

The bacterial plasmid DNA from the bacteria species were extracted using the commercial Zyppy ^{TM} Plasmid Miniprep kit, [Catalog Nos. D4036, D4019, D4020 & D4037- Zymo Research] according to the manufacturer’s instructions, at room temperature. Briefly, 600 µl of each pure bacterial colony freshly grown in nutrient broth were lysed with 100 µl of 7X lysis buffer in 1.5 ml micro-centrifuge tubes and mixed by inverting 4-6 times. 350 µl of cold neutralization buffer was added and mixed till a yellowish precipitate was obtained before centrifuging at 12,000x g for 4 minutes. 900 ul of supernatant was transferred into Zymo-Spin ^{TM} columns placed onto the sterilized collection tubes without disturbing the cell debris pellet and span at 12,000x g for 30 seconds. The flow through was discarded, whereby 200 ul of endo-wash buffer was added to the columns and centrifuged for 30 seconds. This was then followed by addition of 400 ul of Zippy ^{TM} wash buffer to the columns and a span for 1minute. Plasmid DNA was eluted from the column matrix placed onto the sterilized 1.5 ml micro-centrifuge tubes, starting with lower speed (1000x g for 5 minutes) to higher speed (12000x g for 1minute) using 100 ul of Zippy ^{TM} elution buffer in order to ensure maximum quality yield. The eluted plasmid DNA was kept at -20^{o}C, awaiting downstream reactions.

3.8.3.3 Quantification of bacterial plasmid DNA

Nanodrop spectrophotometer (Nanodrop2000 Scientific Thermo Fischer, origin) was used to check the purity at 260/280 nm and concentrations of plasmid DNA extracted from different species of bacteria at 280nm (Rodriguez-Mozaz *et al*., 2015). 2µl of the plasmid DNA samples were loaded onto the Nanodrop machine for the analysis. The quantities of the extracted plasmid DNA for different bacteria species were obtained by using 1.5%

agarose gel electrophoresis (Scientific Thermo Fischer, origin) (Xu *et al*., 2019). 3µl of the DNA mixed with 2µl of loading dye was loaded on the gel tank and the visualization of gel bands done on the ultraviolet analyzer against the 1Kb DNA ladder.

3.8.3.4 Reconstitution of PCR primers for the selected ARGs

A total of 9 antibiotic-resistant genes were investigated in the present study including *qepA*, *qnrA*, *aadA1*, *mphA*, *dfrA5*, *bla*$_{KPC}$, *bla*$_{SHV}$, *sul 1* and *ampC* genes. Each 100 mol of primer pellets (stock solution) were span at 12x g for 1 minute to concentrate all the pellets at the bottom. This was followed by adding the specified amount of nuclease free water and spinning for 15 seconds as per manufacturer's guidelines (Inqaba Biotec, East Africa Limited). The prepared primers solutions were always kept on ice throughout the processing time.

3.8.3.5 Polymerase chain reaction (PCR) amplification

Different antibiotic-resistant genes were amplified on conventional thermocycler PCR (Thermo Fisher Scientific, origin). Isolates of *Escherichia coli*, *Pseudomonas aeruginosa* and *Yersinia enterocolitica* from Huruma quarry influent, *Enterobacter aerogenes* and *Klebsiella oxytoca* detected from Kipkaren bridge, *Citrobacter freundii* obtained from Outspan- Nairobi bridge, *Klebsiella pneumoniae* and *Proteus vulgaris* found at the Moi Teaching and Referral Hospital, *Proteus mirabilis* isolated from the Pioneer bridge and *Salmonella enteriditis* from Eldoret prison were used for the molecular assays. Various polymerase chain reaction (PCR) mixtures were prepared using the PCR components and specified volumes shown on Table 3.1. All the PCR mixtures were always kept on ice throughout the processing time. The PCR reaction volume used was 25 µl inclusive of One *Taq* 2X Master Mix with Standard buffer (12.5µl), forward primer ((1µl), reverse primer

((1µl), nuclease free water (7.5 µl) and plasmid DNA template (3µl) (Table 3.1). A total of 35x cycles were used with varying annealing temperature regime between 53°C-60°C, depending on the type of primer involved. Specific primer gene sequences, the approximated sizes and lengths of amplicon products, specific primer annealing temperatures and general PCR conditions used in the current study were indicated on Appendix 8. Nuclease free water (NFW) was used as a negative control. Each set of the primer was added to bacteria plasmid DNA and run at a time (single primer PCR).

Table 3. 1: Preparation of PCR mixtures

PCR components	Volume used (µl)
One *Taq* 2X Master Mix with Standard buffer	12.5
10 µM Forward Primer	1
10 µM Reverse Primer	1
Nuclease -Free Water (NFW)	7.5
Plasmid DNA template	3
Total	25 µl (per sample)

µl- microliter, µM- micromolar.

3.8.3.6 Gel electrophoresis

Agarose gel electrophoresis was used to assess the success of the PCR, as performed by Wittmeier *et al*., (2022). 2% gel was prepared by dissolving 2 g of agarose powder in 200 ml of commercialized 1X TBE buffer and heated to boil in a microwave for 2-3 minutes while swirling. 1X TBE buffer was prepared from a stock solution by topping up 100 ml of 10X TBE with 900 ml of distilled water. After cooling to 50°C for 10 minutes, 10 µl of Safe View^{TM} Classic Nucleic Acid stain was added (for pre-casting gel) and the gel transferred to the gel tanks with well inserted comb that create wells as the buffer solidifies into a gel. Gel holders without the comb wells were transferred into the electrophoresis tank and filled with 1X TBE buffer to the maximum level, before addition of 30 µl of Safe View^{TM} Classic Nucleic Acid stain, to increase the strength of visualization (for post staining gel). In the first well, 4 µl DNA ladder mixed with 1µl purple (6X) loading dye was added. The rest of the wells were loaded with 3 µl of the PCR product mixed with 2 µl of the same dye. Finally, the larger gel tanks (capacity to carry 16 samples per set) were set at 80 V for 2 hours while smaller gel tanks (one small gel has a capacity to carry 6 samples) were set to 100V for 1hour with a current of 150 AMPs, to ensure that the samples do not flow into the TBE buffer as the current moves along. Some of the amplified ARGs (PCR products) of the same bacteria species (*E. coli, E. aerogenes, C. freundii* and *P. aeruginosa*) were loaded either twice or thrice in different wells to check differences in the strength and qualities of the bands. The obtained DNA gel bands were visualized using UV transilluminator analyzer (UVITEC, origin) and results recorded.

3.9 Data quality control

Standardized guidelines and procedures for sample collection and reagent preparations were strictly followed. The quality of each culture media as well as the manufacturer and expiry dates of all reagents used in the current study were well counter checked before performing any tests. Aseptic conditions were maintained, including autoclaving the prepared culture media, laboratory items used and overnight incubation of cultures at the appropriate temperature for sterilization. The commercial antibiotic disc cartridges were properly sealed to avoid exposure to the light and moisture and then kept at 4^{o}C in the fridge when not in use (recommended conditions from Chemo quip labels). The reagents, samples, primers and specific bacteria cultures were kept at -20^{o}C when not in use.

Bacterial sub -culturing, plasmid DNA extraction, nanodrop quantification, polymerase chain reaction and gel electrophoresis were carried out at the specific conditions as per guidelines of the manufacturer. Amplicon sizes were verified using a molecular DNA ladder.

3.10 Data analysis

All recorded data were coded, exported and analyzed using Microsoft Excel. Descriptive statistics, including means and standard deviations, were used to summarize the data on antibiotic susceptibility tests based on the diameter of the zone of inhibition measured in millimeters (mm). The antibiotic susceptibility test (AST) results were interpreted in line with Clinical and Laboratory Standards Institute guidelines (CLSI, 2023) as summarized on appendix 5, following the consistent use of these guidelines in other related study (Mohamad *et al*., 2022). Bacterial patterns on AST were classified as either susceptible (S), intermediate susceptible (IS), or resistant (R) (Mahmud *et al*., 2023; Teshome *et al*., 2020). The Multi-Antibiotic Resistance Index (MARI) for each bacterial isolate was calculated using the formula: (a/b), where *a* represents the number of antibiotics to which bacteria was resistant and *b* refers to the total number of antibiotics tested against the bacteria (Mir *et al*., 2022; Mohamad *et al*., 2022). Percentage occurrence of each plasmid-mediated antibiotic resistance genes in bacteria was calculated; (*a/b*) x100%, where *a* is the number of bacteria species consisting a particular gene and *b* is the total number of bacteria species (10). Chi-square test was used to determine if there was a significant relationship existing between the phenotypic resistance and genotypic resistance in bacteria isolates. Statistical significance was determined by considering P-values ≤ 0.05 with a 95% confidence interval. The results were presented in figures and tables.

3.11 Ethical approval

This research study was approved by Ethical Review Committee of North Eastern Baraton College located in Nandi county- Kenya, with Approval number: UEAB/ISER/07/03/2024 (Appendix 1). The research permit license was obtained from National Commission for Science, Technology and Innovation, with License No: NACOSTI/P/24/33905 (Appendix 2). Wastewater samples from the restricted-access sites including Moi Teaching and Referral Hospital, Huruma quarry and Kipkenyo boundary wastewater treatment systems were authorized by Eldoret Water and Sanitation Company (ELDOWAS) in Uasin Gishu County-Kenya (Appendix III).

CHAPTER FOUR

RESULTS

4.1 Isolation and identification of Bacteria from River Sosiani and wastewater systems

4.1.1 Analysis of coliform bacteria in water

Total coliforms detected in waste water and River Sosiani from the nine sampling sites were too numerous to count (TNTC). Some of the fecal coliform bacteria detected from Moi Teaching and Referral Hospital, Huruma quarry influent, Kipkenyo boundary influent, Outspan-Nairobi bridge and Eldoret prison were too numerous to count compared to the ones detected at the Kipkaren bridge (235/100ml), Pioner bridge (1,000/100ml), Huruma quarry effluent (1,400/100ml) and Kipkenyo boundary effluent which accounted for 10,000/100ml bacteria coliforms. A summary of the results is presented on Table 4.1 and Appendix 4.

Table 4. 1: Analysis of coliform bacteria from River Sosiani and waste water systems

Sample	Ref. no/ sample code	Water source	Total coliforms	Fecal coliforms
			(CFU/100ml)	
Moi Teaching and Referral Hospital (MTRH)	1384-23/24	Treated wastewater	TNTC/100ml	TNTC/100ml
Huruma quarry influent	1385-23/24	Untreated wastewater	TNTC/100ml	TNTC/100ml
Huruma quarry effluent	1387-23/24	Treated wastewater	TNTC/100ml	1400/100ml
Pioneer bridge	1389-23/24	River	TNTC/100ml	1000/100ml
Kipkaren bridge	1390-23/24	River	TNTC/100ml	235/100ml
Kipkenyo boundary effluent	1391-23/24	Treated wastewater	TNTC/100ml	10000/100ml
Kipkenyo boundary influent	1392-23/24	Untreated wastewater	TNTC/100ml	TNTC/100ml
Outspan-Nairobi bridge	1393-23/24	River	TNTC/100ml	TNTC/100ml
Eldoret prison	1412-23/24	Untreated wastewater	TNTC/100ml	TNTC/100ml

Key: * TNTC- too numerous to count, CFU- colony forming unit, fecal coliforms are bacteria species found in fecal polluted water systems while total coliforms are bacteria species found from all kinds of water polluted systems.

4.1.2 Bacteria sub culturing

Among the 10 gram-negative pathogenic bacteria that were isolated and identified, *Escherichia coli* (*E. coli*) was characterized as deep purple colonies with green metallic sheen, *Enterobacter aerogenes* (*E. aerogenes*) were identified as dry pink colonies, brown mucoid colonies with metallic sheen were *Citrobacter freundii* (*C. freundii*). *Klebsiella pneumoniae* (*K. pneumoniae*) were mucoid pink colonies, *Klebsiella oxytoca* (*K. oxytoca*) were mucoid purple colonies. In addition, *Proteus vulgaris* (*P. vulgaris*) were pinkish colonies, *Proteus mirabilis* (*P. mirabilis*) were whitish colonies, *Pseudomonas aeruginosa* (*P. aeruginosa*) were colorless, *Salmonella enteriditis* (*S. enteriditis*) were characterized as whitish-milky colonies and *Yersinia enterocolitica* (*Y. enterocolitica*) were dark purple colonies without green metallic sheen. Figure 4.1 shows *E. coli, K. pneumoniae, K. oxytoca, P. vulgaris, P. mirabilis* and *Y. enterocolitica* that were isolated and identified on Eosine methylene blue (EMB) agar plates from the total coliforms that were previously cultured on Endo agar media.

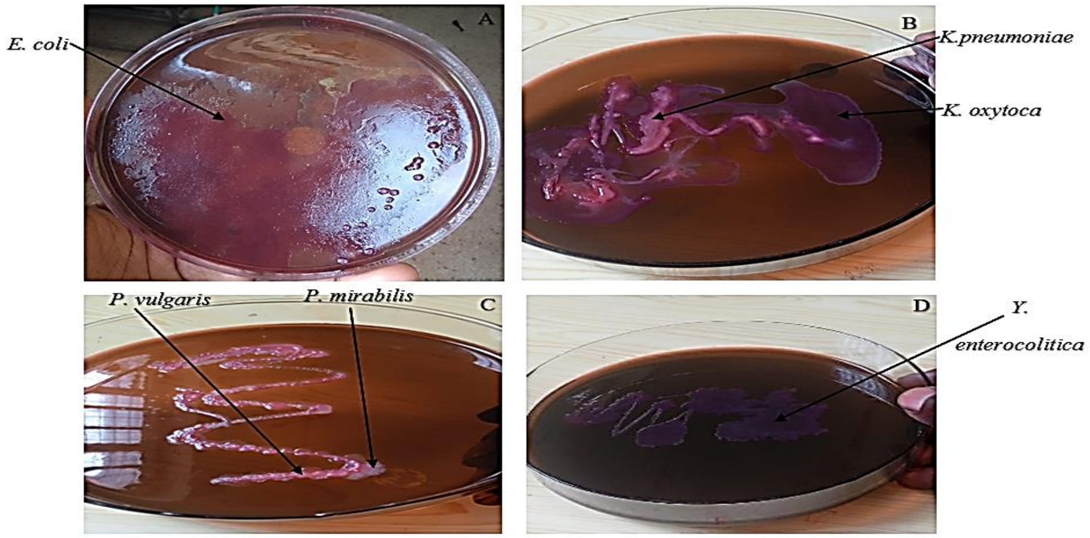


Figure 4. 1: Isolation and identification of bacteria from River Sosiani and waste water systems

Key: A- *Escherichia coli* isolates from Huruma quarry influent had deep purple color with green metallic sheen, B- *Klebsiella pneumoniae* isolates from Moi Teaching and Referral Hospital were mucoid and pinkish colonies while *Klebsiella oxytoca* from Kipkaren bridge were mucoid purple colonies. C stand for *Proteus vulgaris* isolated from MTRH, indicating pinkish color and *Proteus mirabilis from* Pioneer bridge were whitish colonies. D- *Yersinia enterocolitica* from Huruma quarry influent were observed as dark purple colonies without green metallic sheen.

4.1.3 Gram staining and biochemical characteristics

4.1.3.1 Gram staining

All the 10 bacteria including *Escherichia coli*, *Enterobacter aerogenes*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Proteus vulgaris*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella enteriditis* and *Yersinia enterocolitica* were pinkish in color and rod-shaped species confirming the presence of gram-negative bacteria. Figure 4.2 represents rod-shaped *E. coli* isolates from Huruma quarry influent, observed under light microscope at a magnification of 100X.

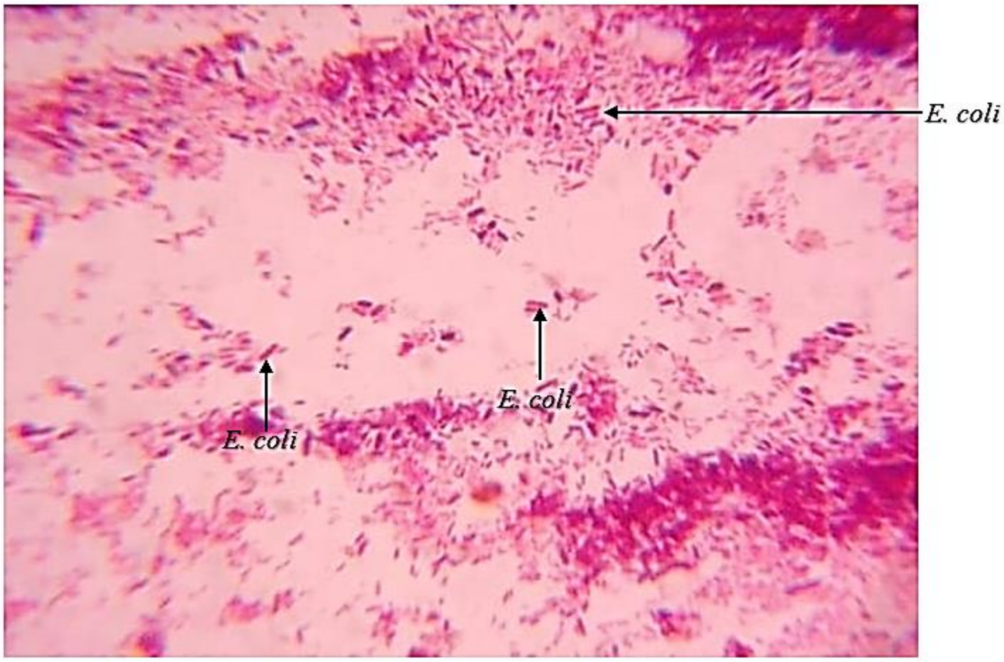


Figure 4. 2: Microscopic view of *E. coli* isolates from Huruma quarry influent site

Key: The gram staining showed that isolates of *Escherichia coli* were rod shaped and pinkish in color.

4.1.3.2 Biochemical characteristics

Bacteria species tested positive for catalase test were *E. coli*, *E. aerogenes, C. freundii, K. pneumoniae*, *K. oxytoca, P. mirabilis*, *P. aeruginosa*, *S. enteriditis* and *Y. enterocolitica.* This indicated that the mentioned bacteria were capable of producing catalase enzyme that breakdown hydrogen peroxide into water and oxygen hence forming the bubbles that were observed. Bacteria species with positive citrate test had blue slant on top of the green butt, showing that they were capable of utilizing citrate as a carbon source. These included *E. coli*, *E. aerogenes, C. freundii*, *K. pneumoniae, K. oxytoca*, *P. vulgaris*, *P. mirabilis* and *P. aeruginosa*. Positive oxidase reaction was observed in *P. aeruginosa,* implying that the species produced cytochrome oxidase enzyme, hence catalyzing the transport of electrons within the species, resulting to the dark purple color. Bacteria species tested positive for indole test were *E. coli, K. oxytoca*, *P. vulgaris* and *Y. enterocolitica*, showing that those bacteria produced tryptophanase enzyme that breakdown tryptophan into different indole groups, represented by the red ring on the surface of the solution. For triple sugar iron test, positive reaction was observed in those species capable of fermenting glucose, lactose and sucrose while producing the hydrogen sulphide gas. These bacteria species were *E. coli, K. pneumoniae, K. oxytoca, P. vulgaris* and *Y. enterocolitica,* having a yellow/blue slant with black butt, and cracks of hydrogen sulphide gas and acid. A summary of the results were shown on Table 4.2.

Table 4. 2: Bacteria species identified on biochemical tests

Biochemical test	**Inference**	**Bacteria identified**	**Reference**
Catalase	Bubble formation	*E. coli*, *E. aerogenes, C. freundii*, *K. pneumoniae, K. oxytoca*, *P. mirabilis, P. aeruginosa, S. enteriditis* and *Y. enterocolitica*	(Yuan *et al*., 2021)
Citrate	Blue slant on top of green butt	*E. coli*, *E. aerogenes*, *C. freundii*, *K. pneumoniae, K. oxytoca*, *P. vulgaris, P. mirabilis* and *P. aeruginosa*	(Shoaib *et al*., 2020)
Oxidase	Purple colonies	*P. aeruginosa*	(Al-hmad &Roodsari, 2016)
Indole	Reddish ring on the surface	*E. coli*, *K. oxytoca*, *P. vulgaris* and *Y. enterocolitica*	(Nieto & Lupton, 2021)
Triple sugar iron	Yellow/blue slant with black butt	*E. coli*, *K. pneumoniae, K. oxytoca, P. vulgaris* and *Y. enterocolitica*	(Masi *et al*., 2021)

Figure 4.3 shows images of the different biochemical tests used in bacteria species identification

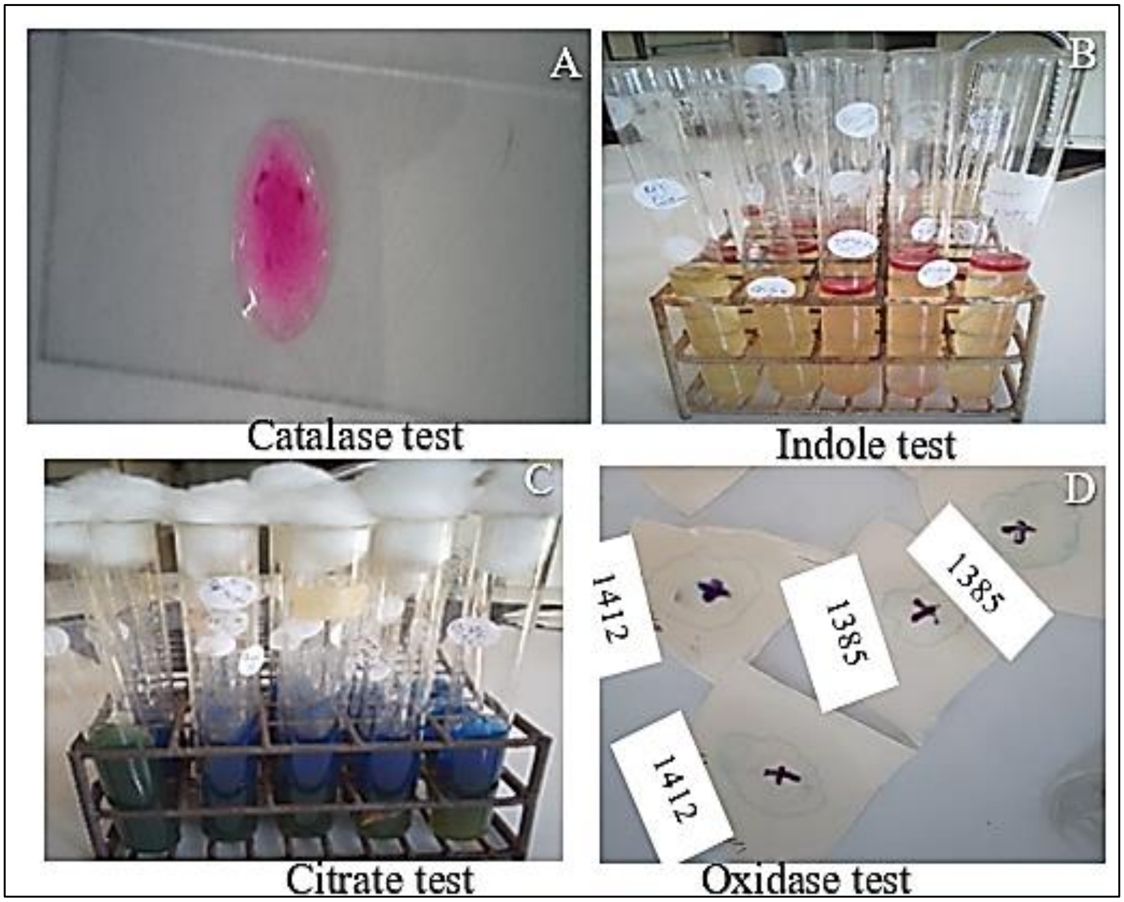


Figure 4. 3: Different biochemical tests used in bacteria species identification

Key: A-catalase test, B-indole test, C-citrate, D-oxidase test showing *P. aeruginosa* isolates from both Huruma quarry influent and Eldoret prison with sample codes 1385 and 1412 respectively.

4.1.4 Distribution of Gram- negative pathogenic bacteria species in River Sosiani and wastewater systems

Out of the nine sampled sites, Kipkaren bridge had a higher count of bacteria, with a total of seven (7) species. These were *E. coli*, *E. aerogenes*, *C. freundii*, *K. pneumoniae*, *K.*

oxytoca, *P. mirabilis* and *P. vulgaris*. This was followed by Huruma quarry influent and the Outspan-Nairobi bridge sites which had 6 bacteria species each. These included *E. coli*, *E. aerogenes, P. mirabilis, P. aeruginosa*, *S. enteriditis and Y. enterocolitica* at the Huruma quarry influent site. The Outspan-Nairobi Road bridge site had *E. coli*, *E. aerogenes*, *C. freundii*, *K. pneumoniae, K. oxytoca* and *P. vulgaris*. On the other hand, the Eldoret prison site was colonized by 5 bacteria species, namely: *E. coli*, *P. vulgaris, P. aeruginosa, S. enteriditis* and *Y. enterocolitica*. Pioneer bridge site also reported 5 species, including *E. coli, E. aerogenes*, *C. freundii*, *P. mirabilis* and *S. enteriditis*. In addition, Moi Teaching and Referral Hospital (MTRH) site constituted 4 species, inclusive of *E. coli*, *E. aerogenes*, *K. oxytoca* and *P. vulgaris*. Huruma quarry effluent, Kipkenyo boundary influent and Kipkenyo boundary effluent sites had the lowest number of bacteria species, with Huruma quarry effluent showing 3 (*E. coli, E. aerogenes and K. pneumoniae*), Kipkenyo boundary influent had 2 species (*E. coli* and *E. aerogenes*) and Kipkenyo boundary effluent with 1 bacteria species (*K. pneumoniae*). A summary of the results was presented on Figure 4.4.

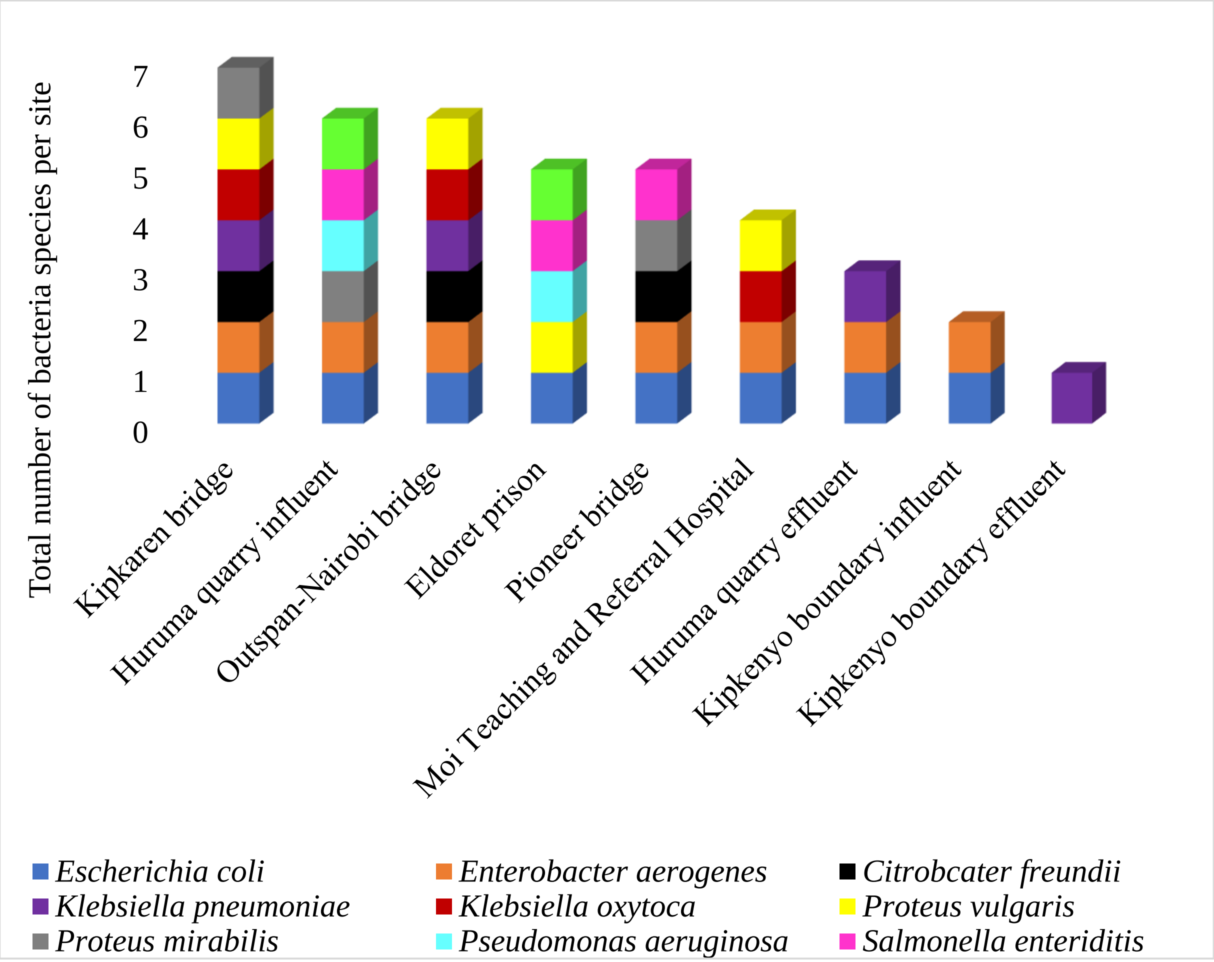


Figure 4. 4: Distribution of Gram- negative pathogenic bacteria species in River Sosiani and wastewater systems

Key: Kipkaren bridge had 7 bacteria species, indicating higher count followed by both Huruma quarry influent and Outspan- Nairobi bridge, each constituting 6 bacteria species. Eldoret prison and Pioneer bridge had 5 bacteria species each compared to MTRH that contained 4 bacteria species. A lower bacteria species count was detected in Huruma quarry effluent (3), Kipkenyo boundary influent (2) and Kipkenyo boundary effluent (1).

4.2 Antibiotic susceptibility test

Bacteria isolated from both river water and waste water showed different antibiotic susceptibility activities. Different zones of inhibition (ZI) were measured in millimeter (mm) (Appendix 6).

4.2.1 *Escherichia coli*

The *E. coli* isolates were susceptible to ciprofloxacin (29±1mm), doxycycline (22.7±1.5mm) and gentamycin (18.7±1.2mm) with larger zones of inhibition. The isolates showed intermediate susceptibility to chloramphenicol (17.3±1.2mm) with smaller zone of inhibition. *Escherichia coli* indicated resistance to azithromycin (9.3±0.6mm), tetracycline (8.7±1.2mm), sulfamethoxazole-trimethoprim (8.7±1.2mm), penicillin (6±0mm), ampicillin (6±0mm), ceftazidime (6±0mm), amoxicillin-clavulanic acid (6±0mm) and streptomycin (6±0mm). Table 4.3 gives a summary of the results.

Table 4. 3:Antibiotic susceptibility profiles *of Escherichia coli*

Escherichia coli was susceptible to 3, intermediate susceptible to 1 and resistant to 8 antibiotics.

Antibiotic activity	**Zone of inhibition (mm)**
Susceptible (S)	
Ciprofloxacin	29±1
Doxycycline	22.7±1.2
Gentamycin	18.7±1.2
Intermediate susceptible (IS)	
Chloramphenicol	17.3±1.2
Resistant (R)	
Azithromycin	9.3±0.6
Tetracycline	8.7±1.2
Sulfamethoxazole-trimethoprim	8.7±1.2
Penicillin	6±0
Ampicillin	6±0
Ceftazidime	6±0
Amoxicillin-Clavulanic acid	6±0
Streptomycin	6±0

Figure 4.5 shows image of antibiotic susceptibility test of *Escherichia coli* subjected to ciprofloxacin (CIP, 30µg), gentamycin (GEN, 10 µg), chloramphenicol (CHL, 30 µg), tetracycline (TET, 30 µg), azithromycin (AZT, 15 µg) and streptomycin (STR, 10 µg).

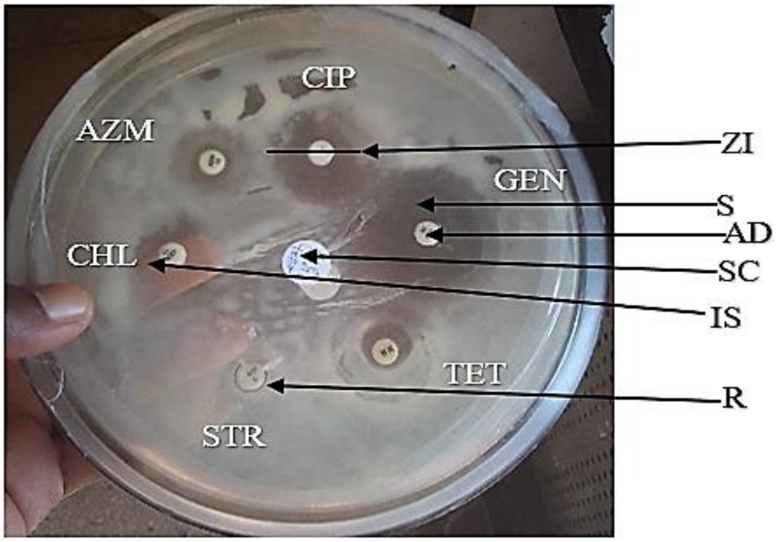


Figure 4. 5: Antibiotic susceptibility tests of *Escherichia coli*

Key: ZI- zone of inhibition measured, S- tested antibiotic showed bacteria susceptibility, AD- antibiotic disc, SC- sample code, IS- tested antibiotic showed bacterial intermediate susceptibility and R- tested antibiotic indicated bacterial resistance. CIP- Ciprofloxacin, GEN- gentamycin, CHL- chloramphenicol, TET- tetracycline, AZT- azithromycin, STR- streptomycin. Figure 4.4 shows that *Escherichia coli* was susceptible to ciprofloxacin and gentamycin, intermediate susceptible to chloramphenicol and resistant to azithromycin, tetracycline and streptomycin.

4.2.2 *Enterobacter aerogenes*

The isolates were susceptible to doxycycline (23.3±2.5mm) with larger zone of inhibition and intermediate susceptible to ciprofloxacin (25±4.4mm) with smaller zone of inhibition. The isolates were resistant to gentamycin (14.3±2.3mm), chloramphenicol (12.3±3.5mm), azithromycin (10.3±2.5mm), streptomycin (7.3±1.2mm), ceftazidime (7.3±1.2mm), tetracycline (6.7±1.2mm), penicillin (6±0mm), ampicillin (6±0mm), amoxicillin-clavulanic acid (6±0mm) and sulfamethoxazole-trimethoprim (6±0mm). The results were shown on Table 4.4.

Table 4. 4: Antibiotic susceptibility profiles of *Enterobacter aerogenes*

Enterobacter aerogenes was susceptible to 1, intermediate susceptible to 1 and resistant to 10 antibiotics.

Antibiotic activity	Zone of inhibition (mm)
Susceptible (S)	
Doxycycline	23.3±2.5
Intermediate susceptible (IS)	
Ciprofloxacin	25±4.4
Resistant (R)	
Gentamycin	14.3±2.3
Chloramphenicol	12.3±3.5
Azithromycin	10.3±2.5
Streptomycin	7.3±1.2
Ceftazidime	7.3±1.2
Tetracycline	6.7±1.2
Penicillin	6±0
Ampicillin	6±0
Amoxicillin-Clavulanic acid	6±0
Sulfamethoxazole- trimethoprim	6±0

4.2.3 *Citrobacter freundii*

Citrobacter freundii was susceptible to ciprofloxacin (29.3±1.2mm), doxycycline (25.3±2.5mm) and gentamycin (20±2mm) with larger zones of inhibition. The isolates also expressed resistance to ceftazidime (14.3±1.5mm), streptomycin (9.3±2.3mm), chloramphenicol (7.3±1.2mm), tetracycline (7±1mm), azithromycin (6.7±1.2mm), penicillin (6±0mm), ampicillin (6±0mm), amoxicillin-clavulanic acid (6±0mm) and sulfamethoxazole- trimethoprim (6±0mm). The results were summarized on Table 4.5.

Table 4. 5: Antibiotic susceptibility profiles of *Citrobacter freundii*

Citrobacter freundii was susceptible to 3 and resistant to 9 antibiotics.

Antibiotic activity	**Zone of inhibition (mm)**
Susceptible (S)	
Ciprofloxacin	29.3±1.2
Doxycycline	25.3±2.5
Gentamycin	20±2
Resistant (R)	
Ceftazidime	14.3±1.5
Streptomycin	9.3±2.3
Chloramphenicol	7.3±1.2
Tetracycline	7±1
Azithromycin	6.7±1.2
Penicillin	6±0
Ampicillin	6±0
Amoxicillin-clavulanic acid	6±0
Sulfamethoxazole-trimethoprim	6±0

4.2.4 *Klebsiella pneumoniae*

Klebsiella pneumoniae were intermediate susceptible to gentamycin (15.3±2.3mm), azithromycin (15±1mm) and doxycycline (11.7±2.9mm) with smaller zones of inhibition.

The isolates were resistant to ciprofloxacin (17.7±1.5mm), chloramphenicol (10.3±2.1mm), tetracycline (10±2mm), ceftazidime (8±2mm), streptomycin (6.7±1.2mm), sulfamethoxazole-trimethoprim (6.7±1.2mm), penicillin (6±0mm), ampicillin (6±0mm) and amoxicillin-clavulanic acid (6±0mm). The results of antibiotic susceptibility tests of *Klebsiella pneumoniae* were presented on Table 4.6.

Table 4. 6: Antibiotic susceptibility profiles of *Klebsiella pneumoniae*

Klebsiella pneumoniae was intermediate susceptible to 3 and resistant to 9 antibiotics.

Antibiotic activity	Zone of inhibition (mm)
Intermediate susceptible (IS)	
Gentamycin	15.3±2.3
Azithromycin	15±1
Doxycycline	11.7±2.9
Resistant (R)	
Ciprofloxacin	17.7±1.5
Chloramphenicol	10.3±2.1
Tetracycline	10±2
Ceftazidime	8±2
Streptomycin	6.7±1.2
Sulfamethoxazole-trimethoprim	6.7±1.2
Penicillin	6±0
Ampicillin	6±0
Amoxicillin-clavulanic acid	6±0

4.2.5 *Klebsiella oxytoca*

Klebsiella oxytoca isolates were susceptible to ciprofloxacin (29.3±2.5mm), gentamycin (26.7±3.2mm) and chloramphenicol (22.3±2.5mm) with larger zones of inhibition and intermediate susceptible to doxycycline (13.7±1.5mm) with smaller zone of inhibition. In addition, they were resistant to azithromycin (10±0mm), streptomycin (10±0mm), tetracyclines (8.7±1.2mm), amoxicillin-clavulanic acid (7.3±1.2mm), penicillin (6±0mm), ampicillin (6±0mm), ceftazidime (6±0mm) and sulfamethoxazole-trimethoprim (6±0mm). The results were summarized on Table 4.7.

Table 4. 7: Antibiotic susceptibility profiles of *Klebsiella oxytoca*

Klebsiella oxytoca was susceptible to 3, intermediate susceptible to 1 and resistant to 8 antibiotics.

Antibiotic activity	Zone of inhibition (mm)
Susceptible (S)	
Ciprofloxacin	29.3±2.5
Gentamycin	26.7±3.2
Chloramphenicol	22.3±2.5
Intermediate susceptible (IS)	
Doxycycline	13.7±1.5
Resistant (R)	
Azithromycin	10±0
Streptomycin	10±0
Tetracycline	8.7±1.2
Amoxicillin-clavulanic acid	7.3±1.2
Penicillin	6±0
Ampicillin	6±0
Ceftazidime	6±0
Sulfamethoxazole-trimethoprim	6±0

4.2.6 *Proteus vulgaris*

Isolates of *P. vulgaris* were susceptible to ciprofloxacin (28.7±1.2mm) and doxycycline (27.7±2.5mm) with larger zones of inhibition. The isolates demonstrated intermediate susceptibility to azithromycin (13.7±2.1mm) with smaller zone of inhibition. Resistance was observed against chloramphenicol (11.3±4.2mm), tetracycline (10±2mm), sulfamethoxazole-trimethoprim (7.3±1.2mm), gentamycin (7.3±1.2mm), ceftazidime (6±1.7mm), amoxicillin-clavulanic acid (6.7±1.2mm), penicillin (6±0mm), ampicillin (6±0mm) and streptomycin (6±0mm). A summary of results presented on Table 4.8.

Table 4. 8: Antibiotic susceptibility profiles of *Proteus vulgaris*

Proteus vulgaris was susceptible to 2, intermediate susceptible to 1 and resistant to 9 antibiotics.

Antibiotics activity	Zone of inhibition (mm)
Susceptible (S)	
Ciprofloxacin	28.7±1.2
Doxycycline	27.7±2.5
Intermediate susceptible (IS)	
Azithromycin	13.7±2.1
Resistant (R)	
Chloramphenicol	11.3±4.2
Sulfamethoxazole-trimethoprim	7.3±1.2
Ceftazidime	6±1.7
Tetracycline	10±2
Gentamycin	7.3±1.2
Amoxicillin-clavulanic acid	6.7±1.2
Penicillin	6±0
Ampicillin	6±0
Streptomycin	6±0

4.2.7 *Proteus mirabilis*

The isolates showed susceptibility to doxycycline (22±2mm) and gentamycin (20±2mm) with larger zones of inhibition. They were intermediate susceptible to ciprofloxacin (22.7±2.1mm) with smaller zone of inhibition. The isolates showed resistance to chloramphenicol (11±1mm), streptomycin (9.3±8.1mm), amoxicillin-clavulanic acid (7.3±1.2mm), sulfamethoxazole-trimethoprim (7.3±1.2mm), tetracycline (6.7±1.2mm), ampicillin (6.7±1.2mm), azithromycin (6±2mm), penicillin (6±0mm), and ceftazidime (6±0mm). The results were indicated on Table 4.9.

Table 4. 9: Antibiotic susceptibility profiles of *Proteus mirabilis*

Proteus mirabilis was susceptible to 2, intermediate susceptible to 1 and resistant to 9 antibiotics.

Antibiotic activity	Zone of inhibition (mm)
Susceptible (S)	
Doxycycline	22±2
Gentamycin	20±2
Intermediate susceptible (IS)	
Ciprofloxacin	22.7±2.1
Resistant (R)	
Chloramphenicol	11±1
Streptomycin	9.3±8.1
Amoxicillin-clavulanic acid	7.3±1.2
Sulfamethoxazole-trimethoprim	7.3±1.2
Tetracycline	6.7±1.2
Ampicillin	6.7±1.2
Azithromycin	6±2
Penicillin	6±0
Ceftazidime	6±0

4.2.8 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa showed intermediate susceptibility to ciprofloxacin (22.3±5.9mm), chloramphenicol (13.3±6.1mm) and doxycycline (11.3±1.5mm) with smaller zones of inhibition. They were resistant to tetracycline (9.3±1.2mm), azithromycin (8.7±1.2mm), ceftazidime (7.3±1.2mm), streptomycin (7.3±2.3mm), gentamycin (6.7±1.5mm), penicillin (6±0mm), ampicillin (6±0mm), amoxicillin-clavulanic acid (6±0mm) and sulfamethoxazole-trimethoprim (6±0mm). A summary of the results was generated on Table 4.10.

Table 4. 10: Antibiotic susceptibility profiles of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa was intermediate susceptible to 3 and resistant to 9 antibiotics.

Antibiotic activity	Zone of inhibition (mm)
Intermediate susceptible (IS)	
Ciprofloxacin	22.3±5.9
Chloramphenicol	13.3±6.1
Doxycycline	11.3±1.5
Resistant (R)	
Ceftazidime	7.3±2.3
Tetracycline	9.3±1.2
Azithromycin	8.7±1.2
Streptomycin	7.3±2.3
Gentamycin	6.7±1.5
Penicillin	6±0
Ampicillin	6±0
Amoxicillin-clavulanic acid	6±0
Sulfamethoxazole-trimethoprim	6±0

4.2.9 *Salmonella enteriditis*

The current work showed that *S. enteriditis* were susceptible to ciprofloxacin (28±0mm) and gentamycin (22.3±3.2mm) with larger zones of inhibition. Also, they were intermediate susceptible to chloramphenicol (16.7±4.2mm) with smaller zone of inhibition. The isolates showed resistance to azithromycin (10.7±1.2mm), doxycycline (9.3±1.2mm), sulfamethoxazole-trimethoprim (9.3±1.2mm), amoxicillin-clavulanic acid (9.3±0.58mm), ceftazidime (8.7±1.2mm), streptomycin (7.3±1.2mm), tetracycline (7.3±1.2mm), penicillin (6±0mm) and ampicillin (6±0mm). The results were shown on Table 4.11.

Table 4. 11: Antibiotic susceptibility profiles of *Salmonella enteriditis*

Salmonella enteriditis were susceptible to 2, intermediate susceptible to 1 and resistant to 9 antibiotics.

Antibiotic activity	Zone of inhibition (mm)
Susceptible (S)	
Ciprofloxacin	28±0
Gentamycin	22.3±3.2
Intermediate susceptible (IS)	
Chloramphenicol	16.7±4.2
Resistant (R)	
Azithromycin	10.7±1.2
Doxycycline	9.3±1.2
Sulfamethoxazole-trimethoprim	9.3±1.2
Amoxicillin-clavulanic acid	9.3±0.58
Ceftazidime	8.7±1.2
Streptomycin	7.3±1.2
Tetracycline	7.3±1.2
Penicillin	6±0
Ampicillin	6±0

4.2.10 *Yersinia enterocolitica*

This study demonstrated that isolates of *Y. enterocolitica* were susceptible to ciprofloxacin (26±0mm), chloramphenicol (22±1mm) and gentamycin (19.3±1.2mm) with larger zones of inhibition. The isolates were intermediate susceptible to azithromycin (14.7±2.3mm) and doxycycline (11±1.7mm) with smaller zones of inhibition. They also showed resistance to sulfamethoxazole-trimethoprim (10±2mm), tetracyclines (6.7±1.2mm), amoxicillin-clavulanic acid (6.7±1.2mm), penicillin (6±0mm), ampicillin (6±0mm), ceftazidime (6±0mm) and streptomycin (6±0mm). The results were presented on Table 4.12.

Table 4. 12: Antibiotic susceptibility profiles of *Yersinia enterocolitica*

Yersinia enterocolitica were susceptible to 3, intermediate susceptible to 2 and resistant to 7 antibiotics.

Antibiotic activity	Zone of inhibition (mm)
Susceptible (S)	
Ciprofloxacin	26±0
Chloramphenicol	22±1
Gentamycin	19.3±1.2
Intermediate susceptible (IS)	
Azithromycin	14.7±2.3
Doxycycline	11±1.7
Resistant (R)	
Sulfamethoxazole-trimethoprim	10±2
Tetracycline	6.7±1.2
Amoxicillin-clavulanic acid	6.7±1.2
Penicillin	6±0
Ampicillin	6±0
Ceftazidime	6±0
Streptomycin	6±0

4.2.11 Determination of Multi antibiotic resistance index (MARI) in bacteria

The findings of this study highlight the varying degrees of Multi-Antibiotic Resistance Indices (MARI) observed among the isolated bacteria. *Enterobacter aerogenes* exhibited the highest MARI of 0.83, followed by *C. freundii*, *K. pneumoniae*, *P. vulgaris*, *P. mirabilis*, *P. aeruginosa*, and *S. enteriditis*, each with a MARI value of 0.75. Both *E. coli* and *K. oxytoca* exhibited a MARI of 0.67 while *Y. enterocolitica* expressed the lowest MARI value of 0.58. The results were summarized on Figure 4.6.

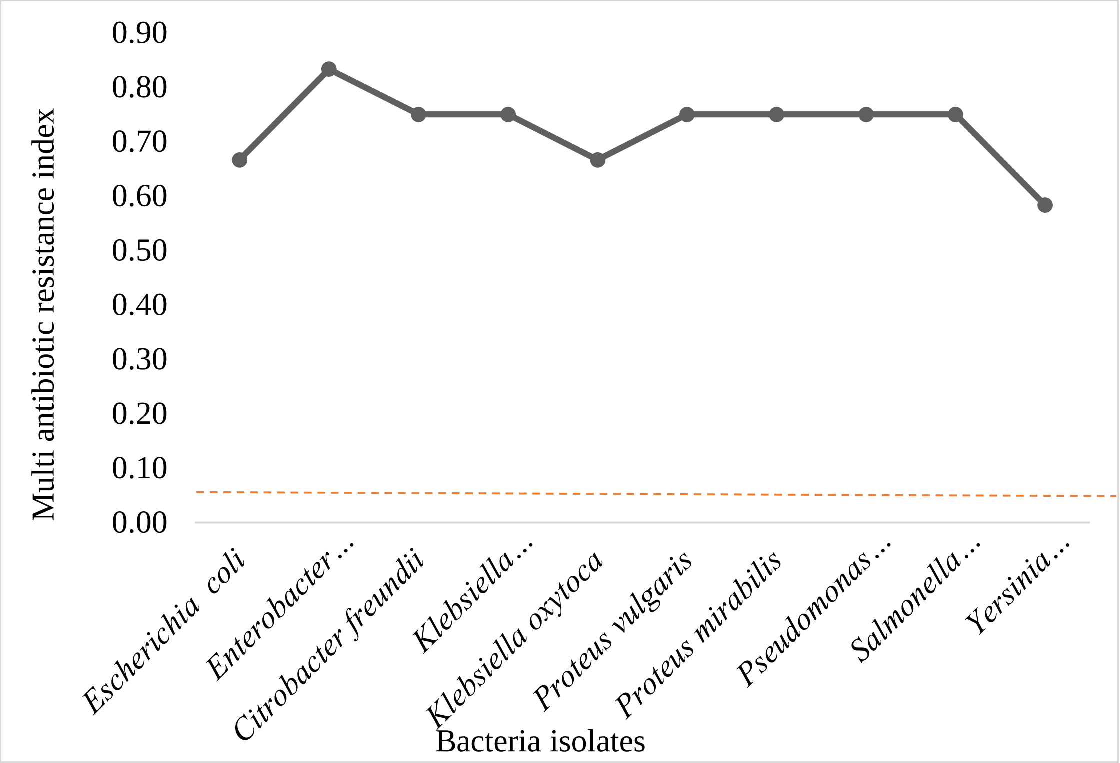


Figure 4.6: Multi antibiotic resistance indices (MARI) of different bacteria species

Key: The highest MARI was observed in *Enterobacter aerogenes* (0.83), followed by *Citrobacter freundii, Klebsiella pneumoniae, Proteus vulgaris, Proteus mirabilis, Pseudomonas aeruginosa* and *Salmonella enteriditis,* each with a MARI value of 0.75. *Escherichia coli* and *Klebsiella oxytoca* showed a MARI of 0.67 while *Yersinia enterocolitica* expressed the lowest MARI of 0.58. All the bacteria species had multi antibiotic resistance index (MARI) greater than 0.2, indicating a higher level of bacteria resistance to multiple tested antibiotics.

4.3 Antibiotic- resistant genes in plasmids of the isolated bacteria

4.3.1 Sub culturing of bacteria

From the subculture of bacteria in liquid nutrient broth, *E. coli, E. aerogenes, C. freundii, K. pneumoniae, K. oxytoca, P. vulgaris, P. mirabilis, P. aeruginosa, S. enteriditis* and *Y. enterocolitica* were obtained.

4.3.2 Determination of purity in the plasmid DNA of Bacteria isolated from River Sosiani and wastewater systems

All the extracted bacterial plasmid DNA from different bacteria including *E. coli, E. aerogenes, C. freundii, K. pneumoniae, K. oxytoca, P. vulgaris, P. mirabilis, P. aeruginosa, S. enteriditis* and *Y. enterocolitica*, were obtained as pure isolates with ratios, ranging from 1.80-1.89 at 260/280nm. A summary of the results was generated on Table 4.13.

Table 4.13: Determination of purity and concentration of plasmid DNA in the Bacteria isolated from River Sosiani and wastewater systems

Antibiotic-resistant bacteria	**Purity level (260/280nm)**
Escherichia coli	1.85
Enterobacter aerogenes	1.89
Citrobacter freundii	1.80
Klebsiella pneumoniae	1.80
Klebsiella oxytoca	1.82
Proteus vulgaris	1.80
Proteus mirabilis	1.80
Pseudomonas aeruginosa	1.80
Salmonella enteriditis	1.80
Yersinia enterocolitica	1.80

Key: * 260/280 is the ratio of absorbance used to assess the purity of the DNA molecule. The recommended value for the pure DNA ranges between 1.8-2.0 while impure DNA is indicated by a value ≤1.6, ng/µl- stand for nanogram per microliter.

4.3.3 Determination of plasmid DNA concentrations of Bacteria isolated from River Sosiani and wastewater systems

Escherichia coli indicated highest concentration of 73.4 ng/µl, followed by *Klebsiella pneumoniae* (66.7 ng/µl), *Enterobacter aerogenes* (61.2 ng/µl), *Proteus vulgaris* (58.5 ng/µl), *Proteus mirabilis* (53.7 ng/µl), *Citrobacter freundii* (50.4 ng/µl), *Pseudomonas aeruginosa* (47.9 ng/µl), *Klebsiella oxytoca* (43.8 ng/µl), *Salmonella enteriditis* (39.8 ng/µl) and *Yersinia enterocolitica* had the lowest concentration of 38.5 ng/µl. The results were summarized on Table 4.14.

Table 4. 14: Determination of plasmid DNA concentrations of Bacteria isolated from River Sosiani and wastewater systems

Plasmid DNA extracted from the *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Proteus vulgaris*, *Proteus mirabilis* and *Citrobacter freundii* had the highest concentration ranging between 73.4-50.4 ng/µl. *Pseudomonas aeruginosa*, *Klebsiella oxytoca*, *Salmonella enteriditis* and *Yersinia enterocolitica*indicated the lowest concentration of the range 47.9-38.5 ng/µl.

Antibiotic-resistant bacteria	**Concentration at 280nm (ng/µl)**
Escherichia coli	73.4
Klebsiella pneumoniae	66.7
Enterobacter aerogenes	61.2
Proteus vulgaris	58.5
Proteus mirabilis	53.7
Citrobacter freundii	50.4
Pseudomonas aeruginosa	47.9
Klebsiella oxytoca	43.8
Salmonella enteriditis	39.8
Yersinia enterocolitica	38.5

4.3.4 Gel electrophoresis

The size of the extracted plasmid DNA for *Escherichia coli*, *Enterobacter aerogenes*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Proteus vulgaris*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella enteriditis* and *Yersinia enterocolitica* were determined on gel (1.5%) as shown on Figure 4.7. The size of the plasmid DNA of all the bacteria species was approximately 2000 base pairs (bp).

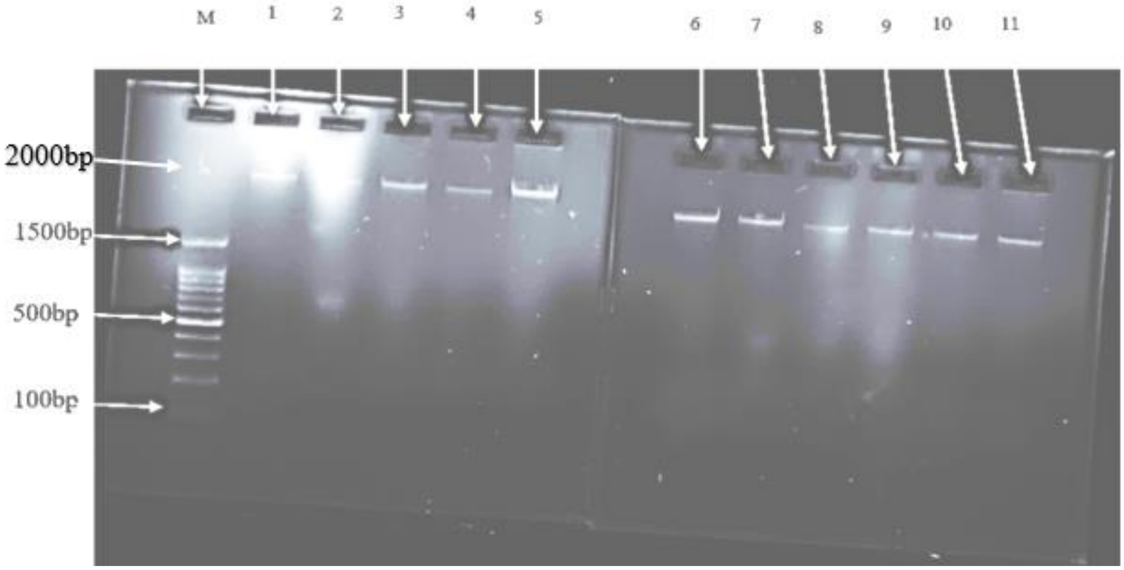


Figure 4. 7: Gel quantification of bacterial plasmid DNA isolated from River Sosiani and wastewater systems

Key: M-DNA ladder, 1&2-*Escherichia coli,* 3-*Enterobacter aerogenes*, 4-*Citrobacter freundii*, 5-*Klebsiella pneumoniae,* 6-*Klebsiella oxytoca,* 7-*Proteus vulgaris,* 8-*Proteus mirabilis*, *9-Pseudomonas aeruginosa,* 10-*Salmonella enteriditis* and 11-*Yersinia enterocolitica.* The approximate size for the plasmid DNA of all the 10 bacteria species was 2000bp.

4.3.5 PCR amplification of the antibiotic- resistant genes in the selected bacterial plasmid DNA

There was no significant relationship between the phenotypic resistance and genotypic resistance detected in the species of *E. coli, E. aerogenes, C. freundii, K. pneumoniae, K. oxytoca, P. vulgaris, P. mirabilis, P. aeruginosa, S. enteriditis* and *Y. enterocolitica* ($P \geq 1$) (Appendix 7). Both *qepA* and *qnrA* genes were detected in all the 10 bacteria species, including *E. coli, E. aerogenes, C. freundii, K. pneumoniae, K. oxytoca, P. vulgaris, P. mirabilis, P. aeruginosa, S. enteriditis* and *Y. enterocolitica* measuring 312bp and 347bp respectively. 70% of bacteria species including *E. coli, E. aerogenes, K. pneumoniae, K. oxytoca, P. mirabilis, P. aeruginosa* and *S. enteriditis* had *dfrA5* gene detected with 279 bp. Also, *AadA1* of 447 bp was identified in 70% of bacteria species: *E. coli, E. aerogenes, C. freundii, K. pneumoniae, P. vulgaris, P. aeruginosa* and *S. enteriditis*. On the other hand, Bla_{KPC} gene measuring 340 bp was also detected in 50% of the bacteria, including *E. coli, E. aerogenes, C. freundii, P. vulgaris* and *Y. enterocolitica*. In addition, 40% of the bacteria had *mphA* gene, detected in *E. coli, E. aerogenes, C. freundii* and *K. pneumoniae* with 410 bp. Moreover, both bla_{SHV} and *sul 1* genes were detected in 30% of bacteria species, namely, *E. coli, E. aerogenes* and *C. freundii* with 867 bp and 338 bp respectively. However, *ampC* gene was not detected in any of the 10 bacterial species (0%). The results were generated on Table 4.15.

Table 4. 15: The occurrence of genes for antibiotic-resistance in plasmids of the selected bacteria isolated from Sosiani River and waste water systems in Eldoret, Uasin Gishu, Kenya

Antibiotic resistance genes were investigated in the plasmid DNA of *Escherichia coli*, *Pseudomonas aeruginosa* and *Yersinia enterocolitica* isolates of Huruma quarry influent, *Enterobacter aerogenes* and *Klebsiella oxytoca* detected from Kipkaren bridge, *Citrobacter freundii* obtained from Outspan- Nairobi bridge, *Klebsiella pneumoniae* and *Proteus vulgaris* found at the Moi Teaching and Referral Hospital, *Proteus mirabilis* isolated from the Pioneer bridge and *Salmonella enteriditis* from Eldoret prison.

Antibiotic-resistant genes	**Size of the gene(bp)**	***E. coli***	***E. aerogenes***	***C. freundii***	***K. pneumoniae***	***K. oxytoca***	***P. vulgaris***	***P. mirabilis***	***P. aeruginosa***	***S. enteriditis***	***Y. entero colitica***	**Percentage availability in the selected bacteria**
QepA	312	√	√	√	√	√	√	√	√	√	√	100%
QnrA	347	√	√	√	√	√	√	√	√	√	√	100%
DfrA5	279	√	√	—	√	√	—	√	√	√	—	70%
AadA1	447	√	√	√	√	—	√	—	√	√	—	70%
Bla$_{KPC}$	340	√	√	√	—	—	√	—	—	—	√	50%
MphA	410	√	√	√	√	—	—	—	—	—	—	40%
Bla$_{SHV}$	867	√	√	√	—	—	—	—	—	—	—	30%
AmpC	191	—	—	—	—	—	—	—	—	—	—	0%
Sul 1	338	√	√	√	—	—	—	—	—	—	—	30%

Key: * √ - antibiotic resistance gene present in that particular bacteria species; ─ -antibiotic resistance gene absent in that particular bacteria species.

4.3.5.1 Plasmid-mediated pentapeptide quinolone resistant gene (*QnrA*)

In the plasmid DNA of the 10 bacteria species including *E. coli, E. aerogenes, C. freundii, K. pneumoniae, K. oxytoca, P. vulgaris, P. mirabilis, P. aeruginosa, S. enteriditis* and *Y. enterocolitica*, Q*nrA* gene was detected with 347bp, as presented on Figure 4.8.

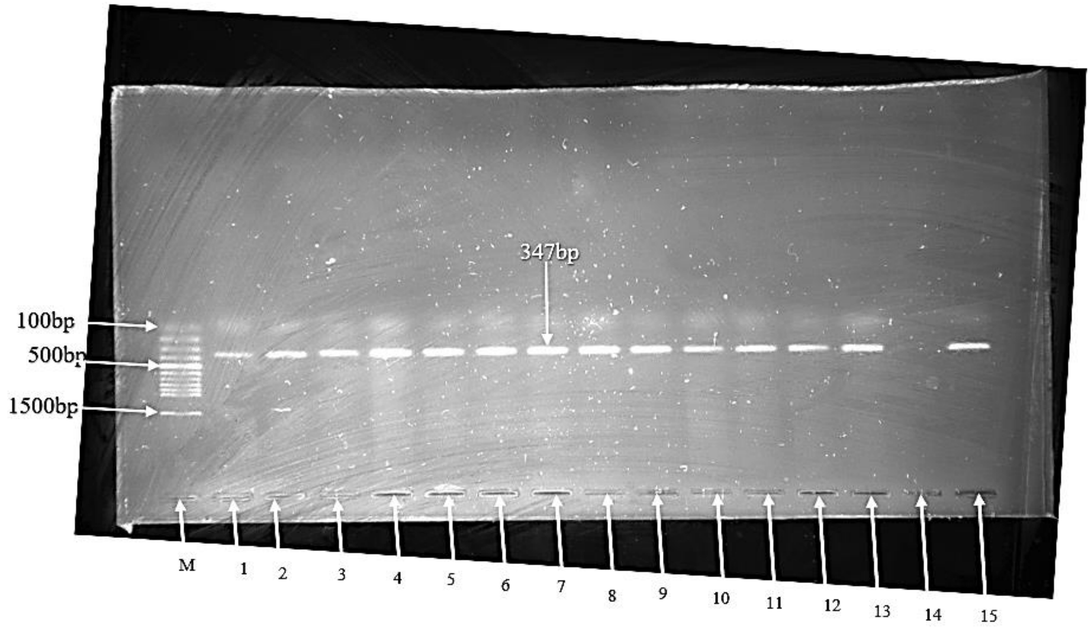


Figure 4. 8: *QnrA* gene detected in different bacteria species from River Sosiani and wastewater systems

Key: M- DNA ladder, (1-3)-*Escherichia coli*, (4&5)-*Enterobacter aerogenes*, (6&7)-*Citrobacter freundii*, 8-*Klebsiella pneumoniae*, 9-*Klebsiella oxytoca*, 10-*Proteus vulgaris*, 11-*Proteus mirabilis*, 12-*Pseudomonas aeruginosa*, 13-*Salmonella enteriditis* and 15-*Yersinia enterocolitica*, 14- nuclease free water.

4.3.5.2 Plasmid-mediated aminoglycoside resistance gene (*AadA1*)

Aminoglycoside resistance gene (*AadA1*) was present in *E. coli*, *E. aerogenes, C. freundii, K. pneumoniae, P. vulgaris, P. aeruginosa* and *S. enteriditis* with 447bp as shown on Figure 4.9.

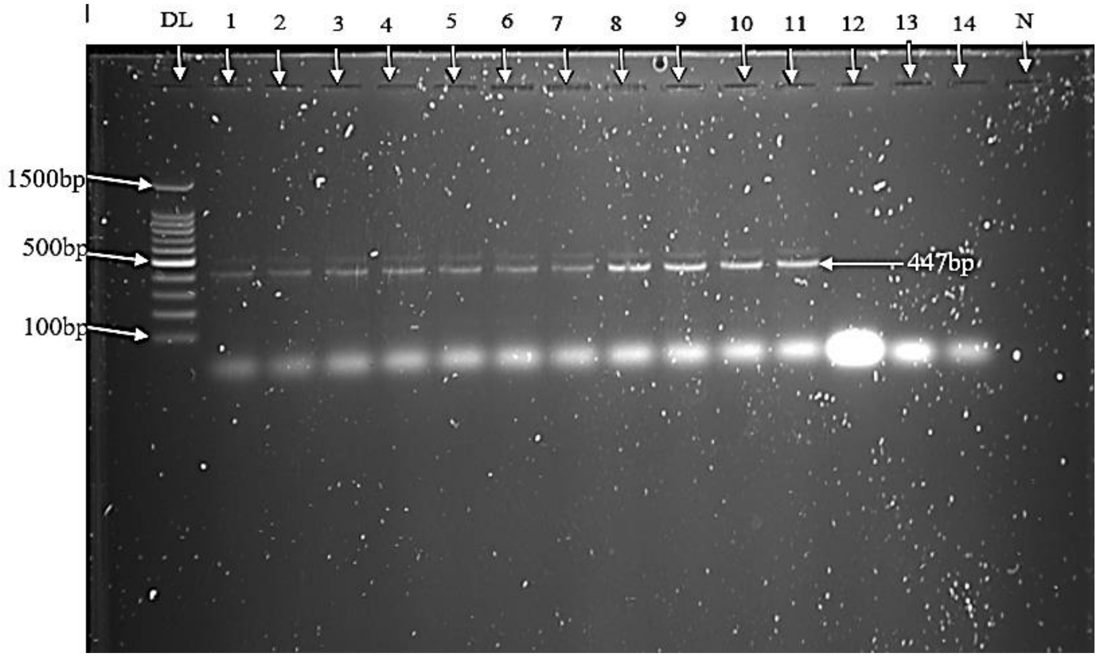


Figure 4. 9: ***AadA1*** **gene detected in different bacteria species from River Sosiani and wastewater systems**

Key: DL- DNA ladder, (1-3)-*E. coli*, (3&4)-*E. aerogenes*, (5&6)-*C. freundii*, 7-*K. pneumoniae* 8-*P. vulgaris*, (9&10)-*P. aeruginosa* and 10-*S. enteriditis*, 15-nuclease free water.

4.3.5.3 Plasmid-mediated macrolide resistance gene (*MphA)*

Plasmid encoded macrolide resistance gene, *MphA*, was detected in *E. coli*, *E. aerogenes*, *C. freundii* and *K. pneumoniae* with 410bp as indicated on Figure 4.10.

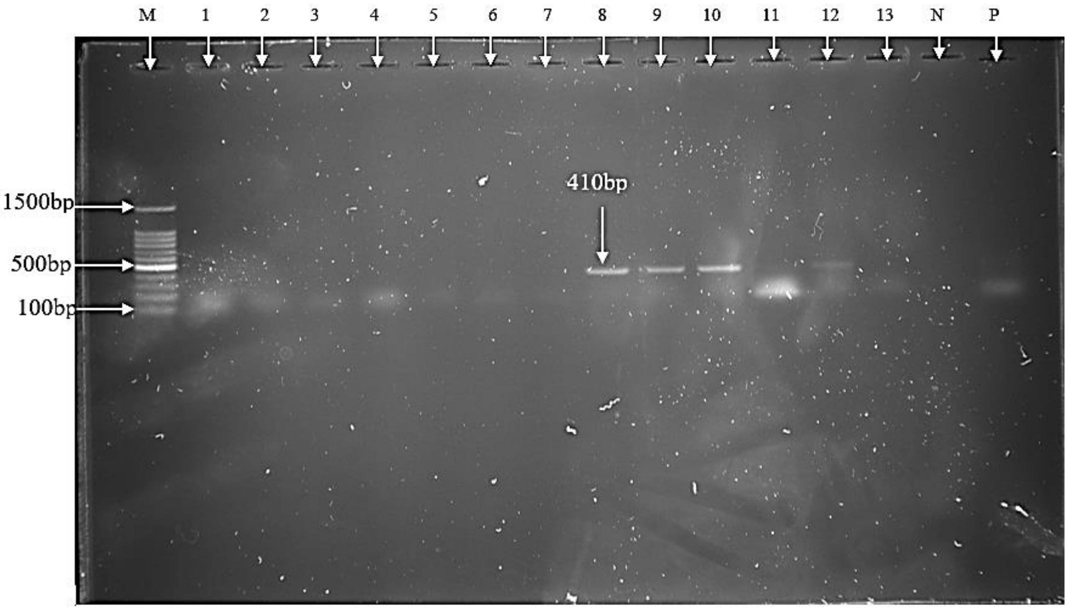


Figure 4. 10*: MphA* gene detected in different bacteria species from River Sosiani and wastewater systems

Key: M-DNA ladder, 8-*E. coli*, 9-*E. aerogenes*, 10-*C. freundii* and 12-*K. pneumoniae,* N-Nuclease free water, P- primers + master mix.

4.3.5.4 Plasmid-mediated carbapenem resistance gene (*Bla*$_{KPC}$)

Carbapenem resistance gene (*bla*$_{KPC}$) was detected in *E. coli*, *E. aerogenes*, *C. freundii*, *P. vulgaris* and *Y. enterocolitica* with 340 base pairs. The results were shown on Figure 4.11.

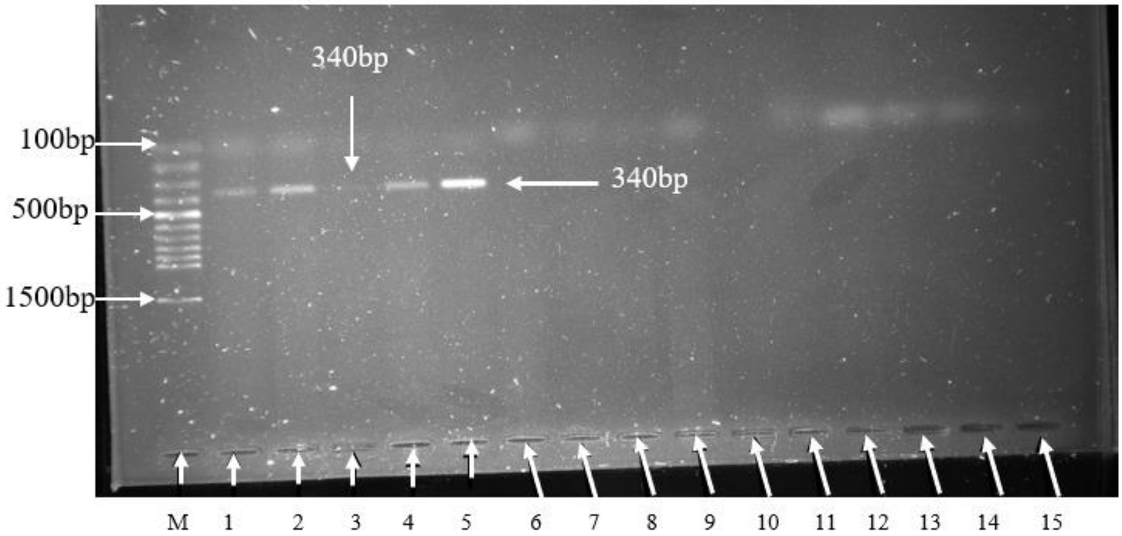


Figure 4. 11: *Bla*$_{KPC}$ gene detected in different bacteria species from River Sosiani and wastewater systems

Key: M- molecular marker, 1- *E. aerogenes,* 2-*Y. enterocolitica,* 3-*C. freundii,* 4- *P. vulgaris,* 5-*E. coli*, 10-blank.

CHAPTER FIVE

DISCUSSION

5.1 Isolation and identification of Bacteria from River Sosiani and wastewater systems

The study showed that all the sampled sites had a very high bacteria load, beyond count (too numerous to count). Some of them were fecal coliforms, indicating that large quantities of fecal matter are often emptied into River Sosiani and the sampled wastewater systems. The presence of fecal coliforms in the water systems suggest that the surrounding communities might be lacking basic sanitary facilities or waste treatment at these facilities is neither effective nor sometimes not carried out at all. Therefore, exposing the general public to the possibility in the rise of pandemic and endemic infections in these regions, especially those people and livestock utilizing water from River Sosiani without proper treatments. Fecal coliforms from aquatic environments are often linked with increased health risks to the surrounding communities as reported in a similar research study (Balkrishna *et al*., 2024). High number of fecal coliforms (10,000 CFU/100ml) were detected in Kipkenyo boundary effluent site than Kipkaren bridge (235 CFU/100ml), exceeding the recommended value by World Health Organization (1000 CFU/100ml) (Aziz *et al*., 2021). Suggesting, that a lot of municipal wastes, medical wastes, agricultural wastes and other wastes released from different farmlands, small scale busy markets, few dispensaries, several primary and secondary schools that surround the Kipkenyo boundary wastewater treatment, seems to harbor enormous bacteria coliforms. Despite both Kipkenyo boundary and Huruma quarry wastewater treatment systems regularly monitored and treated by the Eldoret Water and Sanitation company (ELDOWAS), a higher fecal coliform load was observed in Kipkenyo boundary effluent than the effluent site of Huruma quarry. The variation observed implies that there is effective wastewater treatment processes carried out at Huruma quarry than Kipkenyo, perhaps due to regular use of chemicals including chlorines and other disinfectants. On top of that, Kipkenyo is located in rural settings, having residents whose affordability for the essential resources is low compared to the people employed or carry out different businesses within or nearby Eldoret city. This enable them to afford better sanitary facilities and clean water, reducing fecal and wastewater pollution in the environment, thus maintaing good hygiene. Whereas the increased rates of poverty for people in Kipkenyo might prevent them from affording sanitary facilities and reliable portable water, significantly contributing to large quantities of fecal coliforms obtained in Kipkenyo. High count of total and fecal coliforms detected at the Moi Teaching and Referral Hospital waste discharge screen site might be originating either from the operations of the commercial centers surrounding the hospital or perhaps the medical wastes do not fully undergo proper and regular treatments before discharge. A recent study by Aziz *et al*., (2021) detected higher total coliforms ($40x10^4$ CFU/100ml) than fecal coliforms (10^4 CFU/100ml) from hospital effluents, stating that hospital wastes are regularly discharged into municipal sewers before undergoing proper treatments, corresponding with the current observation in this study.

The study highlights the culture-based method and biochemical tests as the golden and current microbiology standard techniques effective in the isolation and identification of various gram-negative bacteria species from rivers and wastewater systems, consistent with their relevant uses in vast research studies (Chauhan & Jindal, 2020; Shoaib *et al*., 2020; Mahmud *et al*., 2023). A total of ten (10) gram-negative pathogenic bacteria species were found. These were *Escherichia coli, Enterobacter aerogenes, Citrobacter freundii, Klebsiella pneumoniae, Klebsiella oxytoca, Proteus vulgaris, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella enteriditis* and *Yersinia enterocolitica*. Although, gram positive bacteria also do exist in water systems (Sarkar *et al*., 2019; Kamatham *et al*., 2024), they were outside the scope of this study, hence were not considered. Gram-negative bacteria are widely known for spreading bacterial infections to hosts, which are difficult to treat using conventional antibiotics (Zabawa *et al*., 2016). Therefore, it is expected that these pathogenic bacteria also acquire high rate of resistance from the environment. Most of these gram-negative bacteria detected in this study, were similarly reported by other similar studies to have dominated wastewater systems (Verburg *et al*., 2021) and other environments (Agga *et al*., 2015; Prashanthi *et al*., 2021).

The present investigations revealed that among the ten isolated bacteria species, *E. coli* and *E. aerogenes* were the frequently detected bacteria species in all the nine sites. In most of occasions, *E. coli* causes gastrointestinal (diarrhea) and extraintestinal infections (Urinary Tract Infections (UTIs), pneumonia and meningitis while *E. aerogenes* cause nosocomial infections which include UTIs, lower respiratory tract infections, and septicemia (Girlich *et al*., 2020; Maghembe *et al.*, 2024). Therefore, high bacterial species count detected from all the nine points, predicts the public health risks to the residents of Uasin Gishu County, especially the people and livestock that directly use water from River Sosiani. Isolates of *E. coli, E. aerogenes* and *C. freundii* were found in water under all the three bridges sampled, which were; Kipkaren bridge, Pioneer bridge and Outspan-Nairobi bridge along River Sosiani. Of the three bridges along River Sosiani that were sampled, Kipkaren bridge and Outspan-Nairobi bridge sites also had high concentrations of *K.*

pneumoniae and *K. oxytoca*. Different species of bacteria obtained at the sited bridges could be those inevitably down washed and drained into River Sosiani through surface run offs. Other activities from the surrounding establishments, such as guest house operations, car wash sites, petrol filling station around the Pioneer bridge, heavy transport along highways of Outspan-Nairobi bridge and Kipkaren bridge could be the one contributing to high number of bacteria found at the sampled bridges. Additionally, Kipkaren bridge is also surrounded with various motor vehicle service workshops, municipal market and light industries, although they may not directly contribute bacteria to the environment, but their activities may partly linked with bacterial contamination in River Sosiani and release of other toxic substances (Sejkora *et al*., 2011; Mammo *et al*., 2020; Tuvo *et al*., 2023), that trigger antibiotic resistance in bacteria. Presence of bacteria loads at the sea crossing bridges (Li *et al*., 2022), other microbes from the pedestrian bridges in the urban environments (Al-Shaarani *et al*., 2023) and those from the surface landscape leaking into the rivers (Verhougstraete *et al*., 2015) have been severally reported. The study suggests that bridges receive a lot of bacteria emanating from human traffic including waste carriers, tins, packages and plastic bottles that are indiscriminately dumped around, heavy transport vehicles, domestic users who wash and bath directly in the water under these bridges. Although these sources are least documented, they might be possible potential sources of pathogenic bacteria in rivers associated with increased the public health risk of using the water from River Sosiani.

This study showed that untreated wastes had more bacteria species than the treated wastes. Similar isolates of *E. coli, E. aerogenes, K. pneumoniae, K. oxytoca* and *P. vulgaris* were both detected in untreated and treated wastes from wastewater treatment sites, indicating that current treatment methods used do not fully eliminate bacteria from waste water or perhaps there is vigorous regrowth of bacteria after treatment process. Nevertheless, there were more pathogenic bacteria species recorded at the influents than effluent sites, suggesting that waste water treatment systems of Huruma quarry and Kipkenyo boundary often receive raw wastes from urbanization, domestics, animal husbandry and agricultural which contain large bacterial count. Notably, after treatment process, the bacterial count seems to reduce, accounting for fewer bacteria observed at the effluent sites. For instance, at the influent points, *E. coli, E. aerogenes, P. mirabilis, P. aeruginosa* and *S. enteriditis* were detected in both wastewater treatment systems (WWTS), while *K. pneumoniae* was the only isolate observed dominating the effluents of wastewater treatments systems.

Globally, the current applied disinfection technologies in most waste water treatment systems are chlorination, ultraviolet (UV) radiation and ozonation (Barbosa *et al*., 2021). Both Huruma quarry and Kipkenyo boundary wastewater treatment systems use naturally occurring aerobic bacteria to remove the biological contaminants and disinfect the remaining influxes with chlorine before being discharged into River Sosiani. The study observed that Huruma quarry influent had five bacteria species but after treatment process, only three (*E. coli, E. aerogenes and K. pneumoniae*) were detected at its effluent point. This shows that though, action by aerobic bacteria coupled by chlorine destroys some pathogenic bacteria species (*P. mirabilis, P. aeruginosa and S. enteriditis*), it is not effective against some bacteria (*E. coli* and *E. aerogenes*). This as well suggest these bacteria species are resistant to the natural methods of waste water treatment used and may therefore require the use of stronger antibiotics, or more innovative natural remedies that are not only stronger against bacteria, but also environmentally friendly. Barbosa *et al*., (2021) also reported *E. coli* that were found at the influent and effluent sites of the two different WWTS to be resistant to chlorination, which complies with this work. Furthermore, the study suggests that chlorination only is inefficient disinfection method in WWTS, hence it should be coupled with ultra violet (UV) radiation and ozonation (Manaia *et al*., 2018), that penetrates to disrupt bacteria cells and degrade DNA ensuring complete death and elimination of the bacteria. The treated medical wastes from MTRH and untreated wastes from Eldoret prison had *E. coli, P. vulgaris, P. aeruginosa, S. enteriditis* and *Y. enterocolitica*. Worldwide, steam sterilization via autoclaving, incineration, plasma gasification, microwave methods among others are recommended and often used in treatment of medical wastes (Kenny & Priyadarshini, 2021; Mazzei & Specchia, 2023). Moi Teaching and Referral Hospital often uses the incineration method (Njagi *et al*., 2012) which is the most treatment method of medical wastes including those bandages from wounds, syringes, needles, plastic bottles used to store medicines and syrups, cotton wool, gloves, spirit and associated disinfectants. Incineration seems to be a powerful technique operated at extremely high temperatures hence effective in killing the pathogenic bacteria in wastes. Therefore, presence of *E. coli, E. aerogenes, K. oxytoca* and *P. vulgaris* in MTRH waste screen site are likely generated from the neighboring hotels, car wash and other commercial enterprises, hence increasing pathogenic bacteria at the site.

High detection of *S. enteriditis* and *Y. enterocolitica* was recorded in Moi Teaching and Referral Hospital (MTRH) and Eldoret prison. These bacteria have been reported to be associating with poultry, slaughter houses and livestock products (Inns *et al.,* 2017; Zadernowska & Chajęcka-Wierzchowska, 2017; Younis *et al*., 2019). Their detection in the current study seems to suggest that these MTRH and Eldoret prison are constantly receiving large amounts of effluents from poultry, slaughter houses and other animal husbandry sites. MTRH waste disposal screen is surrounded by many residents and located adjacent to the big hotel that serves many people within and outside the hospital. It is possible that the wastes from the slaughtered poultry and meat products get offloaded into the nearby MTRH disposal screen site. Eldoret prison appears to be keeping livestock and poultry, whose wastes (meat products and manure) in addition to those from the neighboring Eldoret GK primary and secondary schools, get disposed into the site. Therefore, contributing to the existence of *S. enteriditis* and *Y. enterocolitica* in the environment. Their presence may predispose residents to infections of zoonotic diseases including the veterinaries, meat consumers, slaughter workers and other subjects handling livestock and poultry products in MTRH and Eldoret prison, which may further spread to other regions. Studies have also reported outbreak of bacterial infections in various schools and prisons caused by *E. coli*, *S. enteriditis* and *Y. enterocolitica* (Greig *et al*., 2011; MacDonald *et al*., 2016; El-Kased & Gamaleldin, 2020). Despite the general improvement of the conditions and facilities in Kenyans' prisons since 2015, congestion due to large number of inmates is still common and seems to contribute significantly to the pathogenic bacteria in the pretreated wastes.

5.2 Antibiotic susceptibility test

The current study reports that *E. coli* was susceptible to doxycycline, ciprofloxacin, gentamycin, and intermediate susceptible to chloramphenicol. Similar work by Gaspar *et al*., (2021) also demonstrates that *E. coli* isolates from wastewater indicated susceptibility to ciprofloxacin and gentamycin. Therefore, these antibiotics seem to be effective in inhibiting the *E. coli* growth in the water systems. This study documents that doxycycline and ciprofloxacin are effective in inhibiting the growth of *E. aerogenes* while ciprofloxacin, doxycycline and gentamycin inhibit the growth of *C. freundii*. The results correlate with study of Kamatham *et al*., (2024) who reported that both *E. aerogenes* and *C. freundii* from waste water samples were susceptible to ciprofloxacin. Thus, ciprofloxacin is a strong antibiotic suggested to be effective in clearing *E. aerogenes* and *C. freundii* isolated from aquatic systems. *Klebsiella pneumoniae* was susceptible to gentamycin, azithromycin and doxycycline, and this was compared to Setiawan *et al*., (2022) study which also investigated the susceptibility of *K. pneumoniae* to ciprofloxacin and doxycycline. The study showed that *K. oxytoca* were susceptible to ciprofloxacin, gentamycin and chloramphenicol. Studies by Sekowska *et al*., (2011) and Sing *et al*., (2016), indicated that *K. oxytoca* were also susceptible to ciprofloxacin, corresponding to the current findings. Also, Subedi *et al*., (2016) work revealed that both *K. oxytoca* and *K. pneumoniae* from clinical samples were susceptible to gentamycin. This comparison highlights that risks associated with *K. pneumoniae* and *K. oxytoca* loads from river basins and wastewater systems might be effectively reduced using the tested antibiotics.

In addition, this study states that *P. vulgaris* growth seems to be fully inhibited by use of doxycycline, ciprofloxacin and less inhibited by azithromycin. *Proteus mirabilis* growth could be highly inhibited by doxycycline, gentamycin and less inhibited by ciprofloxacin. This is supported by Zappa *et al*., (2017) study, which also detected isolates of *P. vulgaris* and *P. mirabilis* being susceptible to gentamycin, ciprofloxacin and azithromycin. Investigation by Serry *et al*., (2018) showed intermediate susceptibility of *P. mirabilis* to ciprofloxacin. The similarity in the results indicates that rivers and wastewater systems in

Kenya and other parts of the global, seem to receive intensive wastes from different sources constituting species of *P. vulgaris* and *P. mirabilis*, and these species are suggested to be eliminated from such environments using doxycycline, ciprofloxacin, gentamycin and azithromycin. *Pseudomonas aeruginosa* were reported to be intermediate susceptible to ciprofloxacin, chloramphenicol and doxycycline. Consistent with other studies, *Pseudomonas aeruginosa* species were reported being susceptible to ciprofloxacin, vancomycin, chloramphenicol, ceftazidime and gentamycin (Teshome *et al*., 2020; Mahmud *et al*., 2023). Management of *Pseudomonas aeruginosa* in the environment may be efficiently and frequently achieved using more of such antibiotics.

This work demonstrated that the growth of *S. enteriditis* in the environment seems to be strongly inhibited by using ciprofloxacin and gentamycin and less inhibited by chloramphenicol. In comparison to some results, the isolates of *S. enteriditis* from underground water sources were susceptible to ciprofloxacin, gentamycin and tetracycline (Hafiane *et al*., 2024). The study shows that *S. enteriditis* is capable of dominating different water sources and could be managed differently, depending on the frequency and strength of the antibiotics. This study reported species of *Y. enterocolitica* being susceptible to ciprofloxacin, azithromycin, gentamycin, chloramphenicol, doxycycline and intermediate susceptible to sulfamethoxazole-trimethoprim, tetracyclines and amoxicillin-clavulanic acid. Similar study found *Y. enterocolitica* being susceptible to ciprofloxacin, chloramphenicol and gentamycin (Roulová *et al*., 2022). This implies that these antibiotics seem to have a higher inhibitory effect against the growth of *Y. enterocolitica* released from various wastes into different water systems around the world. The study documents that variations in the degrees of antibiotic susceptibility levels observed among the 10 bacteria species, perhaps might be influenced by geographical locations and physiological conditions of different water habitats (Malagi *et al*., 2020; Gwenzi *et al*., 2023).

The study demonstrated that *E. coli* was resistant to azithromycin, tetracycline, sulfamethoxazole-trimethoprim, penicillin, ampicillin, ceftazidime, amoxicillin-clavulanic acid and streptomycin. A similar study also reported *E. coli* isolates being resistant to ampicillin, amoxicillin-clavulanic acid and ceftazidime (Asfaw *et al*., 2017). Other studies also detected *E. coli* from the hospital wastewater being resistant to ampicillin, ceftazidime, sulfamethoxazole-trimethoprim (Gaşpar *et al*., 2021), penicillin and tetracyclines (Odonkor & Addo, 2018). Penicillin and its derivatives, amino glycosidic antibiotics, tetracyclines, sulfonamides and cephalosporins, seem to be reducing their strength in the inhibition of *E. coli* growth in various water sources, which could be the reason for the similarity in the resistance reported by other studies. Both *E. aerogenes* and *C. freundii* were resistant to azithromycin, chloramphenicol, tetracycline, sulfamethoxazole-trimethoprim, penicillin, ampicillin, ceftazidime, amoxicillin-clavulanic acid and streptomycin exempt the gentamycin, whose resistance was only observed in *E. aerogenes*. Resistance of *E. aerogenes* and *C. freundii* to several antibiotics is variously reported, being resistant to ceftazidime (Sravani *et al*., 2024), ampicillin (Davin-Regli & PagÃ¨s, 2015), amoxicillin- clavulanic acid and cotrimoxazole (Metri *et al*., 2013). *Citrobacter freundii* was also reported conferring resistance to gentamycin and sulfamethoxazole-trimethoprim (Njoya *et al*., 2021). The consistency in the current results and the other researches suggests that the two bacteria species have developed resistance against a wide range of the tested drugs, rendering them ineffective in the clearing of *E. aerogenes* and *C. freundii*.

High resistance rates were also observed in *K. pneumoniae*, showing resistance to ciprofloxacin, chloramphenicol, tetracycline, sulfamethoxazole-trimethoprim, penicillin, ampicillin, ceftazidime, amoxicillin-clavulanic acid and streptomycin. Also, *K. oxytoca* demonstrated resistance to azithromycin, tetracycline, sulfamethoxazole-trimethoprim, penicillin, ampicillin, ceftazidime, amoxicillin-clavulanic acid and streptomycin. Research work by Subedi *et al*., (2016) detected both *K. oxytoca* and *K. pneumoniae* from clinical samples, being resistant to ampicillin, ceftazidime and ciprofloxacin, which partially agrees with the current observation. Also, the two species acquired resistance to ampicillin, which is similarly reported by Chakraborty *et al*., (2016) and Belachew *et al*., (2018). The findings suggest that *K. oxytoca* and *K. pneumoniae* isolated from various water systems in different geographical sites have adopted strong defense mechanisms to evade the effects of the tested antibiotics.

In this study, *P. vulgaris* demonstrated resistance against chloramphenicol, gentamycin, tetracycline, sulfamethoxazole-trimethoprim, penicillin, ampicillin, ceftazidime, amoxicillin-clavulanic acid and streptomycin. Corresponding study by Ibrahim *et al*., (2023) also detected *P. vulgaris* resistance towards ampicillin, amoxicillin-clavulanic acid, gentamycin, tetracyclines and ciprofloxacin. This indicates the need to recheck the efficacy of these antibiotics to enhance *P. vulgaris* growth inhibition. Resistance in *P. mirabilis* was shown against chloramphenicol, azithromycin, tetracycline, sulfamethoxazole-trimethoprim, penicillin, ampicillin, ceftazidime, amoxicillin-clavulanic acid and streptomycin. These results comply with a study which found *P. mirabilis* being resistant to ampicillin, tetracyclines, chloramphenicol and sulfamethoxazole-trimethoprim (Serry *et al*., 2018). The varying levels of antibiotic resistance in *P. mirabilis* from aquatic ecosystems is suggested to be boosted by the presence of antibiotic residues accumulating in that environment.

This work detected that *P. aeruginosa* conferred resistance to gentamycin, azithromycin, tetracycline, sulfamethoxazole-trimethoprim, penicillin, ampicillin, ceftazidime, amoxicillin-clavulanic acid and streptomycin. A similar study by Nasreen *et al*., (2015) revealed that *P. aeruginosa* were resistant to ampicillin, tetracycline and gentamycin. Also, Asmare *et al*., (2024) findings stated to have found *P. aeruginosa* resistance to sulfamethoxazole-trimethoprim. It seems that *P. aeruginosa* interacts with these antibiotics residues in water systems, hence fostering the resistance observed. This study demonstrated *S. enteriditis* expressing resistance to doxycycline, azithromycin, tetracycline, sulfamethoxazole-trimethoprim, penicillin, ampicillin, ceftazidime, amoxicillin-clavulanic acid and streptomycin. A recent study has also reported *S. enteriditis* resistance to ampicillin, amoxicillin-clavulanic acid, penicillin and ceftazidime, corresponding with this study (Hasan *et al*., 2020). The mentioned antibiotics seem to be ineffective in inhibiting the growth of *S. enteriditis*, due to wide resistance gained against these antibiotics. This might be caused by intensive interaction of the species with antibiotic resistance inducers accumulating in rivers and wastewater systems a result of drug misuse, overuse and poor hygiene. When bacteria encounter particular antibiotics for several times (Rodriguez-Mozaz *et al*., 2015; Zhang *et al*., 2020), it develops resistance mechanisms such as inactivation of the antibiotics, limiting drug uptake, modification of drug targets and extrusion of antibiotics using efflux pumps (Varela *et al*., 2021), which end up evading the antibiotic effects hence emergence of bacteria resistance to antibiotics.

The isolates of *Y. enterocolitica* conferred resistance to tetracycline, sulfamethoxazole-trimethoprim, penicillin, ampicillin, ceftazidime, amoxicillin-clavulanic acid and streptomycin. Corresponding studies also reports increased rates of *Y. enterocolitica* resistance to ampicillin (Thong *et al*., 2018), amoxicillin-clavulanic acid and streptomycin (Odyniec *et al*., 2020). This implies that *Y. enterocolitica* isolated from water systems in distinct regions might have undergone some mutations to form new strains either through changes in environmental conditions or overexposure to different chemicals and pollutants, which trigger the bacteria to be resistant against the commonly used antibiotics. All the 10 bacteria species inclusive of *E. coli, E. aerogenes, C. freundii, K. pneumoniae, K. oxytoca, P. vulgaris, P. mirabilis, P. aeruginosa, S. enteriditis* and *Y. enterocolitica* recorded the highest MAR Index (greater than 0.2, a value recommended by WHO). This showed the isolated bacteria were resistant to several of the tested antibiotics. Suggesting the possibility of high risks associated with antibiotic residue contamination of River Sosiani and wastewater systems, from which the bacteria were isolated.

5.3 Antibiotic-resistant genes in plasmids of the isolated Bacteria

The findings of the current research demonstrated the presence of pentapeptide encoded quinolone (*qnrA*) and efflux pump resistance genes (*qepA*) in *E. coli, E. aerogenes, C. freundii, K. pneumoniae, K. oxytoca, P. vulgaris, P. mirabilis, P. aeruginosa, S. enteriditis* and *Y. enterocolitica*. *QnrA* gene mediates resistance to quinolones through limiting drug uptake via reduction of porins (Hooper & Jacoby, 2015). *QepA* is an efflux pump gene that extrudes quinolones from the bacterial systems before releasing their treatment effects (Elshamy *et al*., 2020). The results concurred with the previous research by Yan *et al*., (2017), who found that the *qepA* and *qnrA* genes were acquired by the most of the bacteria species from aquatic environment. In addition, findings from Ayobola's study in Delta state, Nigeria revealed to have detected 10% occurrence of plasmid-mediated quinolone resistant genes (*qnrA, qnrB, qnrS* and *qepA)* in *E. coli, Salmonella, K. pneumoniae* and *E. clocae,* which was lower detection frequency compared to the current results (Ayobola *et al*., 2021). This suggests that bacteria have evolved intensive resistance mechanisms against quinolone class of antibiotics, thus should be routinely tested to determine their efficacy. The study detected AadA*1* gene (70%) in *E. coli, E. aerogenes, C. freundii, K. pneumoniae, P. vulgaris, P. aeruginosa* and *S. enteriditis*. The gene induces resistance in bacteria through production of aminoglycosidic transferase enzymes (Garneau-Tsodikova & Labby, 2016), which inactivates aminoglycosidic antibiotics. The results comply with Rubab *et al*., (2021) who also detected 70% of *aadA1* gene in *E. coli*. This demonstrates increased bacteria resistance to aminoglycosidic antibiotics including the tested streptomycin, where *aadA1* gene was present in *E. coli*, *E. aerogenes, C. freundii, K. pneumoniae, P. vulgaris, P. aeruginosa, S. enteriditis* and gentamycin, *aadA1* gene being detected in *E. aerogenes P. vulgaris* and *P. aeruginosa.*

From the current results, carbapenem resistance via bla_{KPC} gene was observed in *E. coli, E. aerogenes, C. freundii, P. vulgaris* and *Y. enterocolitica*. Bla_{KPC} gene confers resistance via production of carbapenemases (Meenakshisundaram, 2013) which hydrolyzes carbapenems, resulting to resistance. The current results correspond to Yao *et al*., (2024) findings which expressed 45% of bla_{KPC} resistance in *E. coli, C. freundii, K. pneumoniae, K. oxytoca* and *P. mirabilis,* which was less abundance to the value detected in this work (50%). The results on AST showed that the mentioned bacteria were resistant to ceftazidime, which could have fostered the evolution of bla_{KPC} genes in those bacteria.

Thus, carbapenems and relevant derivatives should be properly designed to enhance fully clearance of the carbapenem resistant bacteria.

This study detected *mphA* gene in 40% of the total bacteria, namely, *E. coli*, *E. aerogenes*, *C. freundii* and *K. pneumoniae*. *MphA* gene foster resistance to macrolides through inducing other target mutations in bacteria (Zhang *et al*., 2024). A study work by Xiang *et al*., (2020) also expressed more azithromycin resistance (79%) triggered by *MphA* gene in *E. coli* thus complying with the current observation. Same bacterial species indicated the trending levels of intermediate susceptibility to the tested azithromycin. It was noted that, the PCR is an effective technique capable of detecting ARGs, even on the plasmids of the bacteria with intermediate susceptibility patterns, for instance, the intermediate susceptibility of *K. pneumoniae* to azithromycin. In addition, Bla_{SHV} gene was also screened at 30%, in *E. coli, E. aerogenes* and *C. freundii*, higher than the 2.7% occurrence of bla_{SHV} gene reported in *E. coli* and *K. pneumoniae* by Ibraham *et al*., (2021). It seems that bacteria dominating rivers and wastewater sites such as *E. coli*, *E. aerogenes* and *C. freundii*, are rapid developing resistance towards several β lactam antibiotics via production of β lactamases (Elshamy *et al*., 2020), inactivating antibiotics such as penicillin, ampicillin, and amoxicillin clavulanic acid, similarly as reported by the current results on antibiotic susceptibility test in bacteria.

Trimethoprim resistance gene (*dfrA5* gene) was discovered in 70% of the total bacteria species count including *E. coli, E. aerogenes, K. pneumoniae, K. oxytoca, P. mirabilis, P. aeruginosa* and *S. enteriditis* while sulfonamide resistance genes (*sul 1* gene) was identified in 30% of species, being *E. coli, E. aerogenes* and *C. freundii*. Notably, the current results on antibiotic susceptibility test also showed sulfamethoxazole-trimethoprim resistance to the same bacteria species of *E. aerogenes, C. freundii, K. oxytoca, P. mirabilis* and *P. aeruginosa*. *DfrA5* gene triggers resistance through modification of dihydrofolate reductase (Jalilian & Hosainzadegan, 2014) while *sul 1* gene mediates resistance in bacteria by changing the enzymic and metabolic pathways in bacteria that interferers with dihydropteroate synthase activity (Jalilian & Hosainzadegan, 2014). A study by Sabri *et al*., (2020) reports *sul1* genes in bacteria species from the wastewater samples and Suhartono *et al*., (2016) study detected *dfrA5* genes at 37%, which was less than that trimethoprim abundance obtained in this study (70%). In concurrence with the results shown, multi antibiotic-resistant (MAR) *E. coli* isolates retrieved from human and other environment samples acquired uneven distribution of ARGs on their plasmids including 18% of *sul1* gene, 27% of *mphA* and 17% of *dfrA5*, that were low values compared to the ones detected in this study (30%, 40% and 70% respectively) (Stephens *et al*., 2020). Globally, this shows the existence of increased levels of bacteria resistance to the sulfonamides and trimethoprim, evolving from the water systems (Gao *et al*., 2012; Suzuki *et al*., 2015). It is much worrying, especially to the public health since sulfamethoxazole-trimethoprim are gradually becoming ineffective due to the widespread of their resistance from the aquatic environments. The results on antibiotic susceptibility testing in bacteria indicated that all the 10 bacteria species were resistant to ampicillin but none of the bacteria contained *ampC* gene upon PCR analysis. It is possible that the ampicillin resistance gene (*ampC*) might have been present on the genomic DNA of the isolated bacteria (intrinsic resistance) however the study targeted ARGs on the plasmid DNA of the isolated bacteria species (extrinsic resistance).

In summary, eight (8) antibiotic-resistant genes were detected on the plasmids of *E. coli*, *E. aerogenes*, *C. freundii*, *K. pneumoniae*, *K. oxytoca*, *P. vulgaris*, *P. mirabilis*, *P. aeruginosa*, *S. enteriditis* and *Y. enterocolitica* isolated from the aquatic sites. These included *qnrA* and *qepA* genes mediating resistance of bacteria against quinolones, *dfrA5* gene for trimethoprim resistance, *aadA1* gene for aminoglycosidic resistance, *mphA* gene encoding macrolide resistance, *blaSHV* gene inducing resistance of bacteria against β-lactams, *blaKPC* gene triggering carbapenem resistance and *sul1* gene mediating sulfonamide resistance in distinct bacteria.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The study detected ten (10) gram-negative bacteria species from River Sosiani and wastewater systems which included; *Escherichia coli, Enterobacter aerogenes, Citrobacter freundii, Klebsiella pneumoniae, Klebsiella oxytoca, Proteus vulgaris, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella enteriditis* and *Yersinia enterocolitica*. These bacteria are pathogenic; hence being a potential threat to the life of humans and animals since they are capable of spreading severe bacterial infections, which often require costly medical interventions.

Most bacterial species isolated in this study were susceptible to ciprofloxacin, doxycycline, gentamycin and chloramphenicol and intermediate susceptible to azithromycin, indicating that these antibiotics have high inhibitory effects against the growth of gram-negative bacteria species. Bacteria species that find their ways into the River Sosiani and wastewater systems, have developed resistance to tetracycline, ceftazidime, penicillin, ampicillin, amoxicillin-clavulanic acid, streptomycin and sulfamethoxazole-trimethoprim. This might increase the prevalence for the outbreak of multi drug resistant infections and more deaths to communities within Uasin Gishu, Kenya.

This study demonstrates that there is intensive horizontal transfer of plasmids carrying genes such as the detected *qnrA*, *qepA*, *aadA1*, *mphA*, bla_{KPC}, bla_{SHV}, *dfrA5* and *sul 1* genes, inducing resistance of bacteria in aquatic environments against quinolones, aminoglycosidic, macrolides, carbapenems, β-lactams, trimethoprim and sulfonamides.

This is due to the consistent interaction of bacteria with antibiotic resistance inducers in the environment. The detected bacteria with ARGs are dangerous, since they are likely to increase public health risks of spreading antibiotic resistance hence causing infections of grave consequences to people and other subjects interacting with these sites.

6.2 Recommendations

According to the findings of the current study, the following recommendations were highlighted;

1. Water from River Sosiani should be considered unsafe for human consumption, animal and farmland activities, since it poses health risks of contacting waterborne diseases associated with the detected pathogenic bacterial loads and therefore, it is recommended for continuous monitoring and appropriate pretreatments before usage.
2. Public and environmental health sectors should consider reevaluating the efficacy of commonly used antibiotics especially the ones indicated low inhibitory effects against the growth of bacteria. This will prevent the rising cases of bacteria resistance to commonly used antibiotics from the different water systems, especially in the developing parts of the world.
3. There is need for the County Government to enforce environment laws on wastewater disposal and treatments within the city, to ensure none of the antibiotic resistance inducers accumulate in water systems. This will reduce prevalence of AMR rising in pathogenic bacteria, hence safeguarding human, animal and ecosystem health.

4. For future studies;

    a) Apart from bacterial resistance against antibiotics, further researches should be carried out from different waterways to determine other microbial resistance to antibiotics inclusive of fungi, viruses and protozoans. This will help to fully understanding the whole spectrum of resistance and develop comprehensive strategies to combat various challenges associated with antimicrobial resistance.

    b) To enhance genetic diversity for the detection of antibiotic resistance gene occurrence, advanced comparable studies to determine chromosomal mediated ARGs from clinical and environmental bacteria isolates are also required in order to fully generate adequate ideal data, that will assist in combating the widespread of AMR.

    c) More investigations are required to check antibiotic residues, chemicals and other environmental factors associated with triggering drug resistance in bacteria from the sampled aquatic sites. With the diverse data, appropriate strategies of combating AMR will be generated.

    d) A study on proteomics should be done to elucidate the structures and functions of different types of proteins encoded by the detected plasmid-mediated ARGs.

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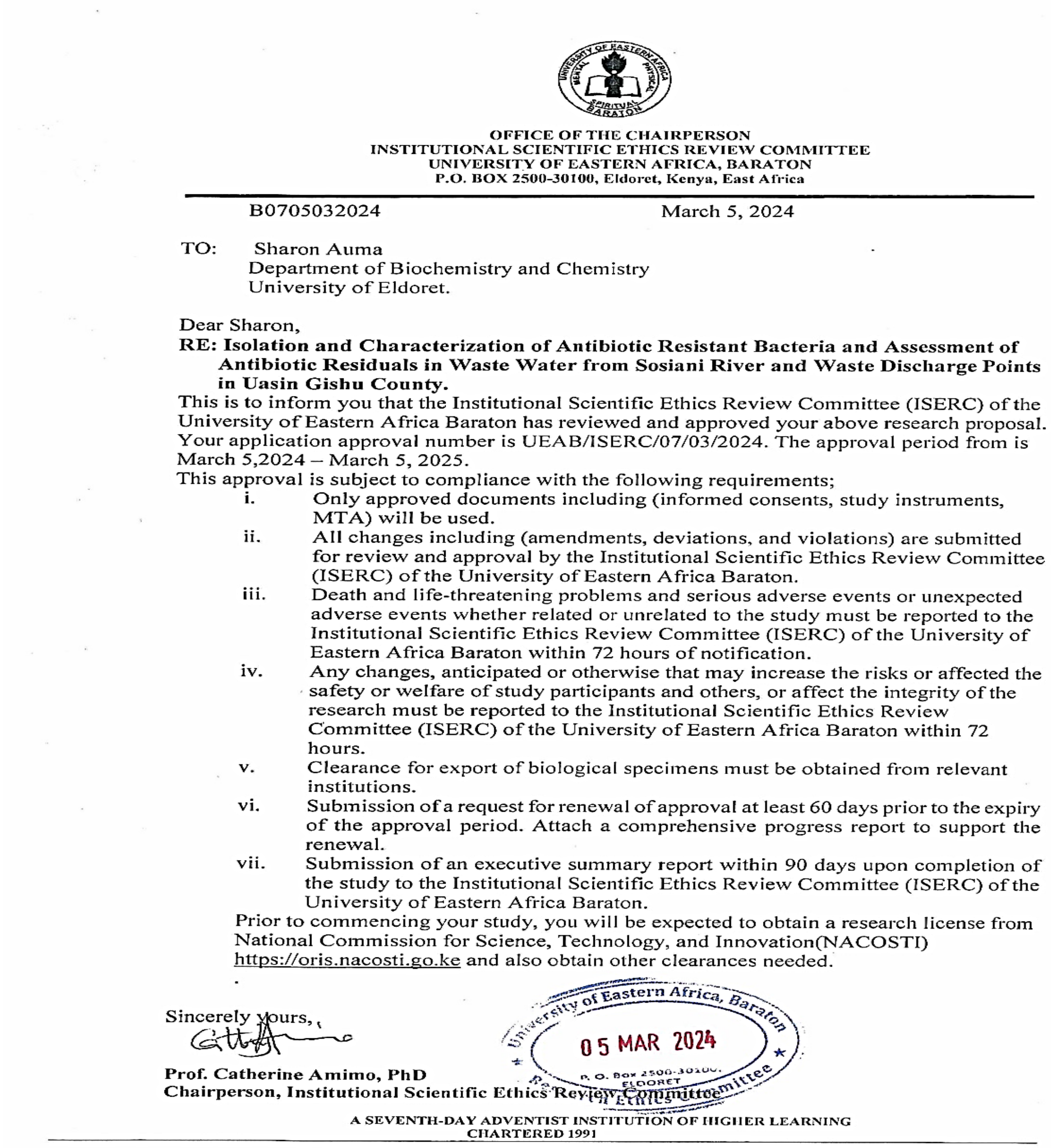
APPENDICES

Appendix I: Ethical approval letter



OFFICE OF THE CHAIRPERSON
INSTITUTIONAL SCIENTIFIC ETHICS REVIEW COMMITTEE
UNIVERSITY OF EASTERN AFRICA, BARATON
P.O. BOX 2500-30100, Eldoret, Kenya, East Africa

B0705032024 March 5, 2024

TO: Sharon Auma
Department of Biochemistry and Chemistry
University of Eldoret.

Dear Sharon,

RE: Isolation and Characterization of Antibiotic Resistant Bacteria and Assessment of Antibiotic Residuals in Waste Water from Sosiani River and Waste Discharge Points in Uasin Gishu County.

This is to inform you that the Institutional Scientific Ethics Review Committee (ISERC) of the University of Eastern Africa Baraton has reviewed and approved your above research proposal. Your application approval number is UEAB/ISERC/07/03/2024. The approval period from is March 5,2024 – March 5, 2025.

This approval is subject to compliance with the following requirements;

i. Only approved documents including (informed consents, study instruments, MTA) will be used.
ii. All changes including (amendments, deviations, and violations) are submitted for review and approval by the Institutional Scientific Ethics Review Committee (ISERC) of the University of Eastern Africa Baraton.
iii. Death and life-threatening problems and serious adverse events or unexpected adverse events whether related or unrelated to the study must be reported to the Institutional Scientific Ethics Review Committee (ISERC) of the University of Eastern Africa Baraton within 72 hours of notification.
iv. Any changes, anticipated or otherwise that may increase the risks or affected the safety or welfare of study participants and others, or affect the integrity of the research must be reported to the Institutional Scientific Ethics Review Committee (ISERC) of the University of Eastern Africa Baraton within 72 hours.
v. Clearance for export of biological specimens must be obtained from relevant institutions.
vi. Submission of a request for renewal of approval at least 60 days prior to the expiry of the approval period. Attach a comprehensive progress report to support the renewal.
vii. Submission of an executive summary report within 90 days upon completion of the study to the Institutional Scientific Ethics Review Committee (ISERC) of the University of Eastern Africa Baraton.

Prior to commencing your study, you will be expected to obtain a research license from National Commission for Science, Technology, and Innovation(NACOSTI) https://oris.nacosti.go.ke and also obtain other clearances needed.

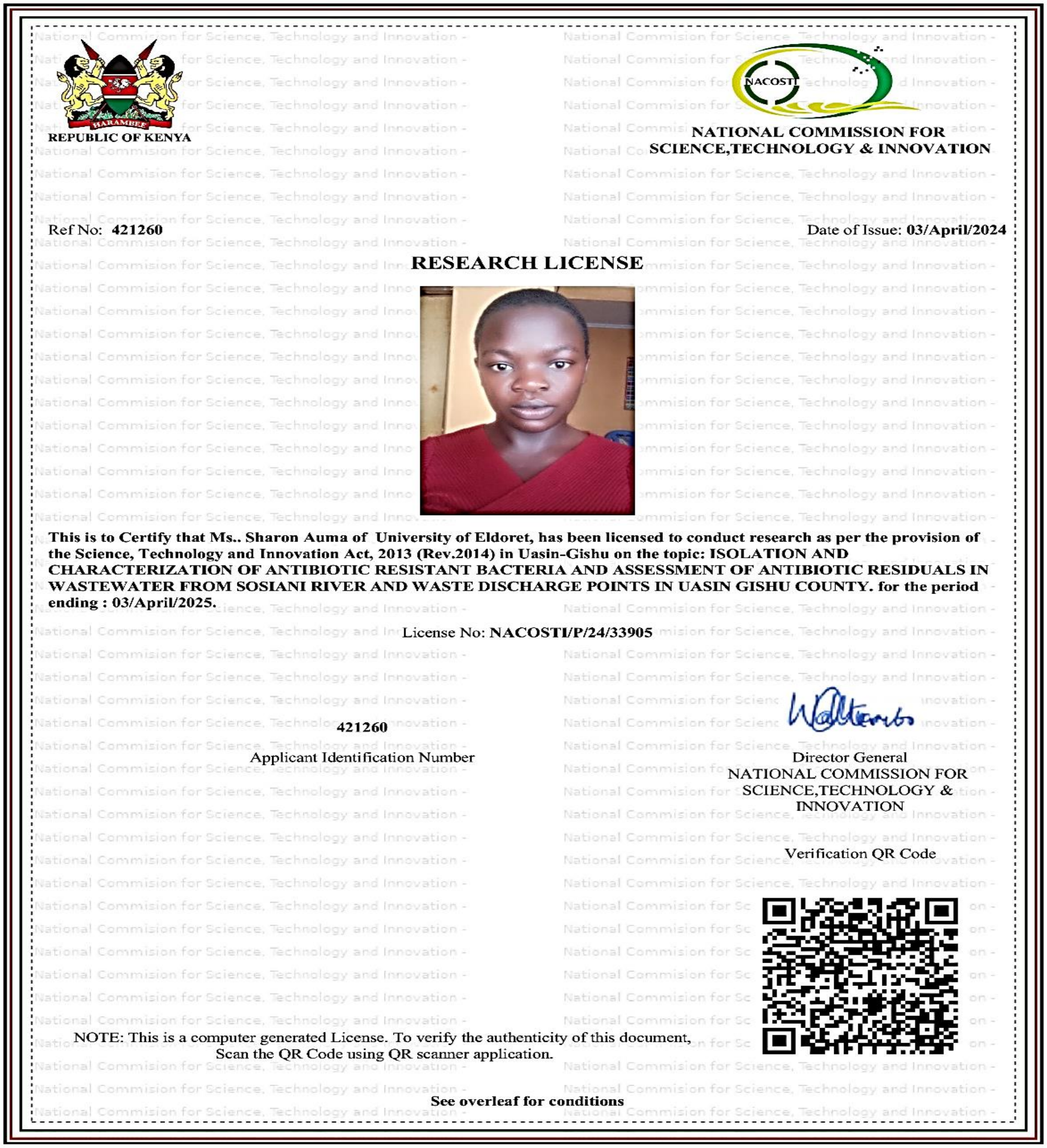
Sincerely yours,

05 MAR 2024

Prof. Catherine Amimo, PhD
Chairperson, Institutional Scientific Ethics Review Committee

A SEVENTH-DAY ADVENTIST INSTITUTION OF HIGHER LEARNING
CHARTERED 1991

Appendix II: NACOSTI Research Permit



REPUBLIC OF KENYA

NATIONAL COMMISSION FOR
SCIENCE,TECHNOLOGY & INNOVATION

Ref No: **421260**

Date of Issue: **03/April/2024**

RESEARCH LICENSE

This is to Certify that Ms.. Sharon Auma of University of Eldoret, has been licensed to conduct research as per the provision of the Science, Technology and Innovation Act, 2013 (Rev.2014) in Uasin-Gishu on the topic: ISOLATION AND CHARACTERIZATION OF ANTIBIOTIC RESISTANT BACTERIA AND ASSESSMENT OF ANTIBIOTIC RESIDUALS IN WASTEWATER FROM SOSIANI RIVER AND WASTE DISCHARGE POINTS IN UASIN GISHU COUNTY. for the period ending : 03/April/2025.

License No: **NACOSTI/P/24/33905**

421260
Applicant Identification Number

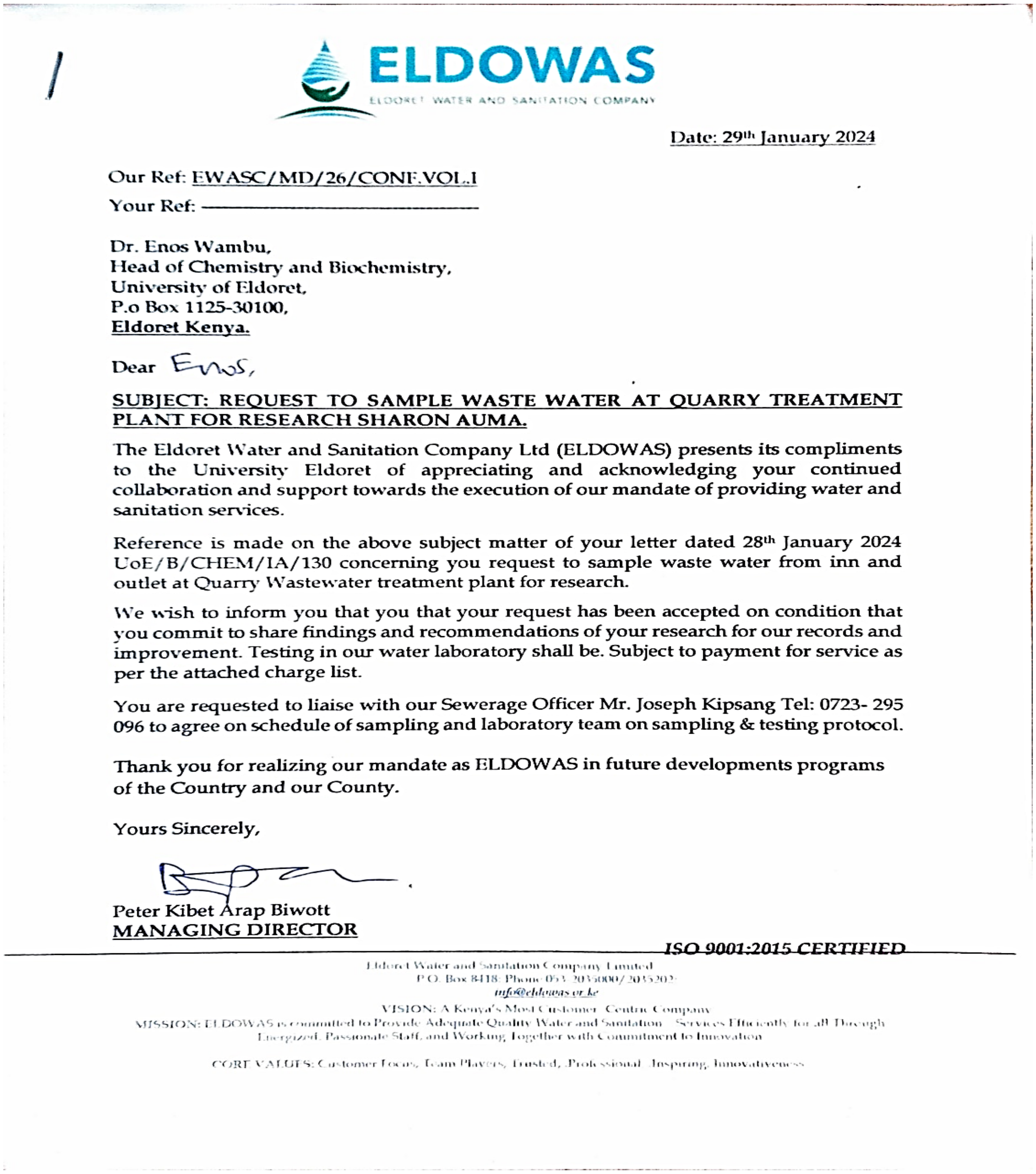
Director General
NATIONAL COMMISSION FOR
SCIENCE,TECHNOLOGY &
INNOVATION

Verification QR Code

NOTE: This is a computer generated License. To verify the authenticity of this document,
Scan the QR Code using QR scanner application.

See overleaf for conditions

Appendix III: Authorized access letter from Eldoret Water and Sanitation Company



ELDOWAS
ELDORET WATER AND SANITATION COMPANY

Date: 29th January 2024

Our Ref: EWASC/MD/26/CONF.VOL.I
Your Ref: ——————————————

Dr. Enos Wambu,
Head of Chemistry and Biochemistry,
University of Eldoret,
P.o Box 1125-30100,
Eldoret Kenya.

Dear Enos,

SUBJECT: REQUEST TO SAMPLE WASTE WATER AT QUARRY TREATMENT PLANT FOR RESEARCH SHARON AUMA.

The Eldoret Water and Sanitation Company Ltd (ELDOWAS) presents its compliments to the University Eldoret of appreciating and acknowledging your continued collaboration and support towards the execution of our mandate of providing water and sanitation services.

Reference is made on the above subject matter of your letter dated 28th January 2024 UoE/B/CHEM/IA/130 concerning you request to sample waste water from inn and outlet at Quarry Wastewater treatment plant for research.

We wish to inform you that you that your request has been accepted on condition that you commit to share findings and recommendations of your research for our records and improvement. Testing in our water laboratory shall be. Subject to payment for service as per the attached charge list.

You are requested to liaise with our Sewerage Officer Mr. Joseph Kipsang Tel: 0723- 295 096 to agree on schedule of sampling and laboratory team on sampling & testing protocol.

Thank you for realizing our mandate as ELDOWAS in future developments programs of the Country and our County.

Yours Sincerely,

Peter Kibet Arap Biwott
MANAGING DIRECTOR

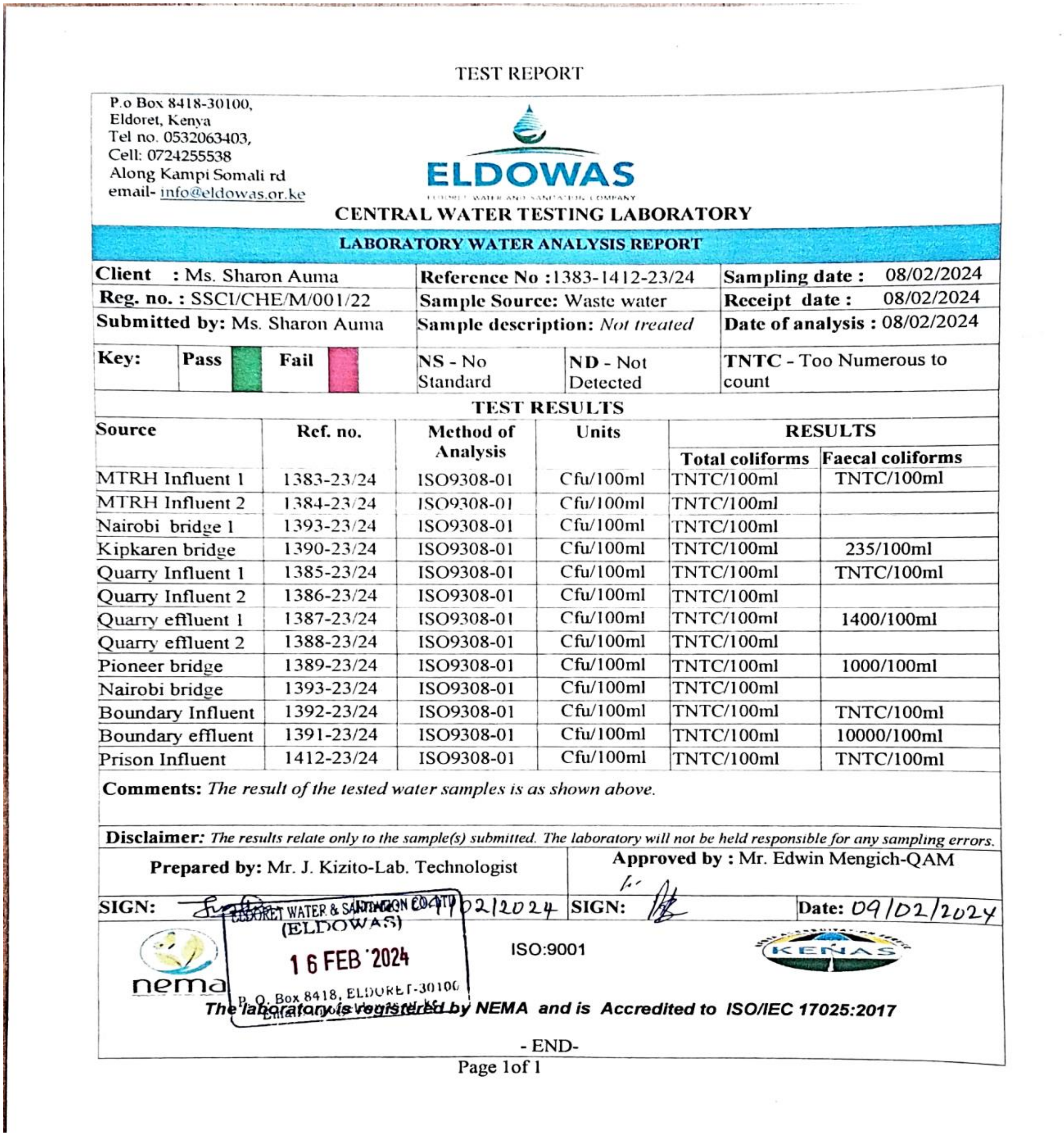
ISO 9001:2015 CERTIFIED

Eldoret Water and Sanitation Company Limited
P.O. Box 8418: Phone 053-2035000/ 2035202
info@eldowas.or.ke

VISION: A Kenya's Most Customer Centric Company
MISSION: ELDOWAS is committed to Provide Adequate Quality Water and Sanitation Services Efficiently for all Through Energized, Passionate Staff, and Working Together with Commitment to Innovation

CORE VALUES: Customer Focus, Team Players, Trusted, Professional, Inspiring, Innovativeness

Appendix IV: Laboratory results on general bacteriological culturing at the ELDOWAS, Kenya



TEST REPORT

P.o Box 8418-30100,
Eldoret, Kenya
Tel no. 0532063403,
Cell: 0724255538
Along Kampi Somali rd
email- info@eldowas.or.ke

ELDOWAS

CENTRAL WATER TESTING LABORATORY

LABORATORY WATER ANALYSIS REPORT

Client : Ms. Sharon Auma	**Reference No** :1383-1412-23/24	**Sampling date :** 08/02/2024
Reg. no. : SSCI/CHE/M/001/22	**Sample Source:** Waste water	**Receipt date :** 08/02/2024
Submitted by: Ms. Sharon Auma	**Sample description:** *Not treated*	**Date of analysis :** 08/02/2024

Key:	**Pass** 🟩	**Fail** 🟥	**NS** - No Standard	**ND** - Not Detected	**TNTC** - Too Numerous to count

TEST RESULTS

Source	Ref. no.	Method of Analysis	Units	RESULTS	
				Total coliforms	Faecal coliforms
MTRH Influent 1	1383-23/24	ISO9308-01	Cfu/100ml	TNTC/100ml	TNTC/100ml
MTRH Influent 2	1384-23/24	ISO9308-01	Cfu/100ml	TNTC/100ml	
Nairobi bridge 1	1393-23/24	ISO9308-01	Cfu/100ml	TNTC/100ml	
Kipkaren bridge	1390-23/24	ISO9308-01	Cfu/100ml	TNTC/100ml	235/100ml
Quarry Influent 1	1385-23/24	ISO9308-01	Cfu/100ml	TNTC/100ml	TNTC/100ml
Quarry Influent 2	1386-23/24	ISO9308-01	Cfu/100ml	TNTC/100ml	
Quarry effluent 1	1387-23/24	ISO9308-01	Cfu/100ml	TNTC/100ml	1400/100ml
Quarry effluent 2	1388-23/24	ISO9308-01	Cfu/100ml	TNTC/100ml	
Pioneer bridge	1389-23/24	ISO9308-01	Cfu/100ml	TNTC/100ml	1000/100ml
Nairobi bridge	1393-23/24	ISO9308-01	Cfu/100ml	TNTC/100ml	
Boundary Influent	1392-23/24	ISO9308-01	Cfu/100ml	TNTC/100ml	TNTC/100ml
Boundary effluent	1391-23/24	ISO9308-01	Cfu/100ml	TNTC/100ml	10000/100ml
Prison Influent	1412-23/24	ISO9308-01	Cfu/100ml	TNTC/100ml	TNTC/100ml

Comments: *The result of the tested water samples is as shown above.*

Disclaimer: *The results relate only to the sample(s) submitted. The laboratory will not be held responsible for any sampling errors.*

Prepared by: Mr. J. Kizito-Lab. Technologist	**Approved by :** Mr. Edwin Mengich-QAM
SIGN: 9/02/2024	**SIGN:** **Date:** 09/02/2024

ELDORET WATER & SANITATION CO. LTD
(ELDOWAS)
16 FEB 2024
P. O. Box 8418, ELDORET-30100

nema

ISO:9001

KENAS

The laboratory is registered by NEMA and is Accredited to ISO/IEC 17025:2017

- END-

Page 1of 1

Appendix V: A summary of break points/ cut off values of different bacteria species tested against specific antibiotics as per Clinical Laboratory Standard Institute guidelines (CLSI, 2023)

1. **Enterobacterales group** (*E. coli*, *E. aerogenes, C. freundii, K. pneumoniae, K. oxytoca, P. vulgaris, P. mirabilis, S. enteriditis* and *Y. enterocolitica)*

Antibiotics (µg)	**Susceptibility**	**Intermediate susceptibility**	**Resistance**
Ciprofloxacin (30)	≥26	22-25	≤21
Doxycycline (30)	≥14	11-13	≤10
Chloramphenicol (50)	≥18	13-17	≤12
Gentamycin (10)	≥18	15-17	≤14
Azithromycin (15)	≥16	11-15	≤10
Tetracycline (30)	≥15	12-14	≤11
Ceftazidime (30)	≥21	18-20	≤ 17
Streptomycin (10)	≥15	12-14	≤ 11
Ampicillin (10)	≥17	14-16	≤13
Amoxicillin-clavulanic acid (10/20)	≥18	14-17	≤13
Penicillin (30)	≥16	13-15	≤12
Sulfamethoxazole-trimethoprim (20)	≥16	11-15	≤10

2. **Pseudomonas species** (*Pseudomonas aeruginosa*)

Antibiotics (µg)	**Susceptibility**	**Intermediate susceptibility**	**Resistance**
Ciprofloxacin (30)	≥25	19-24	≤18
Doxycycline (30)	≥14	11-13	≤10
Chloramphenicol (50)	≥18	13-17	≤12
Gentamycin (10)	≥15	13-14	≤12
Azithromycin (15)	≥23	20-22	≤19
Tetracycline (30)	≥19	15-18	≤14
Ceftazidime (30)	≥18	15-17	≤ 14
Streptomycin (10)	≥15	12-14	≤ 11
Ampicillin (10)	≥17	14-16	≤13
Amoxicillin-clavulanic acid (10/20)	≥18	14-17	≤13
Penicillin (30)	≥16	13-15	≤12
Sulfamethoxazole-trimethoprim (20)	≥16	11-15	≤10

Appendix VI: Mean values and standard deviation of zones of inhibition for different antibiotics tested in various bacteria species

Bacteria species	Set (S)	P	AMP	AMC	SXT	STR	CAZ	TET	AZT	CHL	GEN	DOX	CIP
Escherichia coli	S1	6	6	6	8	6	6	10	9	18	20	21	29
	S2	6	6	6	10	6	6	8	10	16	18	24	30
	S3	6	6	6	8	6	6	8	9	18	18	23	28
	Mean	6	6	6	8.666667	6	6	8.6666667	9.3333333	17.33333333	18.66666667	22.6667	29
	Standard deviation	0	0	0	1.154701	0	0	1.1547005	0.5773503	1.154700538	1.154700538	1.52753	1
Enterobacter aerogenes	S1	6	6	6	6	8	8	8	8	9	12	21	20
	S2	6	6	6	6	6	6	6	10	12	16	26	28
	S3	6	6	6	6	8	8	6	13	16	16	23	27
	Mean	6	6	6	6	7.3333333	7.333333	6.6666667	10.333333	12.33333333	14.66666667	23.3333	25
	Standard deviation	0	0	0	0	1.1547005	1.154701	1.1547005	2.5166115	3.511884584	2.309401077	2.51661	4.3589
Citrobacter freundii	S1	6	6	6	6	8	13	8	6	6	18	25	28
	S2	6	6	6	6	8	16	7	6	8	20	23	30
	S3	6	6	6	6	12	14	6	8	8	22	28	30
	Mean	6	6	6	6	9.3333333	14.33333	7	6.6666667	7.33333333	20	25.3333	29.3333

	Standard deviation	0	0	0	0	2.3094011	1.527525	1	1.1547005	1.154700538	2	2.51661	1.1547
Klebsiella pneumoniae	S1	6	6	6	6	6	10	10	15	11	14	10	16
	S2	6	6	6	8	8	6	8	14	8	18	10	18
	S3	6	6	6	6	6	8	12	16	12	14	15	19
	Mean	6	6	6	6.666667	6.6666667	8	10	15	10.33333333	15.33333333	11.6667	17.6667
	Standard deviation	0	0	0	1.154701	1.1547005	2	2	1	2.081665999	2.309401077	2.88675	1.52753
Klebsiella oxytoca	S1	6	6	8	6	10	6	10	18	20	23	12	27
	S2	6	6	6	6	10	6	8	22	25	28	14	29
	S3	6	6	8	6	10	6	8	20	22	29	15	32
	Mean	6	6	7.333333	6	10	6	8.6666667	20	22.33333333	26.66666667	13.6667	29.3333
	Standard deviation	0	0	1.154701	0	0	0	1.1547005	2	2.516611478	3.214550254	1.52753	2.51661
Proteus vulgaris	S1	6	6	6	6	6	8	8	12	8	8	25	28
	S2	6	6	8	8	6	5	10	13	10	8	28	28
	S3	6	6	6	8	6	5	12	16	16	6	30	30
	Mean	6	6	6.666667	7.333333	6	6	10	13.666667	11.33333333	7.333333333	27.6667	28.6667
	Standard deviation	0	0	1.154701	1.154701	0	1.732051	2	2.081666	4.163331999	1.154700538	2.51661	1.1547
Proteus mirabilis	S1	6	8	6	6	13	6	8	8	10	22	22	22
	S2	6	6	8	6	15	6	6	4	12	20	24	21
	S3	6	6	8	6	0	6	6	6	11	18	20	25
	Mean	6	6.6666667	7.333333	6	9.3333333	6	6.6666667	6	11	20	22	22.6667

	Standard deviation	0	1.154701	1.154701	0	8.1445278	0	1.1547005	2	1	2	2	2.08167
Pseudomonas aeruginosa	S1	6	6	6	6	6	8	10	10	12	5	11	18
	S2	6	6	6	6	6	6	10	8	8	8	13	20
	S3	6	6	6	6	10	8	8	8	20	7	10	29
	Mean	6	6	6	6	7.3333333	7.333333	9.3333333	8.6666667	13.33333333	6.666666667	11.3333	22.3333
	Standard deviation	0	0	0	0	2.3094011	1.154701	1.1547005	1.1547005	6.110100927	1.527525232	1.52753	5.85947
Salmonella enteriditis	S1	6	6	9	10	6	8	8	10	12	20	10	28
	S2	6	6	9	8	8	10	8	10	18	26	10	28
	S3	6	6	10	10	8	8	6	12	20	21	8	28
	Mean	6	6	9.333333	9.333333	7.3333333	8.666667	7.3333333	10.666667	16.66666667	22.33333333	9.33333	28
	Standard deviation	0	0	0.57735	1.154701	1.1547005	1.154701	1.1547005	1.1547005	4.163331999	3.214550254	1.1547	0
Yersinia enterocolitica	S1	6	6	6	8	6	6	12	12	21	18	10	26
	S2	6	6	8	10	6	6	16	16	23	20	10	26
	S3	6	6	6	12	6	6	18	16	22	20	13	26
	Mean	6	6	6.666667	10	6	6	15.333333	14.666667	22	19.33333333	11	26
	Standard deviation	0	0	1.154701	2	0	0	3.0550505	2.3094011	1	1.154700538	1.73205	0

Key: * P-penicillin, AMP-ampicillin, AMC-Amoxicillin-clavulanic acid, SXT- sulfamethoxazole-trimethoprim, STR- streptomycin, CAZ-ceftazidime, TET- Tetracycline, AZT- Azithromycin, CHL-Chloramphenicol, GEN- Gentamycin, DOX- Doxycycline, CIP-Ciprofloxacin.

Appendix VII: Chi-square test analysis on the relationship between phenotypic resistance and genotypic resistance of the bacteria isolates from River Sosiani and wastewater systems

Observed frequencies

		Genotypic resistance							
		qnrA, qepA, dfrA5, aadA1, mphA, blaKPC, blaSHV, sul 1	*qnrA, qepA, aadA1, mphA, blaKPC, blaSHV, sul 1*	*qnrA, qepA, dfrA5, aadA1, mphA*	*qnrA, qepA, dfrA5*	*qnrA, qepA, aadA1, blaKPC*	*Qnr A, qepA, dfrA5, aadA1*	*qnrA, qepA, mphA*	Total
Phenotypic resistance	P, AMP, AMC, SMX-TMP, STR, CAZ, TET	2	1	1	2	1	2	1	10
	Total	2	1	1	2	1	2	1	10

$Chi^{2=}$ 0, df=0, P≥ 1

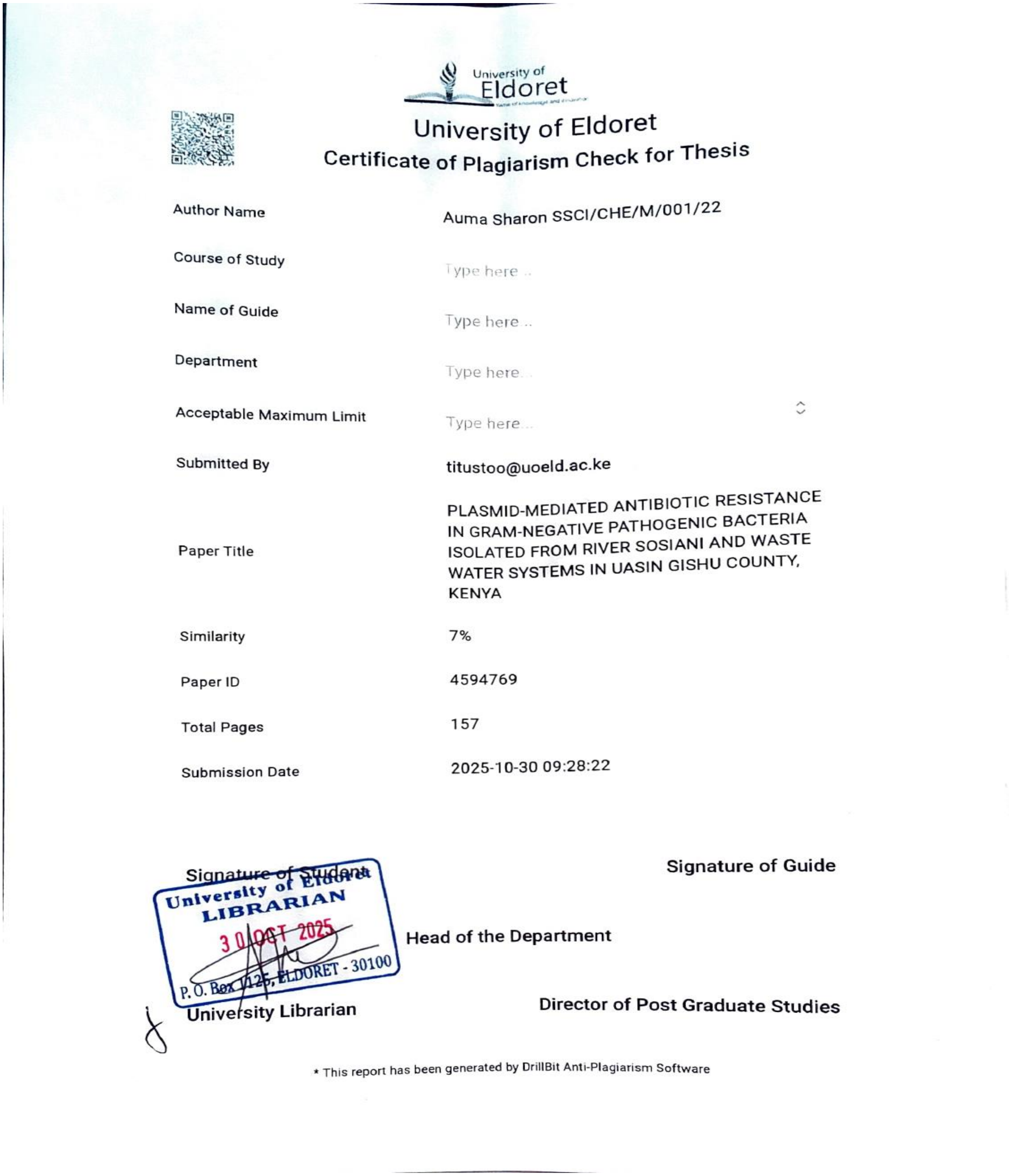
Appendix VIII: Sets of primers and PCR conditions used in the amplification of antibiotic resistance genes in the plasmid DNA of different bacteria species

Gene	**5'Primer modification sequence 3’**	**Expected amplicon product size**	**T_a (ºC)**	**Base length**	**PCR conditions**	**References**
QnrA F	GCCCGCTTCTACAATCAAGT	347	60	20	D_i =94 ºC for 7mis 1x D_f = 94 ºC for 45s ST_a for 45s 35x E_i = 72 ºC for 1min E_f =72 ºC for 7mins 1x R_{T} = 4 ºC for 10mins	(Elshamy *et al.*, 2020)
QnrA R	GGCAGCACTATTACTCCCAAG			21		
QepAF	TCTACGGGCTCAAGCAGTTG	312	55	20		
QepA R	ACAGCGAACCGATGACGAAG			20		
DfrA5F	ACGGAGTGATTGGTTGCGG	279	53	19	D_i = 94 ºC for 5mins 1x D_f =94 ºC for 40s ST_a for 35s 35x E_i =72 ºC for 1min E_f =72 ºC for 7 mins 1x R_T = 4 ºC for 10mins	(Jalilian & Hosainzadegan, 2014; Arabi *et al.*, 2015;)
DfrA5R	CTCTGTAAATCTCCCCGCC			19		
AadA1F	TATCCAGCTAAGCGCGAACT	447	58	20	D_i =95 ºC for 5mins 1x D_f =95 ºC for 35s ST_a for 35s 35x E_i = 72 ºC for 1min E_f =72 ºC for 5min R_T = R_T =4 ºC for 20mins	(Amer *et al.*, 2018; Rubab & Oh, 2021)
AadA1R	ATTTGCCGACTACCTTGGTC			20		
MphAF	ATGCGTGCACTACGCAAAG	410	60	19	D_i =94 ºC for 5mins 1x	

MphAR	CGAGCGGGCTATATCGAC			8	D_f = 94 °C for 30s ST$_a$ for 35s 35x E_i=72°C for 1min E_f = 72 °C for 7mins 1x R_T =4 °C for 20mins	(Zhang *et al.*, 2024)
Bla$_{KPC}$*F*	TGTTGCTGAAGGAGTTGGGC	340	56	20	D_i =94 °C for 5mins 1x D_f =94 °C for 1 min ST$_a$ for 40s 35x E_i = 72 °C for 1min E_f =72 °C for 7 mins 1x R_T= 4 °C for 10mins	(Mlynarcik *et al.*, 2016; Belouad *et al.*, 2023)
Bla$_{KPC}$*R*	ACGACGGCATAGTCATTTGC			20		
Bla$_{SHV}$*F*	GGTTATGCGTTATATTCGCC	867	55	20	D_i =96°C for 5min1x D_f = 96°C for 1min Specific T_a for 45s 35x E_i = 72 °C for 1min E_f = 72 °C for10mins 1x R_T =4 °C for 20min	(Elshamy *et al.*, 2020)
Bla$_{SHV}$*R*	TTAGCGTTGCCAGTGCTC			18		
AmpCFP	AATGGGTTTTCTACGGTCTG	191	60	20		(van Hoek *et al.*, 2015; Rubab & oh, 2021)
AmpCRP	GGGCAGCAAATGTGGAGCAA			20		
Sul 1F	CTTCGATGAGAGCCGGCGGC	338	62	20	D_i =95 °C for 5mins 1x D_f = 95 °C for 45s ST$_a$ for 35s 35x E_i = 72 °C for 1min E_f = 72 °C for 7 mins 1x R_T = 4 °C for 10mins	(Ma *et al.*, 2021; Pavelquesi *et al.*, 2021)
Sul 1R	GCAAGGCGGAAACCCGCGCC			20		

Key: * D_i- Initial denaturation temperature, D_f-Final denaturation temperature ST$_a$ – Specific annealing temperature, E_i- Initial extension temperature, E_f- Final extension temperature, RT - temperature at rest, PCR-polymerase chain reaction.

Appendix IX: Similarity report



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