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ISOLATION AND CHARACTERIZATION OF ENTERIC PATHOGENS FROM THE JUKSKEI RIVER

Luyanda Mkhize

1690192



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A Dissertation submitted to the Faculty of Health Science, University of the Witwatersrand, in fulfillment of the requirements for the degree of

Master of Science in Medicine

In

Clinical Microbiology and Infectious Diseases

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DECLARATION

I, **Luyanda Ntobeko Mkhize**, hereby declare that this dissertation is my own original work, all sources of information or data used in this dissertation have been properly acknowledged and cited in accordance with the University of Witwatersrand standards and guidelines. The dissertation has not been submitted at the University of Witwatersrand or any other University before. It is now being submitted for the degree of Master of Science in Medicine at the University of Witwatersrand, Johannesburg.

Signature: .......... Date: .....**15 January 2025**.....

DEDICATION

IN LOVING MEMORY OF MY AUNT

ZAMAMBO GRACE MKHIZE

1975 - 2020

PRESENTATIONS

Luyanda Mkhize, Musa Marimani, Sanelisiwe Duze. Isolation and characterization of enteric pathogens from the Jukskei River. Presented at the 22nd Biennial Congress of the South African Society for Microbiology (SASM), Protea Hotel Technopark, Stellenbosch, South Africa, 19 September 2023 (Poster).

Luyanda Mkhize, Musa Marimani, Sanelisiwe Duze. Molecular characterization of *Escherichia coli* collected from an urban river in Johannesburg South Africa – A one health approach. Presented at the Faculty of Health Sciences Biennial Research Day 2024, The University of Witwatersrand, South Africa, 05 September 2024 (Poster).

Luyanda Mkhize, Musa Marimani, Sanelisiwe Duze. Molecular characterization of *Escherichia coli*, *Shigella* and *Vibrio cholerae* collected from an urban river in Johannesburg South Africa. Abstract accepted for the 20th International Congress on Infectious Diseases (ICID) 2024 which will be held on the 3rd-6th of December 2024 in Cape Town International Convention Centre (Poster).

ABSTRACT

The Jukskei River is a potential reservoir and vehicle for the transmission of enteric pathogens. It suffers from sewage pollution resulting from inflows of sewage from buildings with no sewage system and the informal settlements along the riverbanks. In South Africa, surveillance of enteric pathogens is limited to clinical specimens, and the presence of these pathogens in urban river water is poorly documented. The current study aimed to isolate and characterize *Escherichia coli* (*E. coli*), *Shigella,* and *Vibrio cholerae* (*V. cholerae*) in the Jukskei River. A total of 80 water samples collected from the Jukskei River were subjected to culture-based methods for the enumeration and isolation of the respective enteric pathogens. Real-time polymerase chain reaction assays targeting *uidA*, *ipaH, and toxR* genes, were used to detect the presence of *E. coli*, *Shigella*, and *V. cholerae* in the water samples and to confirm presumptive *E. coli*, *Shigella*, and *V. cholerae,* respectively. Confirmed isolates were sequenced using the MInION portable nanopore-sequencing device. Reference-based genome assemblies were constructed from the raw reads using the EPI2ME software followed by bioinformatics analysis using the Centre for Genomic Epidemiology website. Bacterial means counts ranged between 4.95 - 6.52 $\log_{10}$CFU/mL, 5.32 - 6.12 $\log_{10}$CFU/mL, and 2.02 - 4.4 $\log_{10}$CFU/mL for *E.coli, Shigella and V. cholerae,* respectively. The *E.coli* isolates were diverse and grouped into 23 different sequence types. The most abundant sequence type was ST1946. The 27 *E. coli* pathogens were further divided into seven different serotypes and 59% of the isolates were O16:H48. 44.4% of the isolates were enterotoxigenic *E. coli*, 3.7% were atypical enteropathogenic *E. coli,* and the remaining 51.9% were non-diarrheagenic *E. coli*. Antimicrobial-resistant genes were detected in 11.1% of the isolates displaying quinolone, macrolides, and aminoglycoside resistance. The 14 *Shigella* isolates were classified into *Shigella flexneri* (85.7%) *Shigella boydii* (7.1%), and *Shigella sonnei* (7.1%). Only one *S. flexneri* isolate harbored *parC*,

varG, and *gyrA* resistance genes. All the *V. cholerae* isolates isolated from the Jukskei River were classified as non-O1/ non-O139. None of the isolates harbored the cholera toxin gene, *ctxA.* However, the isolates possessed other virulent genes including *toxR, vspD, als, hlyA, makA,* and *rtxA.* Importantly, no diversity was observed among the 24 *V. cholerae* isolates as they all belong to ST741. Antimicrobial resistance genes (*parC*, *varG*, and *gyrA*) were detected in 83.3% of the *V. cholerae* isolates. This study unpacks the dangers of the Jukskei River to the communities along its riverbanks. Concerningly, the Jukskei River is known to flood during rainy seasons exposing communities along its banks to a host of deadly bacterial pathogens that can cause diarrheal diseases. Although *V. cholerae* non-O1/non-O139 are not associated with epidemic cholera they can still cause mild to life-threatening illnesses, especially in these already impoverished communities. Therefore, a 'One Health Approach' for the surveillance of enteric pathogens in the country is warranted in the fight against infectious pathogens.

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~Matthew 19:26~

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NOMENCLATURE AND ABBREVIATIONS

- **°C:** degrees Celsius
- **µL:** microliter
- **µm:** micrometer
- **µM:** micromolar
- **7PET:** seventh cholera pandemic
- **A/E:** attaching or effacing
- **AA:** aggregative adherence
- **AAF:** aggregative adherence factor
- **AIEC:** adherent invasive *Escherichia coli*
- **APW:** alkaline peptone water
- **Bp:** base pair
- **BFP:** bundle forming pilus
- **BPW:** buffered peptone water
- **cAMP:** cyclic adenosine monophosphate
- **CD:** Crohn's Disease
- **CEACAM6:** Carcinoembryonic Antigen-related Cell Adhesion Molecule 6
- **CED:** Centre to Enteric Disease
- **CFR:** case per fatality ratio
- **CFU/mL:** colony-forming units per milliliter
- **cgMLST:** core genome multilocus sequence typing
- **CHI3L1:** chitinase 3-like-1
- **CMID:** Clinical Microbiology and Infectious Diseases

- **Cp:** crossing point
- **CTX:** cholera toxin
- **ctxB:** cholera toxin B
- **CU:** chaperone usher
- **DA:** diffuse adherence
- **DAEC:** diffusely adherent *Escherichia coli*
- **DEC:** diarrheagenic *Escherichia coli*
- **DNA:** deoxyribonucleic acid
- **DO:** dissolved oxygen
- **DWAF:** Department of Water Affairs and Forestry
- ***E. coli*:** *Escherichia coli*
- **EAEC:** enteroaggregative *Escherichia coli*
- **EAF:** *Escherichia coli* adherent factor
- **EAST1:** enteroaggregative heat-stable toxin
- **EC:** electrical conductivity
- **EHEC:** enterohaemorrhagic *Escherichia coli*
- **EIEC:** enteroinvasive *Escherichia coli*
- **EPEC:** enteropathogenic *Escherichia coli*
- **ESBL:** Extended-spectrum Beta-lactamase
- **ETEC:** enterotoxigenic *Esherichia coli*
- **G4c:** group 4 capsule
- **GEMS:** Global Enteric Multicentre Study

- **GERM-SA:** Group for Enteric, Respiratory, and Meningeal Diseases Surveillance South Africa
- **GM_1:** monosialotetrahexosylgangliosides
- **HIV/AIDS:** Human Immunodeficiency Virus/ Acquired Immunodeficiency Syndrome
- **HUS:** hemolytic uremic syndrome
- ***ial*:** invasion-associated locus
- **ICE:** integrative and conjugative element
- ***ipaH*:** invasion plasmid antigen H
- **LEE:** locus of enterocyte effacement
- **LMIC:** low- and middle-income country
- **LT:** heat-liable
- **mL:** milliliter
- **MLST:** multilocus sequence typing
- **MSM:** men who have sex with men
- **ng/µL:** nanogram per microliter
- **NCBI:** National Center for Biotechnology Information
- **NICD:** National Institute for Communicable Diseases
- **NTC:** non-template control
- **ORF:** open reading frame
- **ORP:** oxidation-reduction potential
- **PAI:** pathogenicity island
- **PBS:** phosphate-buffered saline
- **PCR:** polymerase chain reaction

- **Per:** plasmid-encoded regulator
- **pINV:** virulence F-plasmid
- **rstR:** repeat sequence transcriptional regulator
- ***S. boydii*:** *Shigella boydii*
- **SAC:** sialic acid catabolism
- **SADC:** Southern African Developing Communities
- **Sd1:** *Shigella dysenterae* type 1
- ***S. flexneri*:** *Shigella flexneri*
- **SRL:** *Shigella* resistance locus
- ***S. sonnei*:** *Shigella sonnei*
- **ST:** heat-liable
- **STEC:** Shiga toxin-producing *Escherichia coli*
- **Stx:** Shiga toxin
- **TDS:** Total dissolved solids
- **TE:** Tris-Ethylenediamine tetra-acetic acid
- **TCBS:** thiosulfate citrate bile-salts sucrose
- **TCP:** toxin co-regulated pilus
- **tcpA:** toxin co-regulated pilus A
- **T4P:** type IV pilus
- **T3SS:** type 3 secretion system
- **T4SS:** type IV secretion system
- **T6SS:** type VI secretion system
- **UC:** Ulcerative Colitis

- **UNGA:** United Nations General Assembly
- **USA:** United States of America
- **UV:** ultraviolet
- **V:** volts
- ***V. cholerae*:** *Vibrio cholerae*
- **VBNC:** Viable but nonculture able
- **VPI-1:** vibrio pathogenicity island 1
- **VPI-2:** vibrio pathogenicity island 2
- **WHO:** World Health Organisation
- **WRC:** Water Research Commission
- **XDR:** extremely drug-resistant
- **XLD:** xylose lysine deoxycholate

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1. INTRODUCTION

Enteric infections pose a significant public health problem worldwide, affecting human health and the economy (Osman et al., 2023). Multiple bacteria, viruses, protists, and helminths cause enteric infections (Brouwer *et al.*, 2018). These pathogens can survive in soil, water, and food and are transmitted via the fecal-oral route through direct and/or indirect contact with the infectious agents which include human-to-human contact, zoonotic, waterborne, and foodborne transmission (Ngure *et al.*, 2013). Clinical features of enteric infections include symptoms such as diarrhea, abdominal pain, vomiting, fever, nausea, malaise, and dehydration (Osman *et al.*, 2023).

Enteric infections are widespread in children in low- and middle-income countries (LMIC) and are the leading cause of death in children under five years with an estimated 1.3 million deaths annually (Asare *et al.*, 2022). People living in LMIC are often faced with challenges that deter them from receiving adequate services including clean water and proper sanitation (Levantesi *et al.*, 2012). A persistent clean water supply is crucial for the survival of a population, particularly a fast-growing community. Unfortunately, inadequate supply leads populations in LMIC to resort to the nearby rivers, which are often polluted, for their domestic water needs (Alhamlan *et al*, 2015).

Generally, river water harbors a vast majority of enteropathogens derived from municipal sewage discharges, rainfall runoffs from agricultural farms, and fecal waste from humans, pets, farm animals, and wildlife (Kinge and Mbewe, 2010). River water can, therefore, be a breeding ground for enteric pathogens causing illnesses ranging from diarrhea, gastroenteritis, cholera, and even typhoid fever (Ledwaba *et al.*, 2018; Bwire *et al.*, 2020). According to the World Health Organization (WHO), it is estimated that 525 000 children die annually due to exposure to

contaminated water (Mugadza *et al.*, 2021). While different countries report varying proportions of the etiology-specific estimates, diarrheagenic *Escherichia coli* (DEC), *Shigella*, and *Vibrio cholerae* remain among the most common pathogens (Hugho *et al.*, 2023).

1.2. *Escherichia coli*

The microorganism *Escherichia coli* is a commensal agent and performs crucial roles as part of the normal human flora. However, under unfavorable conditions, *E. coli* may transition to a pathogenic phase in the human and animal intestines causing morbidity and mortality (Pakbin *et al*., 2021). The mode of transmission for *E. coli* varies from direct to indirect routes, such as direct exposure to infected human or animal feces or through the ingestion of contaminated food or water, respectively (Bolukaoto *et al.*, 2019). The infectious agents that cause diarrhoeal diseases are referred to as diarrhegenic *E. coli* (DEC). The DEC causes illnesses varying from diarrhea and gastroenteritis to foodborne illnesses. Infections are more prevalent in children aged five and younger in developing countries and account for a mortality of about 2 million annually (Alfinete *et al.*, 2022). The DEC pathotypes commonly associated with human disease include (i) enteropathogenic *E. coli* (EPEC), (ii) enterohaemorrhagic *E. coli* (EHEC) or Shiga toxin-producing *E. coli* (STEC), (iii) enteroinvasive *E. coli* (EIEC), (iv) adherent invasive *E. coli* (AIEC), (v) enterotoxigenic *E. coli* (ETEC), (vi) diffusely adherent *E. coli* (DAEC), and the hybrid (vii) enteroaggregative *E. coli* (EAEC) (Acosta *et al.*, 2016).

1.2.1. Enteropathogenic *Escherichia coli*

The EPEC refers to strains of *E. coli* that produce attaching and effacing (A/E) lesions on the host intestinal epithelial barrier (Mare *et al.*, 2021). The A/E lesions enable EPEC to disrupt the effective attachment of cell surfaces in the host intestinal epithelial barrier, resulting in the

effacement of microvilli (Vallance and Finlay, 2000). Importantly, EPEC strains are further classified based on the absence or presence of the plasmid *E. coli* adherent factor (EAF) containing the *bfp* and *per* operons encoding a type IV bundle forming pilus (BFP) and plasmid-encoded regulator (Per), respectively. The BFP is involved in antigenicity, biofilm formation, and autoaggregation of EPEC (Mare *et al.*, 2021). On the other hand, Per is an important transcription activator (Ibarra *et al*., 2003). All EPEC strains contain the *eae* gene, encoding an intimin facilitating adhesion (Zaboon and Al-Hayanni, 2021). Typical EPEC are *eae* and *bfpA* positive, while atypical EPEC are *eae* positive and *bfpA* negative (Snehaa *et al.*, 2021).

Aquatic environments such as rivers, beaches, and lakes are reservoirs for EPEC (Obi *et al.*, 2004; Ebomah *et al*., 2018). Dairy cattle are a natural reservoir of EPEC in developed and developing countries (Eldesoukey *et al.*, 2022; Olawole *et al.*, 2024). The EPEC causes acute watery diarrhea in children younger than two years, and adults in developing countries. Diarrhea is associated with vomiting and fever. Infections can easily progress to persistent diarrhea, often resulting in dehydration and malnutrition (Zhou *et al.*, 2018). In addition, EPEC infections are also found in cases of lactose intolerance and failure to respond to oral rehydration therapy (Blanco Crivelli *et al.*, 2018). In clinical specimens, diarrhea caused by EPEC is often difficult to diagnose because of the possibility of mixed intestinal infections (Mare *et al.*, 2021). This results in EPEC being misdiagnosed as the etiological agent even when it is not the case. EPEC has also been detected in asymptomatic carriers (Kralicek *et al.*, 2022). Antibiotic resistance amongst EPEC has been detected in various studies around the world, with specific emphasis on ampicillin (Kralicek *et al.*, 2022), cephalosporins (Sirous *et al.*, 2020), trimethoprim-sulfamethoxazole (Omolajaiye *et al*., 2020), fluoroquinolones (Dembélé *et al.*, 2020), and aminoglycosides (Eltai *et al.*, 2020).

1.2.1.1. Virulence factors of EPEC

The outer membrane adhesin, intimin, encoded by the *eae* gene facilitates the attachment to the intestinal epithelial barrier (Kaur and Dudeja, 2023). Notably, EPEC has a type 3 secretion system (T3SS) composed of translocation proteins (EspA, EspB, and EspD) that allow the introduction of effector proteins to the host tissues (Santos *et al.*, 2019). The T3SS functions like a molecular syringe injecting >20 effector proteins directly into the host cell cytoplasm affecting cellular function and promoting EPEC survival and colonization (Serapio-Palacios and Finlay, 2020). In particular, EPEC utilizes a locus of enterocyte effacement (LEE) genomic pathogenicity island (Figure 1.1) that encodes most of the genetic elements required in A/E lesions. Interestingly, some atypical EPEC may contain virulent factors not encoded in LEE such as hemolysin and enteroaggregative heat-stable toxin (EAST1) (Mare *et al.*, 2021).

A

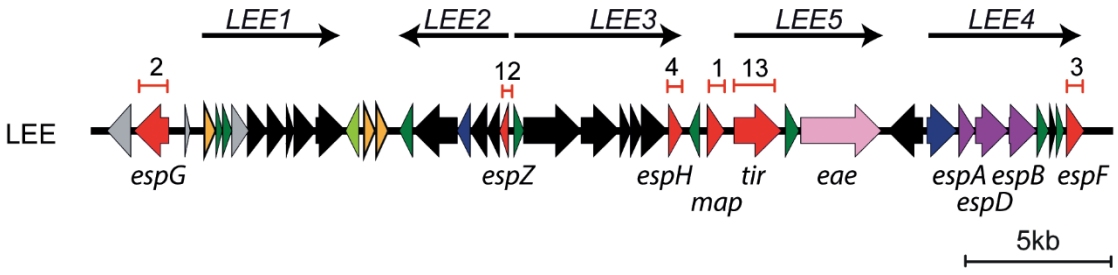

Figure 1.1 Representation of the LEE pathogenicity island. The LEE is a 35.62 Kb pathogenicity island comprising 41 open reading frames (ORFs) in five polycistronic operons (LEE 1 to 5) (Serapio-Palacios and Finlay, 2020). The *esc* genes encode the inner and outer membrane of the T3SS are encoded in LEE 1, 2, and 3 operons. The LEE 4 operon consists of the proteins EspA, EscF, EspB, and EspD. In addition, the LEE5 operon contains the *Tir* and the *eae*

gene and encodes chaperones and effector proteins (EspF, Map, EspG, EspH, and EspZ) (Cepeda-Molero et al., 2017). The effector proteins induce cytotoxicity, reorganization of the cytoskeleton, and electrolyte imbalance resulting in diarrhea (Cepeda-Molero et al., 2017). Non-LEE effectors (NleB, C, D, E, F, and H) inhibit host inflammation and apoptosis (Pollock et al., 2017). The effector genes are labeled in red. The diagram is adapted from (Cepeda-Molero et al., 2017).

1.2.2. Enterohaemorrhagic *Escherichia coli*

The EHEC, also known as Shiga toxin-producing *Escherichia coli*, are major food-borne pathogens causing acute watery diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS). It has been reported that EHEC is one of the subgroups of STEC. Hence, all EHECs are STEC, but not all STECs are EHEC (Bell, 2002). The EHEC strains such as *E. coli* O157:H7 and non-O157 strains have been linked to cause foodborne and waterborne outbreaks worldwide (Bumunang *et al.*, 2019). The most prevalent EHEC is the O157:H7. It has been documented that EHEC utilizes similar pathogenesis mechanisms to that of EPEC (Vallance *et al.*, 2002). Moreover, EHEC also has the LEE pathogenicity island that forms the A/E lesions (Kaper *et al*., 2004).

1.2.2.1. EHEC O157:H7

The EHEC O157:H7 is an important food-borne and water-borne pathogen causing diarrhea, HUS, and hemorrhagic colitis (Goldwater and Bettelheim, 2012). Transmission is through the fecal-oral route and person-to-person via fecal shedding. Cattle are the main reservoir for EHEC O157:H7 and are responsible for the zoonotic transmission of the pathogen through the consumption of undercooked meat and dairy products that are not sufficiently pasteurized (Bolukaoto *et al.*, 2019). Other ruminants, such as goats and sheep, are known reservoirs of EHEC O157:H7 (Eichhorn *et al.*, 2015). Nevertheless, several studies have isolated EHEC O157:H7 in non-ruminants such as

pigs (Ferens and Hovde, 2011; Honish *et al.*, 2017; Essendoubi *et al.*, 2020). In the developed world, EHEC O157:H7 is frequently isolated in the United States of America (USA), Japan, and the United Kingdom (Lim *et al*., 2010). In the USA, EHEC O157:H7 is the major food-borne pathogen and causes over 70 000 illnesses annually (Rahal *et al.*, 2012). Water-borne outbreaks in developed countries persist due to the ingestion of contaminated recreational waters during swimming, or through leaks in drinking water systems (Ramírez-Castillo *et al.*, 2015). In the developing world, particularly in Africa, EHEC O157:H7 has been isolated in various sources across the entire African continent (Gambushe *et al*., 2022). The first human infection by the pathogen was in 1990 in Johannesburg, South Africa (Lupindu, 2018). This was followed by a food-borne outbreak that occurred in 1992. The pathogen has a low infective dose of 10 to 100 colony-forming units (CFU).

It has been documented that EHEC O157:H7 can persist in the environment and is isolated in multiple sources such as water, soil, and food. The protozoa, *Acanthamoeba polyphaga* assists EHEC O157:H7 in survival and replication in the environment and thus serves as a transmission vehicle (Lim *et al*., 2010). The EHEC O157:H7 is an important clinical and environmental pathogen that harbors virulence and antimicrobial resistance genes. The major virulence factors for EHEC O157:H7 are Shiga toxin (*Stx*) 1 and 2, the LEE pathogenicity island, and the plasmid O157 (Lim *et al*., 2010).

1.2.2.2. Non-O157 *Escherichia coli*

There are over 150 non-O157 EHEC O serogroups with the O26, O45, O103, O111, O121, and O145 frequently isolated in severe human diseases (Eichhorn *et al.*, 2015). The non-O157 causes more acute diarrhea than the known O157 strains (Valilis *et al.*, 2018). Non-O157 strains have been linked to food-borne outbreaks globally (Eichhorn *et al.*, 2015). These strains are

predominantly found in cattle. Recently, non-O157 strains are now isolated in pigs (Iwu *et al.*, 2016). The most predominant serotype in animals is the O26 (Chase-Topping *et al.*, 2012). Interestingly, a food-borne outbreak involving undercooked venison from white-tailed deer occurred in Minnesota, USA and the prevalent serotypes were O103 and O145 (Rounds *et al.*, 2012). In the USA alone, non-O157 strains cause an annual 219 000 infections with an etiology ranging from diarrhea, vomiting, abdominal pain, and HUS (Marder *et al.*, 2023). Non-O157 strains are transmitted through fecally contaminated food and water, animals, and from person to person. Generally, non-O157 strains contain at least one Shiga toxin gene to cause infections such as HUS. However, not all non-O157 strains cause infections (Abia *et al.*, 2018). Genomic analysis of certain non-O157 serotypes (O26, O111, and the O103), revealed the T3SS and associated elements as key virulence factors (Ogura *et al.*, 2009). These strains are often underreported due to difficulties in confirming the laboratory results.

1.2.3. Enteroinvasive *Escherichia coli*

The EIEC are the etiological agents of bacillary dysentery in LMIC. Specifically, EIEC uses chromosomal and plasmid-borne genes to invade the human colonic mucosa (Pasqua *et al.*, 2017). In the colon, EIEC replicates intercellularly, spreading the pathogen to other cells and destroying the intestinal epithelial barrier (Kaper *et al*., 2004). Bacillary dysentery in EIEC is similar to that in *Shigella* spp. but is less severe and requires a higher infective dose to cause infection (UdDin and Wahid, 2014; Pasqua *et al.*, 2017). Unfortunately, EIEC and *Shigella* spp. share many phenotypic and genotypic characteristics making the distinction between the two difficult (Lan *et al.*, 2004; Van Den Beld *et al.*, 2019). The EIEC are non-motile, non-lactose fermenting pathogens that complete most of their life cycle inside the host (Sharma *et al.*, 2023). Transmission of EIEC occurs from person to person through the fecal-oral route (Gerba, 2009).

The major virulence factor in EIEC is the large virulent F-type plasmid (pINV) (Figure 1.2) that encodes genes required for the invasion, survival, and diffusion of the pathogen to host cells (Miles *et al.*, 2023). This plasmid is present in almost all EIEC/ *Shigella* pathotypes (Lan *et al.*, 2004). Previously, EIEC caused outbreaks in industrialized countries (Clarke, 2001; Lagerqvist *et al.*, 2020), with the EIEC O96:H19 serotype being the most isolated strain in outbreaks documented in Italy, Spain, and the United Kingdom (Iqbal *et al.*, 2022). The EIEC is a water-borne and food-borne pathogen (Acheson, 2009). In South Africa, EIEC has been isolated across a variety of rivers (Chukwu *et al.*, 2019; Potgieter *et al.*, 2020; Hoorzook *et al.*, 2021).

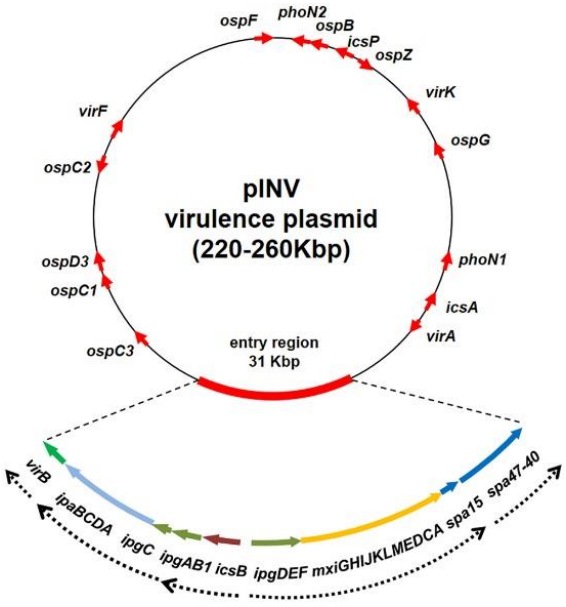

Figure 1.2. Genetic map of the PINV of EIEC and *Shigella* strains. The pINV plasmid contains a 31kb entry region composed of T3SS coding mechanisms (Mxi and Spa), effectors (IpaB, IpaC, and IpaD), chaperones (IpgA, IpgC, IpgE, and Spa15), and transcription regulators (VirB and MxiE) (Pasqua et al., 2017). The red arrows indicate major virulence determinants. Due to the variability in position and number, the *ipaH* genes are not shown. The genetic organization of the entry region is shown in detail, with dashed arrow lines indicating known transcriptional units.

1.2.4. Adherence-Invasive *Escherichia coli*

The AIEC are a subset of *E. coli* that cause inflammatory bowel diseases such as Crohn's Disease (CD) and Ulcerative Colitis (UC) (Lee *et al.*, 2019). Similarly to EIEC, AIEC invades and replicates inside macrophages (Bringer *et al.*, 2012). However, AIEC lacks the T3SS but uses different virulent factors for invasion (Dunne *et al.*, 2013). One mechanism is *FimH*, a type 1 pili that binds to the Carcinoembryonic Antigen-related Cell Adhesion Molecule 6 (CEACAM6) receptor on enterocytes. These receptors are overly expressed on the surface of intestinal epithelial cells of patients with CD (Mansour *et al*., 2023a). Another mechanism is using OmpC, an outer membrane protein that allows for adherence to intestinal cells (Camprubí-Font *et al.*, 2019). The adhesin, *chiA* also assists in adherence by binding to chitinase 3-like-1 (CHI3L1) (Low *et al.*, 2013). The hyper-flagellum facilitates invasion in AIEC (Elhenawy *et al*., 2019). AIEC secretes Vat, a mucinolytic enzyme to further assist with mucosal invasion. The invasin, *IbeA* is crucial for AIEC invasion and colonization of the gut (Mansour *et al*., 2023b). AIEC uses the HtrA protein, a stress response protein for survival in macrophages (Chervy *et al*., 2020). The formation of intracellular biofilms within the macrophages also allows AIEC to survive and replicate within macrophages (Lapaquette *et al*., 2012). In particular, AIEC uses a type IV secretion system (T4SS) to facilitate biofilm formation (Elhenawy *et al.*, 2021).

1.2.5. Enterotoxigenic *Escherichia coli*

The ETEC is the major cause of diarrheal diseases in children under 5 years as well as travelers in LMICs. It accounts for millions of diarrheal episodes and deaths annually (Sukwa *et al.*, 2024). Generally, ETEC infections arise due to the lack of sufficient water and sanitation infrastructure and are thus, caused by ingestion of contaminated food and water (Zhang *et al.*, 2022b). The ETEC strains produce heat-liable (LT) and heat-stable (ST) enterotoxins (Sheikh *et al.*, 2022). The

colonization factors (CF) are a major virulence factor of ETEC that assists the pathogen in exerting toxicity (Vidal *et al.*, 2019). In the host, ETEC colonizes the small intestinal epithelium using adhesins followed by the release of toxins (Zhang *et al.*, 2022a). The CF in ETEC is a group of 25 or more fimbriae or pili that assist in colonization. There are two types of CF, the chaperone-usher (CU) pili and the Type IV pili. The CU pili is found in the majority of Gram-negative bacteria (Del Canto *et al.*, 2017). The type IV pili (T4P) is involved in adhesion, biofilm formation, DNA uptake, and signal transduction (Zhang *et al.*, 2022a). The clinical manifestations of ETEC are similar to those of cholera in that they result in acute watery diarrhea followed by dehydration within a few hours of infection (Fleckenstein *et al.*, 2010).

1.2.6. Diffusely adherent *Escherichia coli*

The DAEC are pathogenic strains of *E. coli* characterized by the presence of a diffuse adherence (DA) pattern on HeLa and Hep-2 epithelial cells where the bacteria uniformly cover the entire cell surface (Javadi *et al.*, 2020a). The DA pattern is due to the expression of adhesins that are further divided into two groups of DAEC, namely the Afa/Dr DAEC and AIDA-I DAEC. The Afa/Dr DAEC contains adhesin-encoding genes responsible for persistent acute diarrhea in children under 6 months and older (Javadi *et al.*, 2020b). By contrast, AIDA-I DAEC is mostly isolated in piglets causing postweaning diarrhea and edema disease (Charbonneau *et al*., 2006). The virulent factor in DAEC is diffuse adherence to the intestinal epithelial of the infected host (Abbasi *et al.*, 2016).

Contaminated food and water, particularly with human or animal feces, is the main transmission vehicle (Mansan-Almeida *et al*., 2013). Asymptomatic carriers are integral in DAEC transmission (Abbasi *et al.*, 2016). Devastatingly, DAEC is a growing concern in developing countries accounting for multiple human infections and hospitalizations (Okeke, 2009). However, DAEC in developing countries is poorly documented and is isolated less frequently. In Mozambique, DAEC

is predominant in children aged 0 to 11 months (Garrine *et al.*, 2020). In Mexico, DAEC has been isolated in river water and irrigation canals and was resistant to several antimicrobials such as sulfamethoxazole-trimethoprim, tetracycline, ampicillin, and, cefotaxime (Canizalez-Roman *et al.*, 2019).

1.2.7. Enteroaggregative *Escherichia coli*

The EAEC are the causative agents of acute and persistent diarrhea in children younger than five years in developing countries and travelers to these endemic countries (Okhuysen and DuPont, 2010). In the absence of diarrhea, EAEC causes growth faltering in children (Aijuka *et al.*, 2018). Infections by EAEC have also been diagnosed in immunocompromised patients, particularly those infected with Human Immunodeficiency Virus (HIV)/Acquired Immunodeficiency Syndrome (AIDS) (Ellis *et al.*, 2020). Outbreaks of EAEC in the developed world have been described previously, as evidenced by a 2011 hybrid O104:H4 EAEC food outbreak in Germany. This hybrid strain comprised of a *Stx* from EHEC (Karch *et al.*, 2012). In South Africa, EAEC is the predominant pathovar in irrigation water and food products such as lettuce (Aijuka *et al.*, 2018).

The EAEC forms aggregative adherence (AA) patterns in human Hep-2 and HeLa cells (Pereira *et al.*, 2008). To elicit pathogenesis, EAEC involves many mechanisms such as adhering to the intestinal epithelial, stimulating mucus secretion, forming biofilms, conferring cytotoxic damage, and inducing mucosal inflammation (Ellis *et al.*, 2020). In particular, EAEC uses aggregative adherence fimbriae (AAF), encoded in the pAA virulence plasmid for adherence to epithelial cells (Boll *et al.*, 2017). The pAA virulent plasmid contains the AggR transcriptional regulator that assists with AAF dispersal (Prieto *et al.*, 2021). The protein involved in colonization (PIC), a serine protease stimulates the secretion of mucus. Cytotoxic damage and colonization of the intestines by

EAEC is accomplished by secretion of the cytotoxin *Pet*, enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 (*EAST-1*), and hemolysin E (*hlyE*) (Navarro-Garcia and Elias, 2011).

1.3. *Shigella* species

The genus *Shigella* consists of Gram-negative, facultative anaerobic bacteria belonging to the Enterobacteriaceae family causing human gastrointestinal infections such as shigellosis (Kinge and Mbewe, 2010). *Shigella* infections account for an estimated 164.7 million cases worldwide with 163.2 million cases isolated in LMIC and 1.1 million deaths annually (Ashkenazi, 2004). The majority of these infections are in children younger than five, travelers to endemic areas, as well as in men who have sex with men (MSM) (Ekdahl and Andersson, 2005; Daskalakis and Blaser, 2007; Tickell *et al.*, 2017). The Global Enteric Multicenter Study (GEMS), a large case-controlled study from 2007 to 2011, revealed *Shigella* as the leading cause of moderate to severe diarrheal diseases in children (Bengtsson *et al.*, 2022). *Shigella* infections are transmitted through the fecal-oral route via person-to-person, ingesting contaminated food and water, and through vectors such as the common housefly (*Musca domestica*) (Ugbogu., 2006; Debnath *et al.*, 2018; Bardsley *et al.*, 2020).

Shigella evolved from *E. coli*, as evidenced by their pathogenicity in that *Shigella* utilizes the same large virulence plasmid (pINV) (Figure 1.2) found in enteroinvasive *E. coli* (Seferbekova *et al.*, 2021). The *Shigella* genus comprises four species namely, *Shigella flexneri*, *Shigella sonnei*, *Shigella boydii*, and *Shigella dysenteriae*, which are further divided into serotypes based on their lipopolysaccharide O-antigen (Kotloff *et al.*, 2018).

1.3.1. *Shigella flexneri*

Shigella flexneri is the leading cause of shigellosis in LMIC (Nisa *et al.*, 2020). *S. flexneri* requires a low infective dose (10 to 100 CFU) and has adapted to withstand the low acidic environment of the stomach, contributing to its success as a global pathogen (Schroeder and Hilbi, 2008). The clinical features of shigellosis caused by *S. flexneri* include acute watery diarrhea, abdominal cramps, high fever, malaise, and nausea. Extreme cases include vomiting, bloody mucous stools, and severe abdominal pain (Leow *et al.*, 2020). An important factor in *S. flexneri* pathogenesis is pyroptotic cell death which allows *S. flexneri* to escape macrophage-mediated killing, induce local inflammation, and invade epithelial cells (Watson *et al.*, 2019).

Furthermore, *S. flexneri* is divided into 19 serotypes and subserotypes (Brengi *et al.*, 2019). Classification is based on the structure and combination of antigenic determinants in the O-antigen of lipopolysaccharides (LPS) (Sadredinamin *et al.*, 2023). *S. flexneri* serotypes are the most diverse among the *Shigella* cluster (Muthuirulandi Sethuvel *et al.*, 2017). In the developing world, the prevalent serotypes are 2a, 1b, 3a, 4a, and 6a (Kotloff *et al.*, 1999).

S. flexneri has dominated endemic-prone areas for a long time and has fostered antimicrobial resistance within *Shigella* spp. (Nisa *et al.*, 2020). According to the Group for Enteric, Respiratory, and Meningeal Diseases Surveillance in South Africa (GERMS-SA), a surveillance subdivision of the National Institute for Communicable Diseases (NICD), *S. flexneri* serotypes 2a and 1b are the predominant serotypes causing invasive and non-invasive shigellosis in mostly children under the age of five (NICD, 2022). In India, a waterborne outbreak of the strain, *S. flexneri* serotype 2a was reported in 2007 following contaminated tap water due to a burst sewage pipeline resulting in 461 cases of diarrhea and shigellosis (Saha *et al.*, 2009). Outbreaks have been reported in China in 1996 (Chiou *et al.*, 2001) and 2015 (Shen *et al.*, 2017). A foodborne outbreak of *S. flexneri* serotype

2a involving tomatoes was reported in the USA in 2001 (Reller *et al.*, 2006). In England, extremely drug-resistant (XDR) *S. flexneri* serotype 2a strains have been isolated among men who have sex with men (MSM) since 2021. These strains harbor the plasmid-encoded Extended-spectrum ß-lactamase (ESBL)-producing gene *bla*$_{\text{CTX-M-27}}$ (Thorley *et al.*, 2023).

1.3.2. *Shigella sonnei*

Shigella sonnei is the causative agent of bacillary dysentery and is commonly found in high-income countries (Torraca *et al*., 2020). However, recent studies have isolated *S. sonnei* in the developing world, particularly in Asia (Shad and Shad, 2021). In South Africa, *S. sonnei* is the most frequently isolated strain among clinical specimens (NICD, 2022) dethroning *S. flexneri*. This is in part due to *S. sonnei* prevalence correlating to the increase in the economic development of a country (Thompson *et al*., 2015). The successful shift to LMIC dominance by *S. sonnei* is through several mechanisms. One mechanism is the natural immunization of populations living in LMICs through exposure to surface water faecally contaminated with *Pleisomonas shigelloides* O17. This pathogen has an O antigen identical to that of *S. sonnei* (Matanza and Clements, 2023). Another mechanism is the use of a free-living amoeba that provides *S. sonnei* with an intracellular environment free of chlorination and sanitation processes through phagocytosis of *S. sonnei* by *Acanthamoeba castellanii* (Jeong *et al.*, 2007; Shad and Shad, 2021). Interestingly, this microbial strain has only one serotype but is further divided into five biotypes based on biochemical properties (a, b, e, f, and g) (Muthuirulandi Sethuvel *et al.*, 2017).

S. sonnei has been identified as a major foodborne pathogen causing outbreaks in USA, Canada, Australia, Denmark, and Spain (Shad and Shad, 2021). Several outbreaks have been documented in California, USA, between 2014 and 2015 (Kozyreva *et al.*, 2016). In high-income countries, *S.*

sonnei infections are usually sexually transmitted particularly in gay and bisexual men/people, and in MSM (Charles *et al.*, 2022). Immunocompromised patients living with HIV are at risk of contracting *S. sonnei* infections (Matanza and Clements, 2023).

1.3.2.1 *Shigella sonnei* virulence factors

S. sonnei encodes a type vi secretion system (T6SS) which allows for propagation, survival efficiency, and commensally targeting *E. coli* (Shad and Shad, 2021). *S. sonnei* must overcome the gut microbiota and mucus layer for successful invasion. The bacterium uses the invasion plasmid antigen (*ipaH*) and invasion-associated locus (*ial*) in the invasion, growth, and survival in the host (Shad and Shad, 2021). The *ipaH* and *ial* genes are critical effectors in the type iii secretion system (T3SS) (Ashida *et al.*, 2007). After initial activation of T3SS, the surface protein Icsa, functions as an adhesin attaching *S. sonnei* to the host tissues. In particular, *S. sonnei* contains a group 4 capsule (g4c) operon that encodes the formation of a group 4 capsule that protects it from serum-mediated killing (Caboni *et al.*, 2015). *S. sonnei* contains antimicrobial-resistant genes and integrons that further assist its survival and emergence (Toro *et al.*, 2022). Another mechanism to increase *S. sonnei* survival is switching to a viable but nonculturable (VBNC) state when conditions are unfavorable for growth (Fakruddin *et al*., 2013).

1.3.3. *Shigella boydii*

Like other *Shigella* spp., *Shigella boydii* is transmitted through contaminated food and water, soil, and fecal matter (Kumar Trivedi, 2015). *S. boydii* has been reported less frequently than other *Shigella* spp. (Akter *et al.*, 2019). There are 19 *S. boydii* serotypes (Grimont *et al.*, 2007). The clinical manifestations of a *S. boydii* infection is shigellosis, HUS, and reactive arthritis (Kumar Trivedi, 2015).

S. boydii is associated with foodborne outbreaks both in LMICs (Yousefi *et al.*, 2018) as well as in high-income countries (Chan and Blaschek, 2005). In South Africa, cases of *S. boydii* serotype 2 were isolated in Mpumalanga province in 2007 following water transmission indicating a potential water outbreak (Smith *et al.*, 2009). Although outbreaks have been reported worldwide in the past, *S. boydii* is the least common species causing shigellosis (El-Gendy *et al.*, 2012).

1.3.4. *Shigella dysenteriae*

Shigella dysenteriae is the leading cause of bacillary dysentery in the developing world (Sváb *et al.*, 2021). The *S. dysenteriae* type 1 (Sd1) strain is the main cause of bacillary dysentery and uses the Shiga toxin (Stx) as its main virulence factor. The Stx is produced in some *E. coli* pathotypes and exists in two main parts, Stx1 and Stx2 (Etcheverría and Padola, 2013).

In the developing world, Sd1 is the pathogen causing a variant of HUS called typical HUS (tHUS) (Grisaru, 2014). An outbreak of HUS involving 81 cases was reported in South Africa between 1994 and 1996 (Bitzan and Lapeyraque, 2016). Cases of HUS have been reported in India, Bangladesh, and South Africa (Amirlak and Amirlak, 2006). The Sd1 is known to cause the highest case per fatality ratio among all ages as compared to other *Shigella* spp. (Zaidi and Estrada-García, 2014). It has been linked to four epidemics in Central America, South Asia, and Central and East Africa (Njamkepo *et al.*, 2016).

1.3.5. Antimicrobial resistance in *Shigella*

The WHO recommends ciprofloxacin, a fluoroquinolone antimicrobial, in the treatment of shigellosis (Williams and Berkley, 2018). However, rampant use of ciprofloxacin has led to antimicrobial resistance in *Shigella* spp. (Aggarwal *et al.*, 2016) thus new antimicrobial therapies

are needed. *Shigella* spp. have distinct lipopolysaccharide O-antigen that determine the serotypes and serotype-specific immunity (Bengtsson *et al.*, 2022).

Shigella spp., parallel to other Enterobacterales, are increasingly becoming resistant to the common antimicrobials. *Shigella* utilizes mobile genetic elements, such as the *Shigella* resistance locus (SRL) to confer resistance to ampicillin, chloramphenicol, streptomycin, and tetracycline (Stenhouse *et al.*, 2023). It has been shown that SRL is present in the majority of *Shigella* spp. in a 66 Kb pathogenicity island (PAI) or alone (Turner *et al.*, 2003).

1.4. *Vibrio cholerae*

V. cholerae is a highly motile, comma-shaped Gram-negative bacterium, with a single flagellum, causing cholera. The global burden of cholera is often underrepresented but is approximately 2.8 million cases annually with 91 000 deaths (Ali *et al.*, 2015; Ilic and Ilic, 2023).

There are over 200 serogroups determined by the O-antigen of the liposaccharide that include both pathogenic and non-pathogenic strains of *V. cholerae*. (Somboonwit *et al.*, 2017). Strains belonging to the O1 and O139 serogroup produce the cholera toxin (CTX), causing cholera and thus epidemics. Conversely, strains lacking CTX are referred to as non-O1/ non-O139 and do not cause cholera but are responsible for gastroenteritis, wound infections, and bacteremia (Montero *et al.*, 2023). Cholera toxin binds to the monosialotetrahexosylgangilioside (GM_1) receptor of host erythrocytes leading to elevated intracellular cyclic adenosine monophosphate (cAMP) levels and resulting in profuse secretory diarrhea (Safa *et al*., 2010).

V. cholerae O1 strains are further classified into two biotypes, *El tor* and *classical,* based on phenotypic and genotypic differences. The Polymyxin B antibiotic is used to determine the

phenotypic differences between the two biotypes where *El tor* is generally resistant whereas *classical* is susceptible (Matson *et al.*, 2010; Safa *et al.*, 2010). *El tor* and *classical* differ in the genotypic expression of their specific genes; *tcpA* (toxin co-regulated pilus A), *ctxB* (cholera toxin B), and *rstR* (repeat sequence transcriptional regulator). A study by (Beyhan *et al.*, 2006), reported that some genes are expressed more than others. The genes encoding virulence were higher in *classical*. Conversely, genes encoding biofilm formation and chemotaxis were higher in *El tor*. These biotypes also differ in their pathogenic potential, survivability, and infection patterns in human hosts. *El tor* strains are associated with asymptomatic infection, increased survival in the environment and a human host, and efficient host-to-host transmission. Whereas *classical* strains have more severe clinical manifestations of cholera (Mandal *et al*., 2011).

The primary transmission of cholera is through the consumption of contaminated water from the environment and secondary transmission is through the fecal-oral route. Primary transmission causes endemic cholera and secondary causes outbreaks (Roskosky *et al.*, 2021). Cholera follows a spatial clustering where the closer an individual is to a cholera case, the more likely to contract the disease (Osei and Duker, 2008; Ruiz-Moreno *et al.*, 2010; Bi *et al.*, 2016; Debes *et al.*, 2016).

1.4.1. The current cholera pandemic

There have been seven cholera pandemics since 1817 (Hu *et al.*, 2016). The seventh cholera pandemic was first reported in Indonesia in 1961 and was only found in Africa in 1970. *V. cholerae* serotype O1 biotype *El Tor* (7PET), was the etiological agent responsible for this pandemic causing 3 to 5 million cases yearly (Weill *et al.*, 2017). In 2008, Zimbabwe experienced a cholera epidemic accounting for approximately 90 000 cholera cases and over 4000 deaths (Islam *et al.*, 2011).

Oppositely, some locally-spreading *V. cholerae* strains cause syndromic cholera and do not lead to pandemic cholera (Deen *et al*., 2020).

1.4.2. Recent cholera outbreak in South Africa

Cholera cases are often underreported in most countries due to several factors. Mild cases are not reported, patients die before reaching treatment facilities, and stools may not be routinely cultured, thus *V. cholerae* is not isolated. Another factor is that affected countries fail to disclose cases for fear of affecting tourism and exports (Zuckerman *et al*., 2007; Ganesan *et al*., 2020). The average case per fatality ratio (CFR) of cholera in Africa has increased over the years from 1.9% in 2021 to 2.3% in 2023 (WHO, 2023). South Africa is not endemic to cholera, cases are mainly imported from the neighboring countries undergoing an outbreak (Zimbabwe, Zambia, Malawi, etc.) (Smith *et al.*, 2021). A recently documented cholera outbreak in South Africa occurred in 2023. This cholera outbreak was reported in the Gauteng Province, involving an import-related case following travel to Malawi (NICD, 2023). Amongst the cultured *V. cholera* isolates, some were linked to the consumption of untreated water from the Jukskei and Klip Rivers (NICD, 2023). The *V. cholera* O1 serotype Ogawa was the strain implicated in the cholera outbreak.

1.4.3. *V. cholerae* pathogenesis

V. cholerae exists in the environment as a free-living pathogen, colonizes fish or crustaceans, or forms associations with algae (Vezzulli *et al.*, 2010; Halpern and Izhaki, 2017; Ahmed *et al.*, 2018; Baker-Austin *et al.*, 2018). *V. cholerae* has developed different survival mechanisms in the host and estuarine waters. Part of this strategy is to switch from motile to sessile lifestyles in response to chemical and physical stimuli. This is essentially the formation of biofilms (Silva and Benitez, 2016). Adhesion to surfaces, both in humans and the environment, plays a role in the success of *V.*

cholerae as a pathogen and an environmental organism. Biofilms are complex bacterial populations of a single strain or more that attach to a surface predominantly a phytoplankton, and interact with each other for nutrient and metabolic by-product circulation and to confer antimicrobial resistance (Islam *et al.*, 2007). Biofilms play a role in the success of *V. cholerae* in the environment from predation by eukaryotes, as well as the limitation of nutrients (Teschler *et al.*, 2015).

V. cholerae, among other bacteria, enter a viable but nonculturable (VBNC) state when conditions become unfavorable for growth. This includes nutrient limitations, changes in temperature, heightened salinity levels, or low pH (FernÁndez-Delgado *et al.*, 2015). The VBNC state is a condition in which bacteria maintain limited metabolic activity, but remain undetectable in traditional culture media (Wu *et al*., 2016). To survive in the VBNC state, bacteria change their morphology. Specifically, *V. cholerae* transitions from a curved rod shape to a coccoid, thus enhancing its survival and persistence in the environment (Alam *et al.*, 2007).

1.4.5. *V. cholerae* virulence factors

Two virulence factors are responsible for pathogenesis in *V. cholerae,* CTX and the toxin-coregulated pilus (TCP). CTX alters the host cell signal transduction pathways leading to diarrhea. Conversely, the TCP is a vital component in *V. cholerae* colonization in host intestinal epithelium (Hsiao and Zhu, 2020). The TCP is encoded by genes in the TCP cluster of the *Vibrio* Pathogenicity Island 1 (VPI-1) (Figure 1.3A) and promotes invasion of the host in the gastrointestinal tract by using the CTX prophage (Kumar *et al.*, 2020). All the toxigenic *V. cholerae* harbor the CTX prophage and VPI-1, the *V. cholerae* O1 (Zhou *et al.*, 2014) and *V. cholerae* O139 (Dorman *et al.*, 2019).

The DNA VPI-1 fragment is approximately 41.3 Kb in length and harbors 31 genes (VC0817 to VC0847) with known and unknown functions. The presence of VPI-1 in non-O1 and non-O139 has been demonstrated to convert a non-toxigenic strain to a toxigenic variant (Kumar *et al*., 2020). The VPI-1 has seven key constituents, *attL*, *vpiT*, *AldA* cluster, TCP cluster, ACF gene cluster, *int*, and *attR* (Figure 1.3A). The *Vibrio* pathogenicity island 2 (Figure 1.3B) is a 57 Kb gene region present in toxigenic isolates of *V. cholerae* containing 52 open reading frames (ORFs) (VC1758 to VC1809) (Jermyn and Boyd, 2002). The VPI-2 has a sialic acid catabolism (SAC) cluster that enables *V. cholerae* to grow solely on sialic acid as a carbon source (Boyd *et al.*, 2015). The bacterial neuraminidase enzyme encoded by the *nanH* gene catalyzes the removal of sialic acid and is a critical factor in microbial pathogenesis. A substantial increase in the sensitivity of CTX in host cells is observed following this enzymatic reaction (Jermyn and Boyd, 2002; Chen *et al.*, 2022).

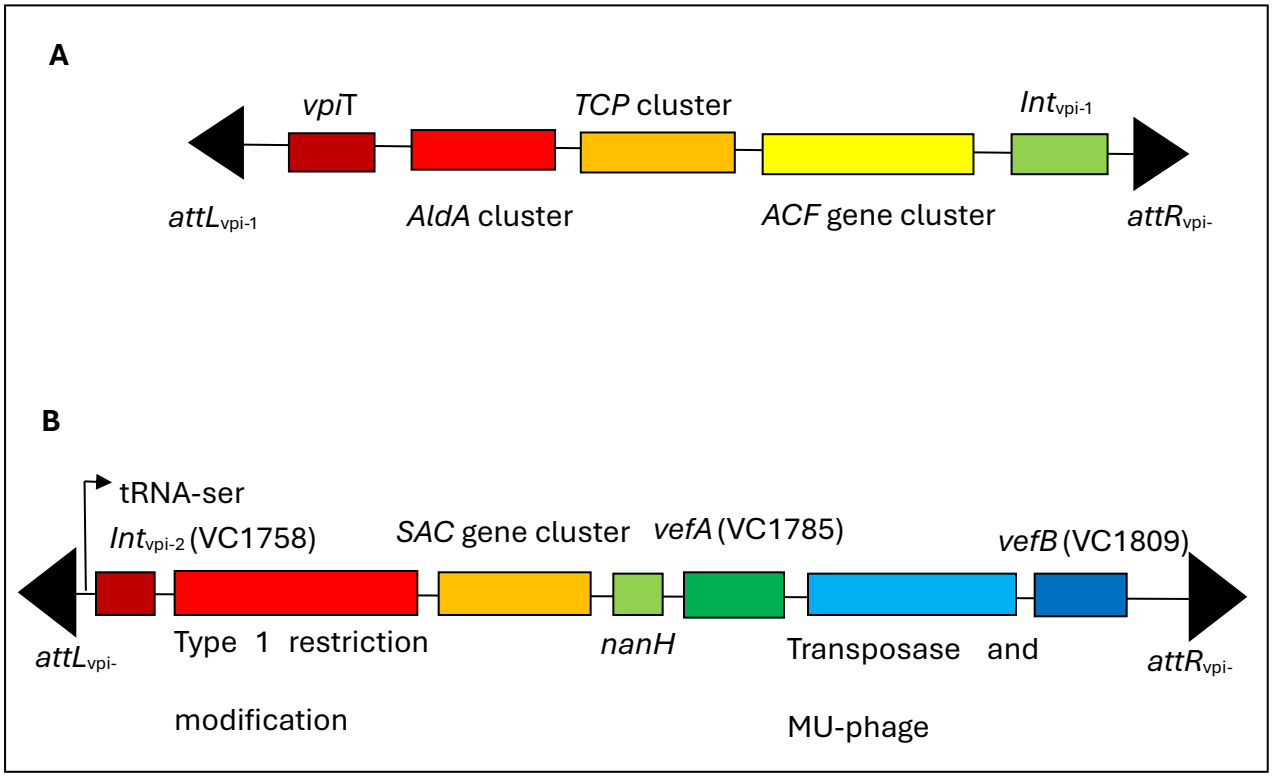

Figure 1. 3. The outline of *V. cholerae* pathogenicity island VPI-1 [A] and VPI-2 [B]. The VPI-1 is a DNA fragment of approximately 41.3Kb harboring 31 genes (VC0817 to VC0847) with known and unknown functions. The Vibrio pathogenicity island 2 is a 57 Kb gene region, present in toxigenic isolates of *V. cholerae* containing 52 open reading frames (ORFs) (VC1758 to VC1809).

1.4.4. Antimicrobial resistance patterns in *V. cholerae*

V. cholerae has developed resistance against the common antibiotics, these are chemical compounds that inhibit growth or kill the bacterial species. Antibiotics target various sites in bacteria to illicit bactericidal or bacteriostatic effects. They can (i) inhibit cell wall synthesis, (ii) interrupt cell membrane integrity, (iii) prevent DNA, RNA, and protein synthesis, and (iv) interrupt

essential cellular metabolic pathways (Das *et al*., 2020). Many bacteria, including *V. cholerae*, have developed mechanisms to resist the effects of antibiotics. These include (i) alteration of the target DNA gyrase, (ii) increased efflux (i.e. export of drug outside microorganism), (iii) fluoroquinolone inactivation (by an aminoglycoside N-acetyltransferase), and (iv) protection of targets by DNA binding proteins (known as Qnr) (Holmes *et al*., 2016).

Cholera, the disease caused by the pathogen *V. cholerae* is treated with a variety of antimicrobials (often resulting in resistance), oral rehydration solution- a mixture of salts and glucose, and the WHO-recommended- oral cholera vaccine (Sousa *et al.,* 2020). Tetracycline and quinolones are the most widely used antibiotics for treating cholera symptoms. Their widespread use has led to the development of resistance by some *V. cholerae* strains making the treatment of cholera difficult (Kitaoka *et al.*, 2011).

V. cholerae can acquire resistance genes from contact with other bacteria through mobile genetic elements. Subsequently, these resistance genes are transferred to the commensal microorganisms in the gastrointestinal tract and intestines (Gladkikh *et al.*, 2020). Antimicrobial resistance in *V. cholerae* is mediated by efflux pumps, chromosomal mutations, and acquisition of conjugative plasmids, transposons, integrative and conjugative elements (ICE) such as SXT elements carrying antibiotic resistance genes (Lepuschitz *et al.*, 2019).

1.5. Motivation for this study

In 2010, the United Nations General Assembly (UNGA) declared access to clean water and sanitation a human right. However, 2.1 billion people and a further 4.5 billion, are without adequate water and sanitation, respectively (Magana-Arachchi and Wanigatunge, 2020). Thus, many people, especially in developing countries, are exposed to agents of enteritic waterborne outbreaks. Fecal

contamination of drinking water through human and animal sewage poured into water distribution systems and surface water is the common transmission pattern of waterborne enteric pathogens (Park *et al.*, 2018). The WHO has reported a staggering two million cases of waterborne diseases annually (WHO, 2020). Many developing countries, like South Africa, are often faced with a high incidence of diarrhea, particularly in immunocompromised individuals and infants (Aijuka *et al.*, 2018). Diarrheal diseases are notifiable in South Africa and surveillance for enteric pathogens is active. However, surveillance is focused on clinical samples. Unfortunately, the role of the environment, in particular river water cannot be neglected as they may contribute to ongoing transmissions of enteric pathogens. Therefore, it is important to understand the population structure of the enteric pathogens in these environments.

The Jukskei River in Johannesburg, South Africa is one of the largest urban rivers in the province of Gauteng, the most populated province in the country. This river passes through an array of urban and informal settlements affecting the overall water quality and posing a serious public health risk to the neighboring population which may be susceptible to waterborne enteric pathogens (Bolukaoto *et al.*, 2019; Hoorzook *et al.*, 2021). A study by Hoorzook *et al.*, 2021, reported that the Jukskei River is contaminated by sewage and effluent. The Jukskei River is therefore a potential reservoir and transmission vehicle for enteric pathogens.

1.6. Aim

This study aimed to detect the presence of enteric bacterial pathogens, specifically, *Escherichia coli*, *Shigella,* and *Vibrio cholerae,* in the Jukskei River in Johannesburg, South Africa.

1.7. Objectives

- To determine the physio-chemical properties of the Jukskei River.
- To perform a quantitative and qualitative analysis of *Escherichia coli*, *Shigella,* and *Vibrio cholerae* in the Jukskei River.
- To determine the virulence of *Escherichia coli* isolated from the Jukskei River
- To characterize *Escherichia coli*, *Shigella* species, and *Vibrio cholerae* isolated from the Jukskei River using Whole Genome Sequencing.
- To determine the evolutionary relationship between the *Escherichia coli*, *Shigella,* and *Vibrio cholerae* from the Jukskei River and those isolated from clinical, food, and/or environmental samples.

CHAPTER 2: MATERIALS AND METHODS

2.1. Ethical clearance

This study was approved by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand and an ethical weaver (W-CBP-220530-02) is attached in Appendix A.

2.2. Study area and sampling site

The Jukskei River is one of the largest rivers in Johannesburg, Gauteng province of the Republic of South Africa. This river rises from an underground spring in the Bezuidenhout Valley, East of Johannesburg, and passes along a range of urban settlements such as Alexandra Township, Buccleuch, and Midrand before it confluences with the Crocodile River and flows into the Hartbeespoort Dam. The Jukskei River receives effluent from industries, stormwater runoff, and an overflow of sewage as it flows northwards. The overflow is caused by increased pressure on the available infrastructure and poor management of the existing structural resources or lack thereof. Thus, blocked sewers, and hijacked buildings with no drainage systems add to this influx. Periodic flooding is also a prominent problem with the Jukskei River further posing public health risks to many poor communities along its banks.

Five distinct sites were identified along the Jukskei River (Figure 2.1). These sites were selected according to the developments, industries, and population information, and to encapsulate the differences in these environments through regular collection of samples.

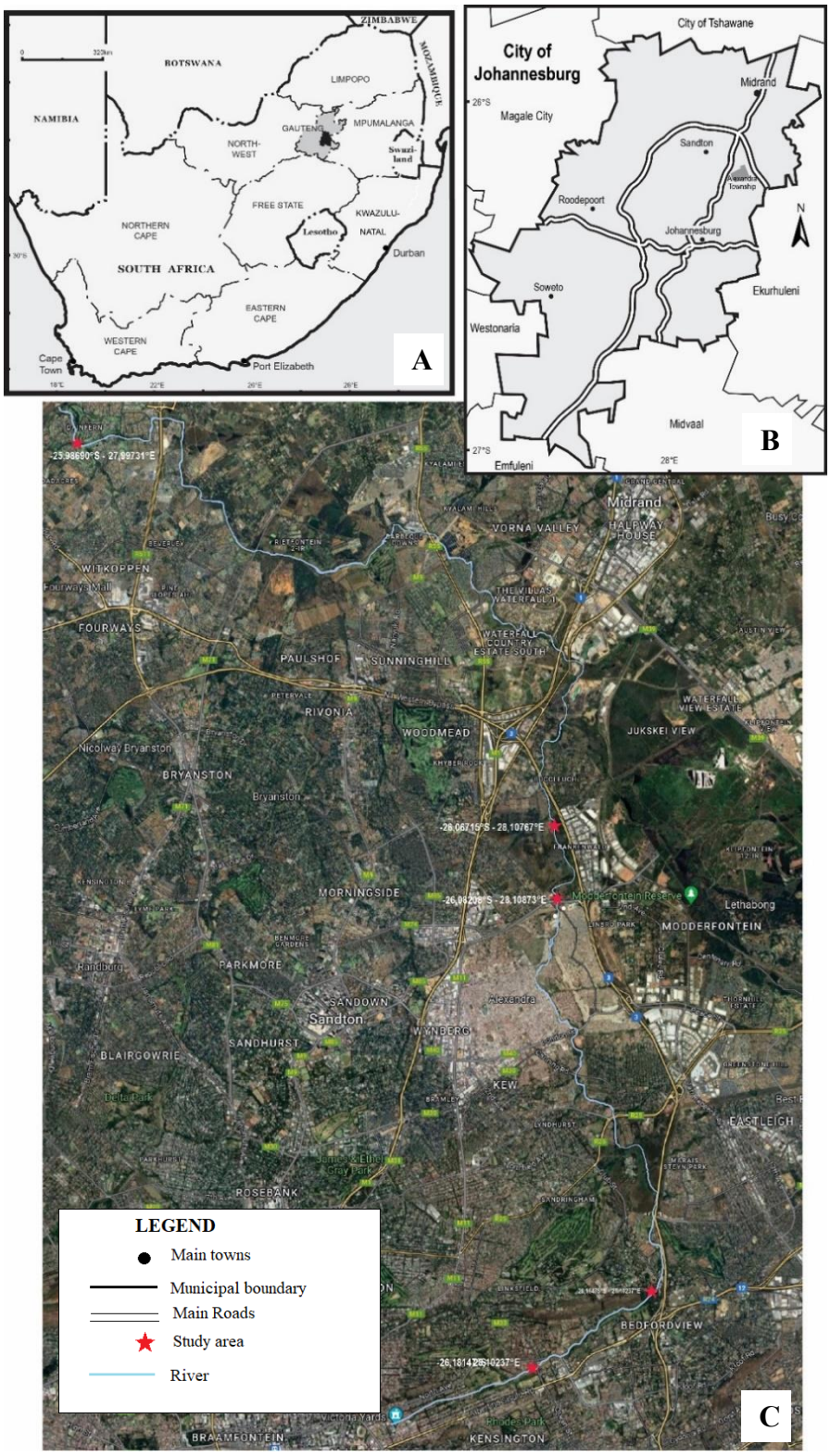

Figure 2.1. The Jukskei River is in the Gauteng Province represented by the light blue line [A] within Johannesburg [B]. The five geographic sampling locations along the river are marked with red stars [C].

2.3. Sample collection

A total of 80 water samples were collected across five different collection sites along the Jukskei River from January to November 2023 in sterile 2-litre bottles (Plastpro Scientific, South Africa). During sampling, the temperature at the collection point was measured using a portable thermometer. The samples were stored in cooler boxes with ice (with an approximate temperature of 2 to 8 °C), and transported to the Department of Clinical Microbiology and Infectious Disease (CMID), University of the Witwatersrand Medical School. After each sample collection, the following physio-chemical parameters were analyzed: temperature, pH, oxidation-reduction potential (mV), dissolved oxygen saturation (%), dissolved oxygen concentration in parts per million (ppm), electrical conductivity (ms/cm), resistivity (mΩ/cm), total dissolved solids (ppm), and salinity in practical salinity unit (psu). The physio-chemical parameters were outsourced to the School of Chemistry in the Department of Environmental and Analytical Chemistry at the University of the Witwatersrand Main Campus.

2.4. Quantitative analysis of the water samples

A total of 1 Litre of water samples were filtered through a 0.45 µm pore size sterile membrane filter (Labex, South Africa). The filters were aseptically transferred to a 50 mL centrifuge tube (Separations, South Africa) containing 10 mL of phosphate buffer saline (PBS, pH 7.0) (Merck, Darmstadt, Germany). The filters were washed by scraping the bacteria off using inoculation loops and vortexed for 10 minutes to dislodge bacteria from the membrane. Ten-fold serial dilutions were prepared from the filtered water samples. One hundred microliters of each dilution were inoculated onto MacConkey-Sorbitol Agar (Merck, Darmstadt, Germany) for *E. coli*, Thiosulfate citrate bile-salts sucrose (TCBS) Agar (Merck, Darmstadt, Germany) for *V. cholerae* and Xylose Lysine Deoxycholate (XLD) agar (Merck, Darmstadt, Germany) for *Shigella* species. All the plates were

incubated at 37°C for 24 hours, the presumptive colonies enumerated, and total viable counts expressed as $\log_{10}$ colony forming units per milliliter (CFU/mL) of the sample (Appendix C).

2.5. Qualitative analysis of the water samples

One Litre of water samples were filtered through 0.45 µm pore size sterile membrane filters. The filters were aseptically transferred to sterile 50 mL centrifuge tubes containing 10 mL PBS. The filters were washed by scraping the bacteria off using inoculation loops and vortexed for 10 minutes to dislodge bacteria from the membrane. Subsequently, 100 µL was inoculated onto MacConkey-Sorbitol Agar, TCBS Agar, and XLD agar, and incubated overnight at 37°C.

A further 1 mL of the filtered water was added onto two centrifuge tubes containing 9 mL of buffered peptone water (BPW) (Merck, Darmstadt, Germany) and alkaline peptone water (APW) (Merck, Darmstadt, Germany) to enrich for *Shigella* and *V. cholera,* respectively. These were incubated at 37°C for 24 hours. Following incubation, 100 µL of each solution was inoculated on XLD and TCBS agar and incubated at 37°C for 24 hours. The presumptive colonies grown on these selective media were further inoculated on nutrient agar (Merck, Darmstadt, Germany) for biochemical testing and genomic DNA extraction. Oxidase (Merck, Darmstadt, Germany) and indole (Thermo Fisher Scientific, MA, USA) were used to perform the biochemical tests. For the oxidase test, a well-grown single colony was spread onto the reaction zone of an oxidase strip using an inoculation loop. A color change or lack thereof was observed after 60 seconds and recorded according to Table 2.1 below. For the indole test, a drop of indole was added to the filter paper, and a colony spread onto the filter paper containing the indole. A color change was observed after 10 seconds and recorded as depicted in Table 2.1.

Table 2.1. Oxidase and indole biochemical tests indicate positive and negative results according to color change for each tested pathogen.

	Biochemical Test			
Pathogen	Oxidase positive (Purple)	Oxidase negative (Light pink)	Indole positive (Blue)	Indole negative (Pink)
E. coli	-	+	+	-
Shigella	-	+	-	-
V. cholerae	+	-	+	-

2.6. Genomic DNA extraction from filtered water samples

A total of 10 mL of filtered water samples with the filters were used in genomic DNA extraction following the E.Z.N.A water DNA extraction kit standard protocol (Omega Bio-tek, GA, USA .). DNA concentration and purity were determined by measuring the absorbance ratio at A260/280 nm using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, MA, USA) and stored at -20°C until further analysis.

2.7. Genomic DNA extraction from bacterial culture

The genomic DNA of the biochemically confirmed isolates was extracted using the boiling method. Briefly, 400 µL of Tris-EDTA (TE) buffer (Merck, Darmstadt, Germany) was transferred into a 1.5 mL Eppendorf tube. A loopful of culture was added to the TE buffer, vortexed, and boiled at 95°C for 25 minutes using a heating block. The sample was then centrifuged at 12 000 × g for 3 minutes (Eppendorf centrifuge 5417C, Merck, NJ, USA). Subsequently, 20 µL of supernatant was added onto a new 1.5 mL Eppendorf containing 80 µL of TE buffer, vortexed, and centrifuged

at 12 000 × g for 1 minute. The DNA concentration and purity were assessed using the NanoDrop 2000 spectrophotometer set at the absorbance ratio A260/280 nm. The genomic DNA samples were kept at -20°C until use.

2.8. Conventional PCR analysis

Conventional polymerase chain reaction (PCR) assays were applied to confirm the presence of *E. coli*, *Shigella*, and *V. cholerae* in the collected water samples. Specifically, PCR was performed using primer pairs adapted from previous closely related studies (Anastasi *et al.*, 2010) (Kinge and Mbewe, 2010)(Koskela *et al.*, 2009). The assays were optimized using known species (*Escherichia coli* ATCC 35218, *Vibrio cholerae* NCTC 8021, and *Shigella flexneri* ATCC 12022) from stock cultures sourced from the Infection Control laboratory within the University of the Witwatersrand Medical School. These known species were subsequently included as positive controls to validate accurate DNA amplification. The PCR reactions were performed in an Applied Biosystem MiniAmp thermal cycler (Thermo Fisher Scientific, MA, USA), and the total volume for each PCR reaction was 25 µL. Each reaction consisted of 12.5 µL of 2× PCR master mix (Thermo Fisher Scientific, MA, USA), 10 µM per primer (Integrated DNA Technologies, IA, USA), 6.5 µL of nuclease-free water (Thermo Fisher Scientific, MA, USA), and 5 µL of extracted genomic DNA (10-100 ng/µL). The primer sequences used for conventional PCR amplification are shown in Table 2.2. The thermal cycling conditions employed for PCR amplifications were initial activation of 95°C for 15 minutes, 35 cycles of denaturation at 94°C for 45 seconds, variable annealing conditions, extension at 68°C for 2 minutes, and a final extension at 72°C for 5 minutes. The annealing conditions were primer specific; *uidA* gene was amplified at 56°C for 30 seconds, *ipaH* gene at 55°C for 1.5 minutes, and *toxR* gene at 61°C for 1 minute. A positive and a no-template control were included in each reaction for quality control. The strains were positive

controls for the respective gene targets: *Escherichia coli* ATCC 35218, *Vibrio cholerae* NCTC 8021, and *Shigella flexneri* ATCC 12022. Amplicons were separated on a 2% (w/v) agarose gel stained with 10 µL of GelRed (Thermo Fisher Scientific, MA, USA). The amplified PCR products were electrophoresed at 110 V for 45 minutes. A 100 bp molecular weight marker (Anatech, South Africa) was used as a reference to estimate the size of the generated PCR amplicons. Finally, the PCR amplicons were viewed under UV transillumination, and electrophoretic images were captured using the Gel Doc EZ system (Universal Hood II Gel Doc System, Bio-Rad, CA, USA).

Table 2.2. Target genes and primer sequences used to amplify *E. coli*, *Shigella*, and *V. cholerae*.

Pathogen	Target gene	Primer sequence (5' – 3')	Product size (bp)	References
E. coli	*uidA*	F: CTG GTA TCA GCG CGA AGT CT R: AGC GGG TAG ATA TCA CAC TC	556	(Anastasi *et al.*, 2010)
Shigella	*ipaH*	F: CCT TGA CCG CCT TTC CGA TA R: CAG CCA CCC TCT GAG GTA CT	606	(Kinge and Mbewe, 2010)
V. cholerae	*toxR*	F: TGG CAT CGT TAG GGT TAG CAA R: CAT TCA CAG CCC TGA AGT TTC A	68	(Koskela *et al.*, 2009)

F: forward primer; R: reverse primer

2.9. Real-time PCR analysis of *uidA*, *ipah*, *toxR* genes

Real-time PCR assays were optimized for *E. coli*, *Shigella*, and *V. cholerae* identification using primer pairs illustrated in Table 2.2. A total reaction volume of 20 µL comprised of 2× PowerTrack SYBR Green Master Mix (Thermo Fisher Scientific, MA, USA), 10 µM of each primer, 7 µL nuclease-free water, and 2 µL of template DNA (10-100 ng/µL) was used for all amplifications. Amplification reactions were performed in a Roche LightCycler 480II (Roche Diagnostics, Germany) following 5 programs: enzyme activation, denaturation, annealing, elongation, and melting curve. The cycling conditions included an initial enzyme activation step of 95°C for 2 minutes. This was followed by 40 cycles of denaturation set at 95°C for 2 minutes followed by variable annealing specific for each gene and an elongation step at 72°C for 1 minute. The annealing conditions were as follows; *uidA* gene was specifically amplified at 56°C for 30 seconds, *ipaH* gene at 55°C for 30 seconds, and *toxR* gene at 55°C for 1 minute. Amplification plots and crossing point (Cp) values were recorded after 40 cycles. A positive control and a no-template control were included in each reaction for quality assurance. The positive controls were *Escherichia coli* ATCC 35218, *Shigella flexneri* ATCC 12022, and *Vibrio cholerae* NCTC 8021. In addition, melting curve analysis was employed to confirm the specificity of all real-time PCR amplifications.

2.10. Detecting virulence genes in *E. coli* isolates

Five multiplex PCR assays (Reaction A-E) targeting variable virulence genes in *E. coli* were performed using primer sequences adapted from (Alfinete *et al.*, 2022). The PCR assays were optimized using isolates retrieved from the National Institute of Communicable Diseases (NICD) before conducting the experimental work. Genomic DNA from culture-confirmed *E. coli* isolates was extracted using the QIAmp DNA Kit (Qiagen, GmbH, Germany), and the concentration and

purity were evaluated using the NanoDrop 2000 spectrophotometer set at A260/280 nm. The multiplex PCR assays contained a total volume of 25 µL depending on the number of primers per reaction. For reactions A and B (Table 2.3 and Table 2.4), 12.5 µL of PCR master mix 2× (Thermo Fisher Scientific, MA, USA), 0.5 µL primer mix (10 µM), and 9.5 µL of genomic DNA (10-100 ng/µL). Reactions C and D (Table 2.5 and Table 2.6) contained 12.5 µL of PCR master mix 2× (Thermo Fisher Scientific, MA, USA), 0.5 µL primer mix, 0.5 µL of nuclease-free water, and 10 µL of genomic DNA. Reaction E (Table 2.7) was composed of, 12.5 µL of PCR master mix 2× (Thermo Fisher Scientific, MA, USA), 0.5 µL of a 0.1 µM *lt1* primer, 1.5 µL of nuclease-free water, and 10 µL of genomic DNA. The NICD samples used in optimization were included as a positive control and a no-template control was included in every run as a negative control for quality control.

Reaction A

Table 2.3. Primer sequences of the multiplex PCR assay to detect different virulence genes encoding EHEC strains.

Pathovar	Target gene	Primer sequence (5' – 3')	Product size (bp)	References
EHEC	*eaeA*	F: CTG AAC GGC GAT TAC GCG AA R: CCA GAC GAT ACG ATC CAG	917	(Aranda *et al.,* 2004)
	stx1	F: ACA CTG GAT GAT CTC AGT GG R: CTG AAT CCC CCT CCA TTA TG	614	(Omar and Barnard, 2014)

	stx2	F: CCA TGA CAA CGG ACA GCA GTT R: CCT GTC AAC TGA GCA CTT TG	779	(Omar and Barnard, 2014)

EHEC, enterohemorrhagic *E. coli*; F: forward primer; R: reverse primer

Reaction B

Table 2.4. Primer sequences of the multiplex PCR assay to detect different virulence genes encoding for EIEC and EAEC.

Pathovar	**Target gene**	**Primer sequence (5' – 3')**	**Product size (bp)**	**References**
EIEC	*ial*	F: GGT ATG ATG ATG ATG AGT CCA R: GGA GGC CAA CAA TTA TTT CC	650	(López-Saucedo *et al.*, 2003)
EAEC	*eagg*	F: AGA CTC TGG CGA AAG ACT GTA TC R: ATG GCT GTC TGT AAT AGA TGA GAA C	194	(Pass *et al.*, 2000)
***E. coli* toxin**	*asta*	F: CCA TGA CAA CGG ACA GCA GTT R: CCT GTC AAC TGA GCA CTT TG	106	(Kimata *et al.*, 2005)

EIEC, enteroinvasive *E coli*; EAEC, enteroaggregative *E coli*; F: forward primer; R: reverse primer

Reaction C

Table 2.5. Primer sequences for the duplex PCR assay to detect different virulence genes encoding for EPEC.

Pathovar	Target gene	Primer sequence (5' – 3')	Product size (bp)	References
Atypical EPEC	*eaeA*	F: CTG AAC GGC GAT TAC GCG AA R: CCA GAC GAT ACG ATC CAG	917	(Aranda *et al.*, 2004)
Typical EPEC	*bfpA*	F: AAT GGT GCT TGC GCT TGC TGC R: TAT TAA CAC CGT AGC CTT TCG CTG AAG TAC CT	410	(Omar and Barnard, 2014)

EPEC, enteropathogenic *E coli*; F: forward primer; R: reverse primer

Reaction D

Table 2.6. Primer sequences for the duplex PCR assay to detect different virulence genes encoding for EHEC.

Pathovar	Target gene	Primer sequence (5' – 3')	Product size (bp)
EHEC of serogroup O157	*rfbE*	F: CGG ACA TCC ATG TGA TAT GG R: TTG CCT ATG TAC AGC TAA TCC	259

EHEC of serogroup O111	*wbdI*	F: TAG AGA AAT TAT CAA GTT AGT TCC R: ATA GTT ATG AAC ATC TTG TTT AGC	406

EHEC, enterohemorrhagic *E. coli*; F: forward primer; R: reverse primer

Reaction E

Table 2.7. Primer sequences for the PCR assay to detect and amplify virulence genes encoding for ETEC.

Pathovar	Target gene	Primer sequence (5' – 3')	Product size (bp)	References
ETEC	*Lt1*	F: TGG ATT CAT CAT GCA CCA CAA GG R: CCA TTT CTC TTT TGC CTG CCA TC	360	(Pass *et al.*, 2000)

ETEC, enterotoxigenic *E. coli*; F: forward primer; R: reverse primer

For all PCR assays, amplification was performed in the Applied Biosystems MiniAmp thermal cycler by applying the cycling conditions: an initial activation step at 95°C for 15 minutes, 35 cycles of denaturation at 94°C for 45 seconds, annealing at 54°C for 45 seconds, extension at 68°C

for 2 minutes, and a final extension at 72°C for 5 minutes. Positive controls and a no-template control were included in each reaction for quality control to determine the specificity and sensitivity of the PCR assay. The amplicons were separated on a 2% (w/v) agarose gel stained with 10 µL of GelRed. These PCR amplicons were electrophoresed by applying 110 volts for 45 minutes. The size of the PCR products was evaluated using a 100 bp molecular weight marker. Finally, the PCR amplicons were viewed under UV transillumination, and electrophoretic images were captured using the Gel Doc EZ system.

2.11. Serotyping *V. cholerae* isolates

The serotyping procedure was performed using the slide agglutination technique according to the antiserum manufacturer's instructions (Mast Group, UK). In summary, a pure culture of *V. cholerae* was inoculated on nutrient agar (Merck, Darmstadt, Germany) and incubated for 24 hours at 37°C. A loop of bacterial culture was emulsified in 10 µL of 0.85% saline solution (Sigma Aldrich, MO, USA) on a glass slide against a dark background. Thirty microliters of antiserum (Davies Diagnostics, South Africa) were placed onto the emulsified isolate. The reagents were mixed by tilting the slide simultaneously for 60 seconds until agglutination was observed on the slide. The agglutination reactions were repeated three times per antiserum (Ogawa, Inaba, and Bengal) and the reaction outcomes were recorded as indicated in Table 2.8.

Table 2.8. Agglutination reactions of *V. cholerae* per antiserum.

V. cholerae O1 serotype	Ogawa antisera	Inaba antisera	O139 (Bengal) antisera
Ogawa	+	-	-
Inaba	-	+	-

Hikojima	+	+	-
O139	-	-	+
Non-O1	-	-	-

2.12. Detecting toxin production in *V. cholerae* isolates using real-time PCR

A real-time PCR assay targeting the *ctxA* gene was performed to detect toxin production in *V. cholerae*. The primer sequences targeting *V. cholerae* enterotoxin adapted from a study by (Koskela *et al.*, 2009) are listed in Table 2.9. Briefly, genomic DNA concentration and purity were assessed using the NanoDrop 2000 spectrophotometer. For the PCR reaction, 10 µL of 2× PowerTrack SYBR Green Master Mix (Thermo Fisher Scientific, MA, USA), 0.5 µL of yellow buffer (Thermo Fisher Scientific, MA, USA), 1 µL of each primer (10 µM), 5.5 µL of nuclease-free water, and 2 µL of genomic DNA were added to make a final volume of 20 µL. Amplification was performed in a Roche LightCycler 480II (Roche Diagnostics, Germany) . The *V. cholerae* O1 serotype Ogawa (NCTC 8021) reference strain was used as a positive control and no template control was included in each reaction for quality validation. Amplification plots as well as Cp values were observed after 40 cycles.

Table 2.9. Primer sequences for the detection of *V. cholerae* enterotoxin.

Pathogen	Target gene	Primer sequence (5' – 3')	Product size (bp)	References
V. cholerae	*ctxA*	*F*: ACT CAC TCT GTC CTC TTG GCA TAA	67	(Koskela *et al.*, 2009)

R: GCA GAT TCT AGA CCT CCT GAT GAA AT

F: forward primer; R: reverse primer

2.13. Whole genome sequencing (WGS) of *E. coli*, *V. cholerae*, and *Shigella* isolates

Genomic DNA was extracted from culture-confirmed *E. coli*, *V. cholerae*, and *Shigella* isolates using the QIAmp DNA Kit (Qiagen, GmbH, Germany). The concentrations of genomic DNA were determined using the Qubit dsDNA assay HS (High Sensitivity) Assay Kit (Thermo Fisher, MA, USA). The DNA libraries were prepared using the Rapid Barcoding Kit 24 V14 (SQK114.24) (Oxford Nanopore) and loaded into the MInION flow cell (Oxford Nanopore) following the manufacturer's protocol. Sequencing reactions were performed on the MinKNOW Mk1B platform using the MinKNOW Software 23.11.2 (Oxford Nanopore). Reference-based genome assemblies were constructed from the raw reads using the bacterial genome workflow on the EPI2ME software 5.1.10. The reference genomes used to map the raw reads were retrieved from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/datasets/genome/) and are listed in Table 2.10.

Table 2.10. List of the reference strains with genome accession number used for genome assembly.

Reference strain	Accession number
***Escherichia coli* strain K-12 sub strain MG1655**	U00096.3
***Shigella flexneri* 2a strain 301**	AE005674.2
***Shigella sonnei* strain SE6-1**	CP055292.1
***Shigella boydii* strain FDAARGOS_1139**	CP068090.1
***Vibrio cholerae* strain RFB16**	CP043554.1

2.14. Bioinformatic tools for analysis of WGS data

2.14.1 Species confirmation

Species identification of the assembled genomes was performed using SpeciesFinder 2.0 (https://cge.food.dtu.dk/services/SpeciesFinder/) (Larsen *et al.*, 2014) and PathogenWatch 19.6 (https://cgps.gitbook.io/pathogenwatch/). Identification of pathogenicity towards a human host was determined using PathogenFinder 1.1 software (https://cge.food.dtu.dk/services/PathogenFinder/) (Cosentino *et al.*, 2013).

2.14.2. Bacterial Typing

The assembled genomes were analyzed to identify the sequence types using the MLST 2.0.9 program (https://cge.food.dtu.dk/services/MLST/) as well as core genome MLST using the cgMLST 1.2 software (https://cge.food.dtu.dk/services/cgMLSTFinder/). The serotypes were determined using SerotypeFinder 2.0 software (https://cge.food.dtu.dk/services/SerotypeFinder/) for *E. coli* and *Shigella*; and CholeraeFinder 1.0 (https://cge.food.dtu.dk/services/CholeraeFinder/) for *V. cholerae*. FimTyper 1.0 (https://cge.food.dtu.dk/services/FimTyper/) (Roer *et al.*, 2017) was used to predict the *E. coli* Fim types.

2.14.3. Antimicrobial resistance, virulence genes, and pathogenic islands

Identification of acquired antibiotic-resistant genes among *E. coli, Shigella,* and *V. cholerae* genomes was performed using ResFinder 4.4.3 software (https://cge.food.dtu.dk/services/ResFinder/) (Camacho *et al.*, 2009; Bortolaia *et al.*, 2020) and KmerResistance 2.2 software (https://cge.food.dtu.dk/services/KmerResistance/) (Clausen *et al.*, 2016; Clausen, Aarestrup and Lund, 2018). Additionally, the presence of virulence genes was

surveyed using the VirulenceFinder 2.0.5 software (https://cge.food.dtu.dk/services/VirulenceFinder/) (Joensen *et al.*, 2014; Malberg Tetzschner *et al.*, 2020). Lastly, the *V. cholerae* genomes were screened for the presence of Pathogenic Islands using CholeraeFinder 1.0 software (https://cge.food.dtu.dk/services/CholeraeFinder/).

2.14.4. Phylogenetic analysis

To examine the relationship between the isolates collected from the Jukskei River and those from human, environmental, and food samples in South Africa, a total of 33 genomes available from Enterobase (https://enterobase.warwick.ac.uk/) were selected at random for comparative analysis. These genomes included 10 *E. coli*, 6 *S. flexneri*, 1 *S. sonnei*, and 16 *V. cholerae*. Detailed information is depicted in Table 2.11.

Table 2.11. Genome information of the 33 bacterial strains from the Enterobase database.

Species	Assembly Barcode	Serotype	Source	Location	Sample collection date
Escherichia coli	ESC_IC2195AA	O127 H6	Human	South Africa	2023
Escherichia coli	ESC_CC1101AA	O157	Human	South Africa	2021
Escherichia coli	ESC_DC9937AA	O5	Human	South Africa	2020
Escherichia coli	ESC_EC0538AA	O1	Human	South Africa	2022
Escherichia coli	ESC_FC6463AA	O3	Food	South Africa	2021
Escherichia coli	ESC_JC7512AA	O26	Human	South Africa	2023
Escherichia coli	ESC_FC6464AA	O91	Human	South Africa	2022

Escherichia coli	ESC_IC2199AA	O6	Human	South Africa	2023
Escherichia coli	ESC_JC7513AA	O151	Human	South Africa	2023
Escherichia coli	ESC_CC5230AA	O63	Human	South Africa	2022
Shigella sonnei	ESC_MC5129AA	-	Human	South Africa	2023
Shigella flexneri	ESC_NC4245AA	Type 2a	Human	South Africa	2024
Shigella flexneri	ESC_LB7644AA	2a	Human	South Africa	2020
Shigella flexneri	ESC_NC4243AA	Type 1B	Human	South Africa	2024
Shigella flexneri	ESC_LB7643AA	2a	Human	South Africa	2020
Shigella flexneri	ESC_NC4242AA	Type 3a	Human	South Africa	2024
Shigella flexneri	ESC_HB6236AA	Sf6	Human	South Africa	2019
Vibrio cholerae	VIB_EA6538AA	O1	Human	South Africa	2023
Vibrio cholerae	VIB_EA6539AA	O1	Human	South Africa	2023
Vibrio cholerae	VIB_EA7921AA	O1	Human	South Africa	2024
Vibrio cholerae	VIB_EA7922AA	O1	Human	South Africa	2024
Vibrio cholerae	VIB_EA6212AA	O1	Human	Souh Africa	2023
Vibrio cholerae	VIB_EA6213AA	O1	Human	South Africa	2023
Vibrio cholerae	VIB_EA6214AA	O1	Human	South Africa	2023
Vibrio cholerae	VIB_EA6216AA	O1	Human	South Africa	2023
Vibrio cholerae	VIB_DA9044AA	Non O1	Environment	South Africa	2020
Vibrio cholerae	VIB_EA0364AA	Non O1	Environment	South Africa	2020
Vibrio cholerae	VIB_EA0366AA	Non O1	Food	South Africa	2020
Vibrio cholerae	VIB_EA0367AA	Non O1	Food	South Africa	2020
Vibrio cholerae	VIB_EA0373AA	Non O1	Food	South Africa	2019

Vibrio cholerae	VIB_EA0374AA	Non O1	Human	South Africa	2019
Vibrio cholerae	VIB_EA0376AA	Non O1	Environment	South Africa	2019
Vibrio cholerae	VIB_EA0377AA	Non O1	Environment	South Africa	2019

For each species, the nucleotide sequences were aligned using a multiple sequence alignment tool DNAMAN version 4.03 (Lynnon Biosoft, CA, USA). Thereafter, a neighbour-joining tree was constructed using DNAMAN version 4.0 with a bootstrapping stringency of 1000. The phylogenetic trees were viewed using TreeViewer.

2.15. Statistical analysis

The data were collected and captured using Microsoft Excel® 2010 data software. For bacterial enumeration, statistical analysis was performed using the non-parametric test with the GraphPad Prism software (GraphPad Software Inc, CA, USA) as the data was not normally distributed. Bacterial counts from the quantitative analysis were expressed as ± standard deviation of the mean and differences in bacterial counts between the sampling sites were assessed by one-way ANOVA followed by Dunn's multiple comparison analysis test. Data were regarded as statistically significant when $p < 0.05$. Descriptive statistics were used to describe qualitative data.

CHAPTER 3: RESULTS

3.1. Physio-chemical analysis of the water samples from Jukskei River

A total of 80 water samples were collected from the Jukskei River from January to November 2023 along five different collection sites (Two samples per collection site eight times). The physio-chemical properties of the water samples at the different collection sites are listed in Table 3.1 below. The temperature ranged between 19 to 23°C. The average pH of the water samples ranged between 6.93 and 7.4, with the highest pH levels observed in Dainfern. The oxidative reduction potential in the water samples across the collection sites ranged between 239.7 and 263.7 mV. The amount of oxygen in the water measured as dissolved oxygen saturation, was at an average between 3 and 4.7% across all five collection sites. The average electrical conductivity was lower in Bruma and Gilloolys at 0.14 ms/cm and 0.19 ms/cm, respectively, and slightly higher in Malboro, Bucchluech, and Dainfern ranging between 0.3 and 0.4 ms/cm. Lastly, the total dissolved solids were 70 ppm in Bruma, 93.9 ppm in Gilloolys, 180.3 ppm in Malboro, 193.2 ppm in Buccleuch, and 181 ppm in Dainfern.

Table 3.1. Mean values of physio-chemical properties of water samples per location site.

Physio-chemical Properties									
Location	Temperature (°C)	pH	ORP (mV)	DO Sat. (%)	DO Conc. (mg/L)	EC (ms/m)	Resistivity (mΩ/cm)	TDS (mg/L)	Salinity (psu)
Bruma	19	7.31	263.7	4.7	0.44	14	0.0071	70	0.01
Giloolly's	19	6.97	251	4.1	0.4	19	0.0053	93.3	0.09
Marlboro	20	6.93	258.7	3	0.28	36	0.0028	180.3	0.18
Buccluech	20	6.94	256.8	3.1	0.3	40	0.0026	193.2	0.2
Dainfern	23	7.4	239.7	4.1	0.4	30	0.0036	181	0.16

ORP, Oxidation Reduction Potential; DO Sat., Dissolved Oxygen Saturation; DO Conc., Dissolved Oxygen Concentration; EC, Electrical Conductivity; TDS, Total Dissolved Solids. *Red highlighted values indicate parameters falling below stipulated guidelines by the DWAF.

3.2. Quantification of microbial contamination levels in the Jukskei River

3.2.1. Enumeration of *E. coli*

All 80 water samples were contaminated with *E. coli* at different microbial loads across the five collection sites. The mean counts for *E. coli* were 5.23 $\log_{10}$ CFU/mL in Bruma, 4.95 $\log_{10}$ CFU/mL in Giloollys, 6.52 $\log_{10}$ CFU/mL in Marlboro, 5.95 $\log_{10}$ CFU/mL in Buccleuch, and 4.94 $\log_{10}$ CFU/mL in Dainfern (Figure 3.1). These counts exceeds the acceptable levels for *E. coli* in drinking water which should be 0 CFU/ml (DWAF, 1996). The p-value as measured by the one-way ANOVA was 0.00655 (Appendix D) across all sites indicating statistical significance (p<0.05). According to Dunn's multiple comparison test which identifies which pairs of means are

significantly different from each other (Dinno, 2015), statistical significance was found between Giloollys-Marlboro as well as Marlboro-Dainfern (Appendix D).

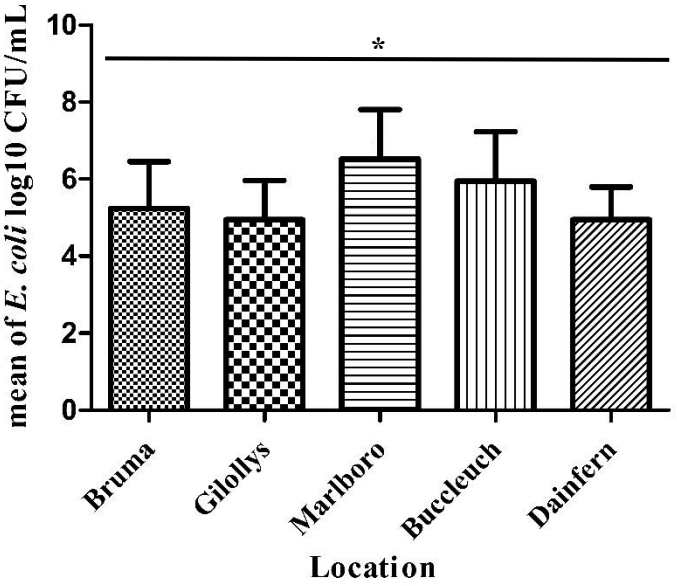

Figure 3.1. Counts of presumptive *E. coli* isolates (in log10 CFU/mL) on MacConkey-Sorbitol agar per location. The * indicates statistical significance (p<0.05) amongst all locations. Statistical significance was observed between Giloolly-Marlboro and Marlboro-Dainfern.

3.2.2. Enumeration of *Shigella*

The water samples collected from the Jukskei River were also contaminated with different concentrations of *Shigella* across the five collection sites. The mean counts for *Shigella* in Bruma, Giloollys, Marlboro, Buccleuch, and Dainfern were 5.38 $\log_{10}$ CFU/mL, 5.32 $\log_{10}$ CFU/mL, 6.12 $\log_{10}$ CFU/mL, 5.46 $\log_{10}$ CFU/mL, and 5.35 $\log_{10}$ CFU/mL respectively (Figure 3.2). Marlboro had the highest contamination levels compared to the other collection sites. The p-value determined by one-way ANOVA was 0.0019 (Appendix D) indicating statistical significance in the microbial load at the different collection sites. Upon performing a Dunn's multiple comparison

analysis significant differences were observed between Bruma-Marlboro, Giloollys-Marlboro, and Marlboro-Dainfern (Appendix D). According to the WHO, Shigella should not be detected in drinking water (WHO, 2022).

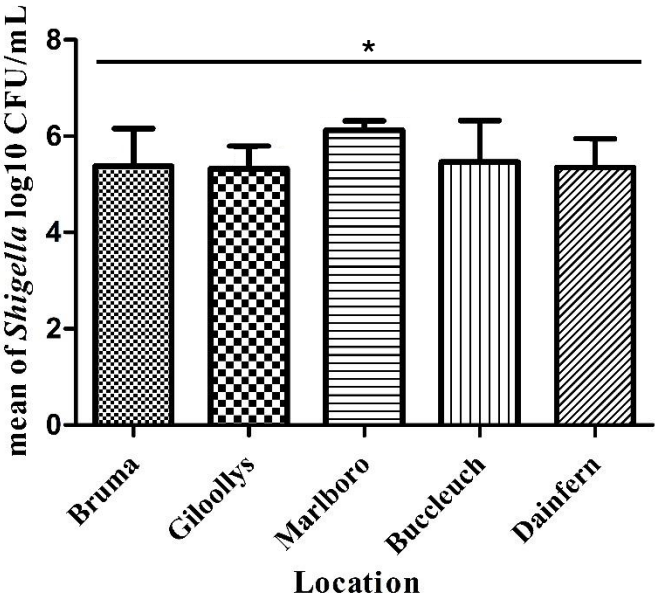

Figure 3.2. Counts of presumed *Shigella* colonies (in log10 CFU/mL) on Xylose Lysine Deoxycholate (XLD) agar per location. The * indicates statistical significance (p<0.05) amongst all locations. Statistical significance was observed between Bruma-Giloollys, between Giloollys-Marlboro, and between Marlbor-Dainfern.

3.2.3. Enumeration of *V. cholerae*

Vibrio cholerae was also detected in varying loads across the five collection sites along the Jukskei River. However, the contamination levels were much lower compared to *E. coli* and *Shigella*. The mean counts for *V. cholerae* were 2.4 $\log_{10}$ CFU/mL in Bruma, 2.02 $\log_{10}$ CFU/mL in Giloollys, 4.4 $\log_{10}$ CFU/mL in Marlboro, 3.88 $\log_{10}$ CFU/mL Buccleuch, and 2.3 $\log_{10}$ CFU/mL in Dainfern (Figure 3.3). Overall Marlboro had the highest microbial counts compared to the other collection

sites. Moreover, there was a statistically significant difference between the *V. cholerae* counts in the five collection sites with a p-value of 0.0489 (Appendix D). According to the WHO, *V. cholerae* should not be detected in drinking water (WHO, 2022).

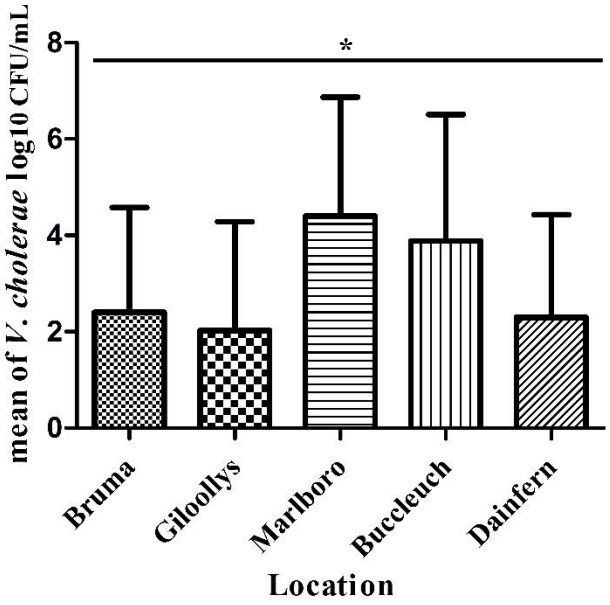

Figure 3.3. Counts of presumed *V. cholerae* colonies (in log10 CFU/mL) on Thiosulfate Citrate Bile salt Sucrose (TCBS) agar per location. The * is indicative of statistical significance (p<0.05).

3.3. Qualitative detection of *E. coli*, *Shigella* isolates, and *V. cholerae* from water samples

3.3.1. Detection using real-time PCR of *uidA*, *ipaH*, and *toxR* genes

The *uidA, ipaH,* and *toxR* genes were detected in all 80 water samples collected from the five different collection sites along the Jukskei River using real-time PCR (Figure 3.4 to 3.6) confirming the presence of *E. coli*, *Shigella,* and *V. cholerae* in the Jukskei River. Furthermore, all water samples also detected the *ctxA* gene which codes for toxin production in *V. cholerae* (Figure

3.7). The Cp values ranged between 19 and 32 for the *uidA* gene (Figure 3.4), 16 and 32 for the *ipaH* gene (Figure 3.5), 27 and 30 for the *toxR* gene (Figure 3.6), and 22 and 30 for the *ctxA* gene (Figure 3.7) (Appendix F).

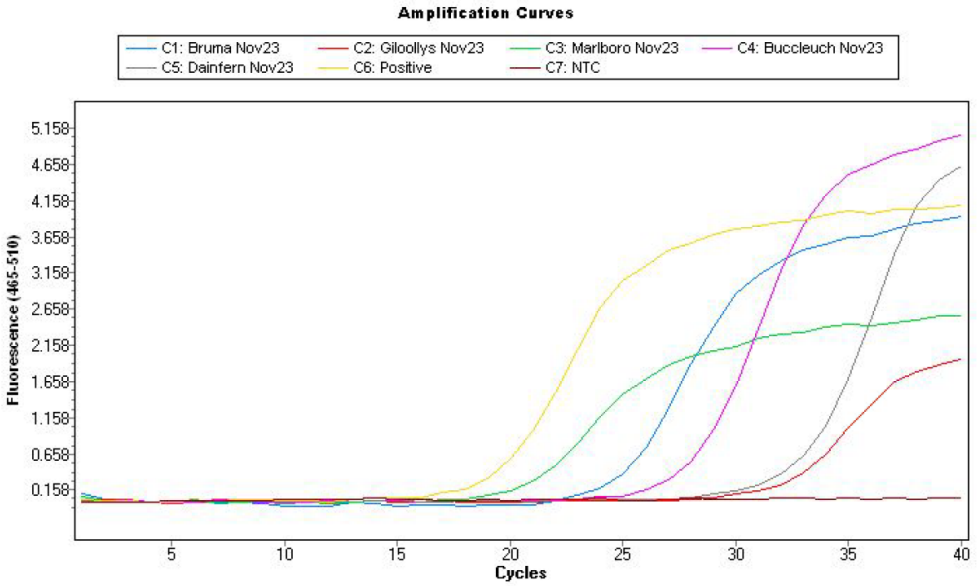

Figure 3.4. Amplification curves indicating amplification of the *E. coli uidA* gene by real-time PCR. C1 to C5 are the water samples showing positive results, C6 is the positive control and C7 is the no template control.

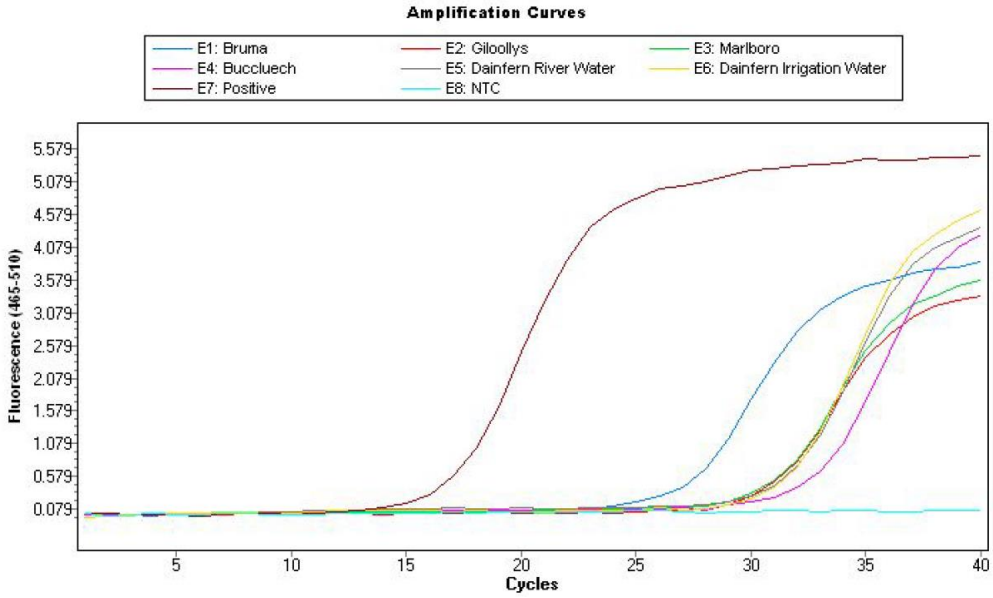

Figure 3.5. Amplification curves indicating amplification of the *ipaH* gene of *Shigella* isolates from water samples using real-time PCR. E1 to E6 are the water samples showing positive results, E7 is the positive control, and E8 is the no-template control.

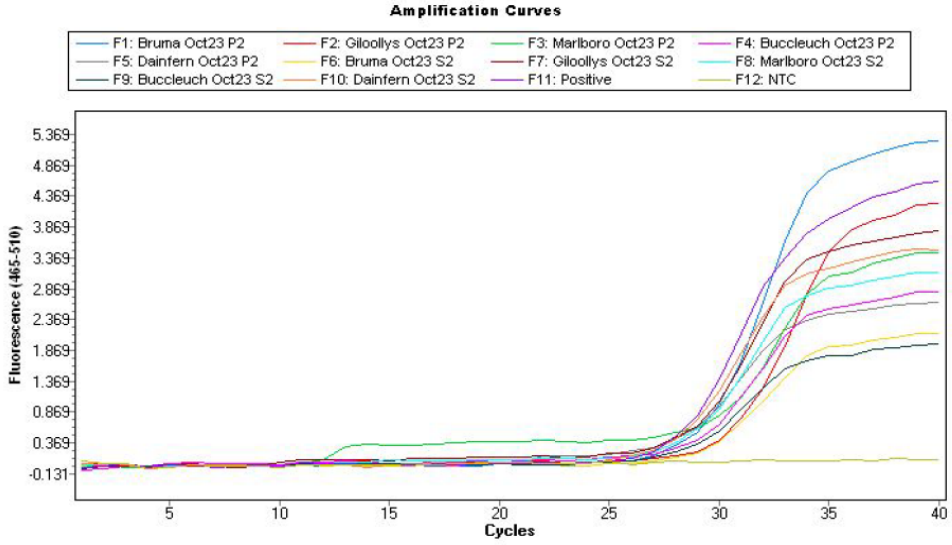

Figure 3.6. Amplification curves indicating amplification of the *toxR* gene of *V. cholerae* from water samples using real-time PCR. F1 to F10 are the water samples indicating positive results, F11 is the positive control, and F12 is the no template control.

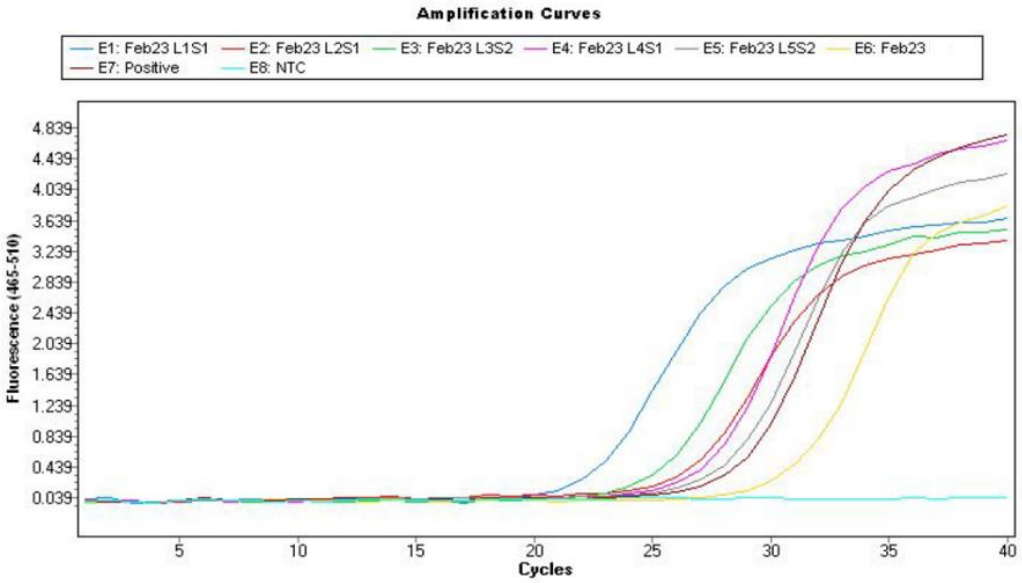

Figure 3.7. Amplification of the *ctxA* gene for *V. cholerae* detection from water samples using real-time PCR. E1 to E6 are the water samples indicating positive results. E1 is Bruma, E2 is Giloollys, E3 is Marlboro, E4 is Buccleuch, E5 is Dainfern, and E6 is Dainfern sample 2. E7 is the positive control, and E8 is the no-template control.

3.3.2. Confirmation of presumptive *E. coli*, *Shigella,* and *V. cholerae* using biochemical tests

From the 80 water samples collected from January to November 2023, a total of 43 presumptive *E. coli* on MacConkey-sorbitol agar (Figure 3.8C), 28 presumptive *shigella* on XLD agar (Figure 3.8D), and 38 presumptive *V. cholerae* on TCBS agar (Figure 3.8A) underwent biochemical tests for confirmation. Of the 43 *E. coli*, 16 were oxidase positive and 27 were oxidase negative. 27 *E. coli* were indole positive and 16 were indole negative (Table 3.7). For presumptive *Shigella*, 16 isolates were oxidase negative and 12 oxidase positive. Twelve isolates were oxidase positive and 16 were indole positive (Table 3.7). Lastly, the 43 *V. cholerae* were colorless colonies on aesculin bile agar (Figure 3.8B). Moreover, 24 *V. cholerae* isolates were oxidase positive and 14 were

oxidase negative. Twenty-four were indole positive and 14 were indole negative (Table 3.7). Thus, an overall of 27 *E. coli*, 16 *Shigella*, and 24 *V. cholerae*, were confirmed using biochemical tests.

Table 3.2. Indole and oxidase tests of presumptive *E. coli*, *Shigella*, and *V. cholerae* isolates.

Presumptive colonies		**Indole**		**Oxidase**	
Isolate		Positive	Negative	Positive	Negative
E. coli	43	**27**	16	16	**27**
Shigella	28	12	**16**	12	**16**
V. cholerae	38	**24**	14	**24**	14

E. coli is positive for indole and negative oxidase; *Shigella is* negative for indole and oxidase*; V. cholerae* is positive for indole and oxidase.

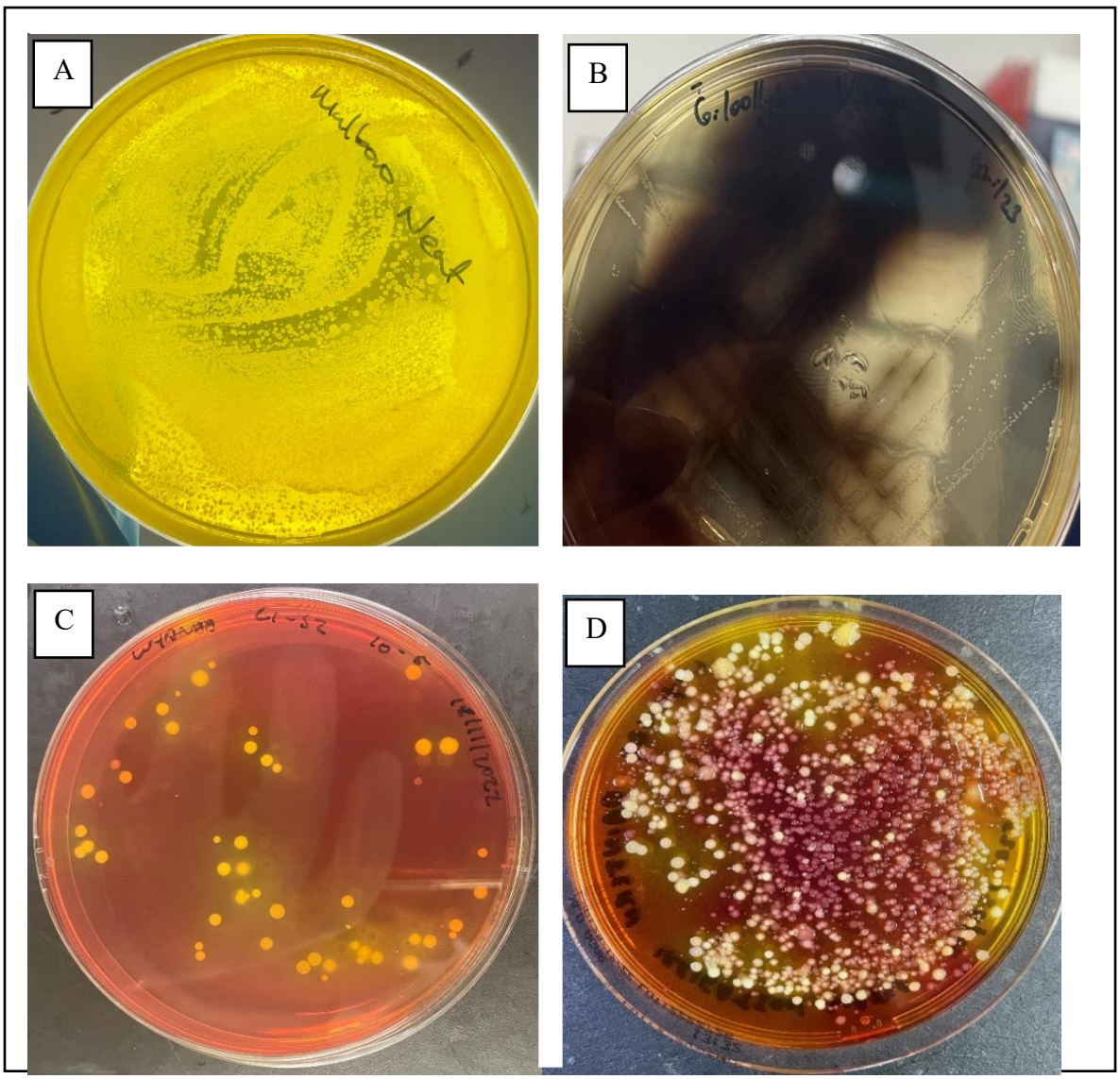

Figure 3.8. *V. cholerae* spread on TCBS agar [A]. *V. cholerae* on aesculine bile agar [B]. *E. coli* on MacConkey-Sorbitol agat [C]. Mixed plate of *Shigella* and *E. coli* on XLD agar [D].

3.3.3. Confirmation of *E. coli*, *Shigella*, and *V. cholerae* using conventional PCR

The 27 *E. coli*, 16 *Shigella,* and 24 *V. cholerae* were confirmed further using conventional PCR assays targeting the *uidA* gene (556 bp), *ipaH* gene (606 bp), and *toxR* gene (68 bp). The *uidA* gene was detected in all 27 *E. coli* isolates (Figure 3.9), the *ipaH* gene was detected in 14 *Shigella*

isolates except for 2 isolates which were negative for the *ipaH* gene (Figure 3.10), and 24 *V. cholerae* isolates were positive for the *toxR* gene (Figure 3.11).

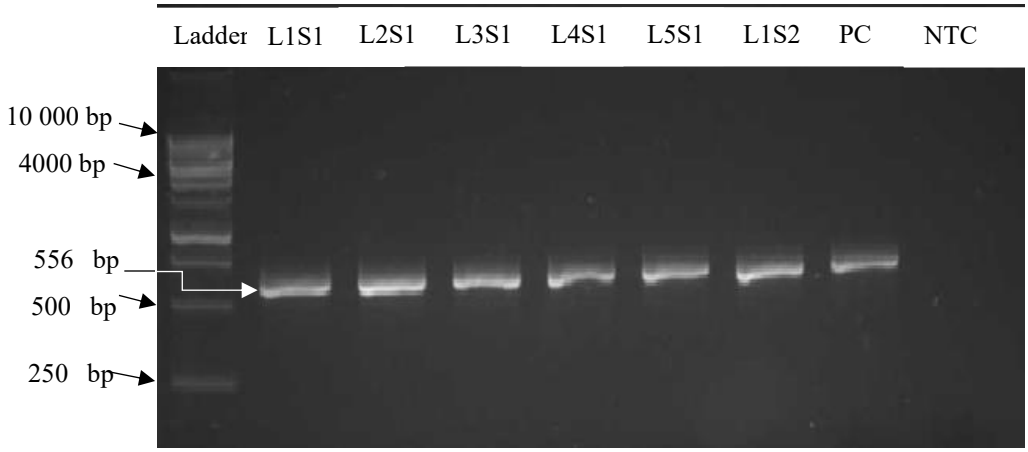

Figure 3.9. A 2% (w/v) agarose gel showing amplification of the *uidA* gene (556 bp) for the confirmation of *E. coli* isolates cultured on nutrient agar. A 1kb DNA molecular weight marker (Promega) was used to size the PCR products (Lane 1). The lanes; L1S1, L2S1, L3S1, L4S1, L5S1, and L1S2 are samples isolated from Bruma, Giloollys, Marlboro, Buccleuch, and Dainfern, respectively. Lane 7 is the positive control (*Escherichia coli* ATCC 35218) and Lane 8 is the NTC (No template control).

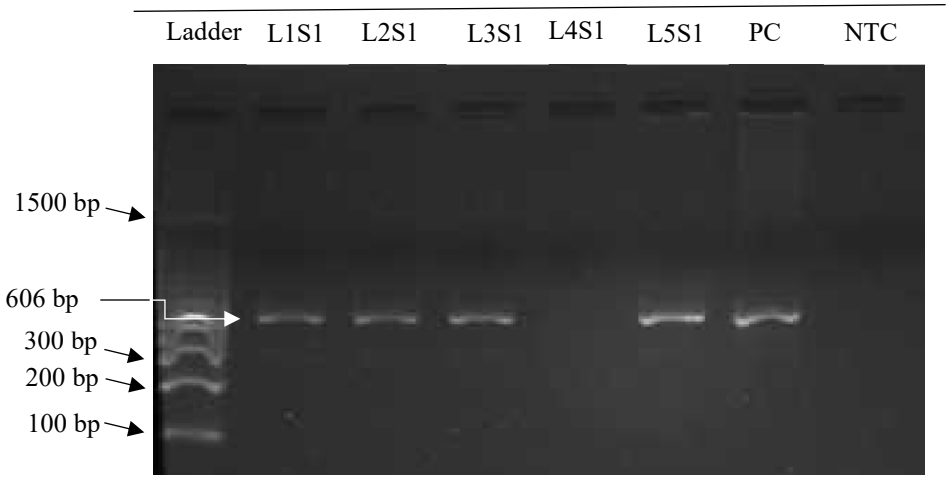

Figure 3.10. A 2% (w/v) agarose gel showing amplification of the *ipaH* gene (606 bp) of *Shigella* isolates cultured on nutrient agar. A 100 bp DNA molecular weight marker (Promega) was used to size the PCR products (Lane 1). The lanes; L1S1, L2S1, L3S1, L4S1, and L5S1 are isolated water samples in Bruma, Giloollys, Marlboro, Buccleuch, and Dainfern, respectively. Lane 6 is the positive control (*Shigella flexneri* ATCC 8021) and lane 7 is the NTC.

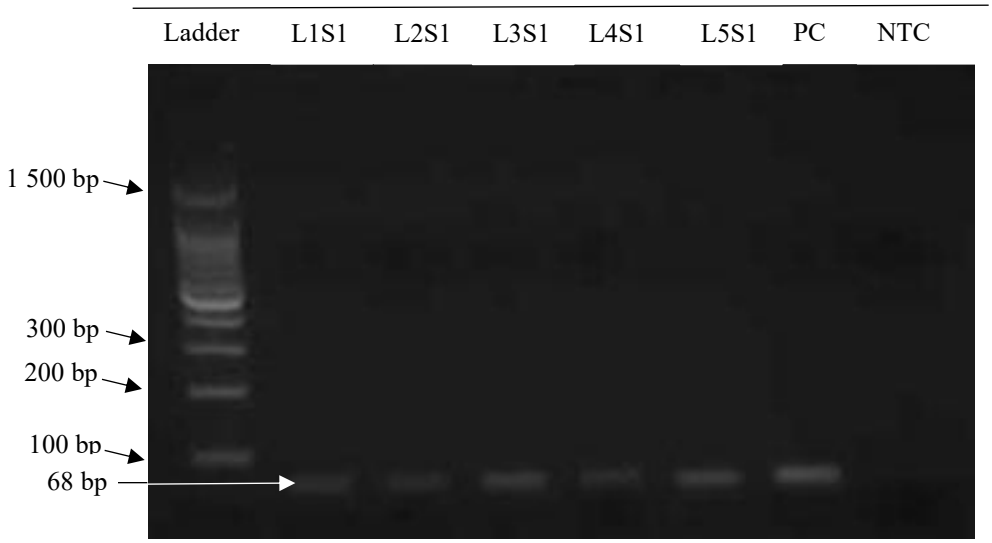

Figure 3.11. A 2% (w/v) agarose gel showing amplification of the *toxR* gene (68 bp) for *V. cholerae* identification from presumptive colonies. A 100 bp DNA molecular weight marker (Promega) was used to size the PCR products (Lane 1). The lanes; L1S1, L2S1, L3S1, L4S1, and L5S1 are water samples isolated in Bruma, Giloollys, Marlboro, Buccleuch, and Dainfern, respectively. Lane 6 is the positive control (*Vibrio cholerae* NCTC 8021) and lane 7 is the NTC.

3.4. Characterization of *E. coli* isolates from the Jukskei River

3.4.1. *Escherichia coli* pathotypes

The 27 confirmed *E. coli* isolates underwent multiplex PCR assays to determine the virulence genes associated with Diarrheagenic *E. coli*. Of the 27 *E. coli* isolates, 12 were positive for the *Lt1* gene, found in enterotoxigenic *E. coli* (ETEC). The 12 ETEC isolates were collected from Marlboro and Buccleuch (Table 3.3). One *E. coli* isolate was positive for the *eaeA* gene and thus

regarded as an atypical enteropathogenic *E. coli* (EPEC). The remaining *E. coli* isolates were negative for all the virulent genes associated with diarrheagenic *E. coli* (Table 3.3).

Table 3.3. *E. coli* isolates with results of virulence genes multiplex PCR assays.

	Pathovar										
	EHEC			**EHEC O157**	**EHEC O11**	**EIEC**	**EAEC**	**ETEC**	**EPEC**		***E. coli* toxin**
Isolate	*eaeA*	*Stx1*	*Stx2*	*rfbE*	*wbdl*	*Ial*	*eagg*	*Lt1*	*eaeA*	*bfpA*	*asta*
***E. coli* Bruma February 2023**	-	-	-	-	-	-	-	-	-	-	-
***E. coli* Bruma April 2023**	-	-	-	-	-	-	-	-	-	-	-
***E. coli* Bruma July 2023**	-	-	-	-	-	-	-	-	-	-	-
***E. coli* Bruma October 2023**	-	-	-	-	-	-	-	-	-	-	-
***E. coli* Bruma November 2023**	-	-	-	-	-	-	-	-	-	-	-
***E. coli* Giloollys February 2023**	-	-	-	-	-	-	-	-	-	-	-
***E. coli* Giloollys April 2023**	-	-	-	-	-	-	-	-	-	-	-
***E. coli* Giloollys May 2023**	-	-	-	-	-	-	-	-	-	-	-

***E. coli* Giloollys July**	-	-	-	-	-	-	-	-	-	-	-
***E. coli* Giloollys October 2023**	-	-	-	-	-	-	-	-	-	-	-
***E. coli* Giloollys November 2023**	-	-	-	-	-	-	-	-	-	-	-
***E. coli* Marlboro February 2023**	-	-	-	-	-	-	-	+	-	-	-
***E. coli* Marlboro April 2023**	-	-	-	-	-	-	-	+	-	-	-
***E. coli* Marlboro May 2023**	-	-	-	-	-	-	-	+	-	-	-
***E. coli* Marlboro July 2023**	+	-	-	-	-	-	-	+	+	-	-
***E. coli* Marlboro November**	-	-	-	-	-	-	-	+	-	-	-
***E. coli* Buccleuch February 2023**	-	-	-	-	-	-	-	+	-	-	-
***E. coli* Buccleuch April 2023**	-	-	-	-	-	-	-	+	-	-	-
***E. coli* Buccleuch May 2023**	-	-	-	-	-	-	-	+	-	-	-

E. coli **Buccleuch July 2023**	-	-	-	-	-	-	-	+	-	-	-
E. coli **Buccleuch October 2023**	-	-	-	-	-	-	-	+	-	-	-
E. coli **Buccleuch November 2023**	-	-	-	-	-	-	-	+	-	-	-
E. coli **Dainfern February 2023**	-	-	-	-	-	-	-	-	-	-	-
E. coli **Dainfern April 2023**	-	-	-	-	-	-	-	-	-	-	-
E. coli **Dainfern July 2023**	-	-	-	-	-	-	-	-	-	-	-
E. coli **Dainfern October 2023**	-	-	-	-	-	-	-	-	-	-	-
E. coli **Dainfern November 2023**	-	-	-	-	-	-	-	-	-	-	-

EHEC, enterohemorrhagic *E. coli*; EIEC, enteroinvasive *E. coli*; EAEC, enteroaggregative *E coli;* ETEC, enterotoxigenic *E. coli;* EPEC, enteropathogenic *E. coli.*

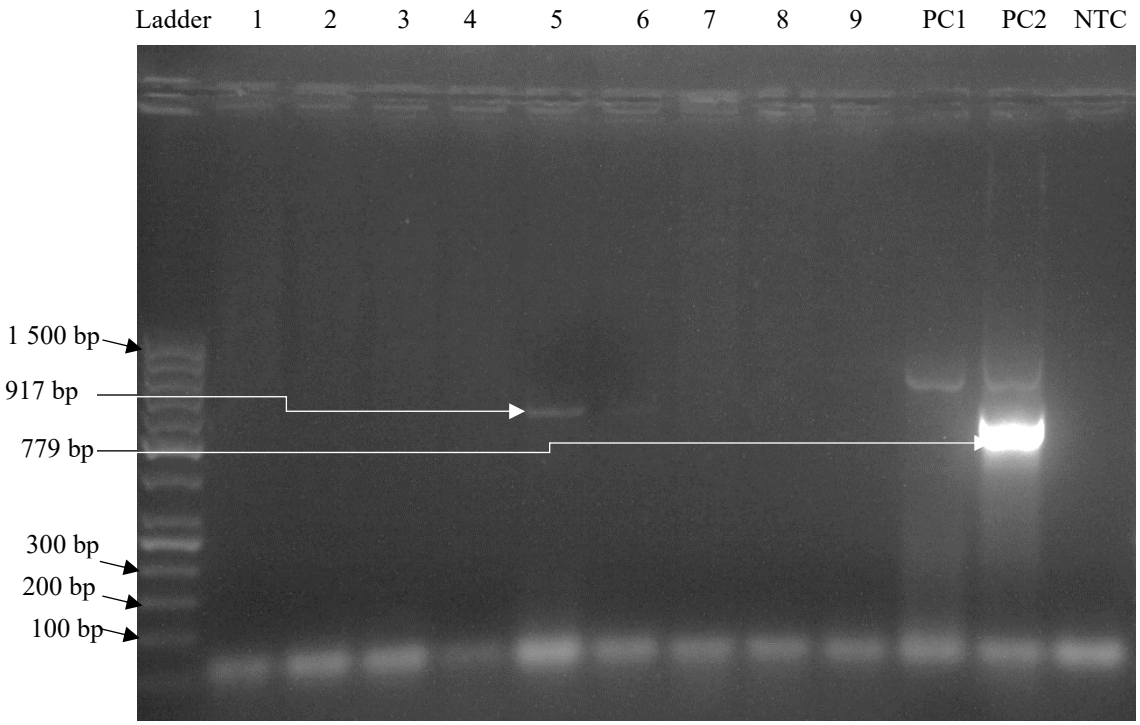

Figure 3.12. A 2% (w/v) agarose gel of a multiplex amplification of the *eaeA* gene (917 bp), *Stx1* gene (617 bp), and *Stx2* gene (779) for the identification of EHEC and atypical EPEC from *E. coli* isolates. A 100 bp DNA molecular weight marker (Promega) was used to size the PCR products (Lane 1). Samples 1, 2, 3, 4, 6, 7,8, and 9 were negative for the EHEC. These were *E. coli* the following isolates: *E. coli* Giloollys July 2023, *E. coli* Giloollys October 2023, *E. coli* Marlboro February 2023, *E. coli* Marlboro April 2023, *E. coli* Marlboro November 2023, *E. coli* Buccleuch February 2023, *E. coli* Buccleuch April 2023, and *E. coli* Buccleuch May 2023. Sample 5- *E. coli* Marlboro July 2023, was positive for the *eaeA* gene. PC1 is the EHEC positive control with the *eaeA* PC2 is the EHEC positive control with *eaeA* and *stx2* gene present. Lane 13 is the NTC.

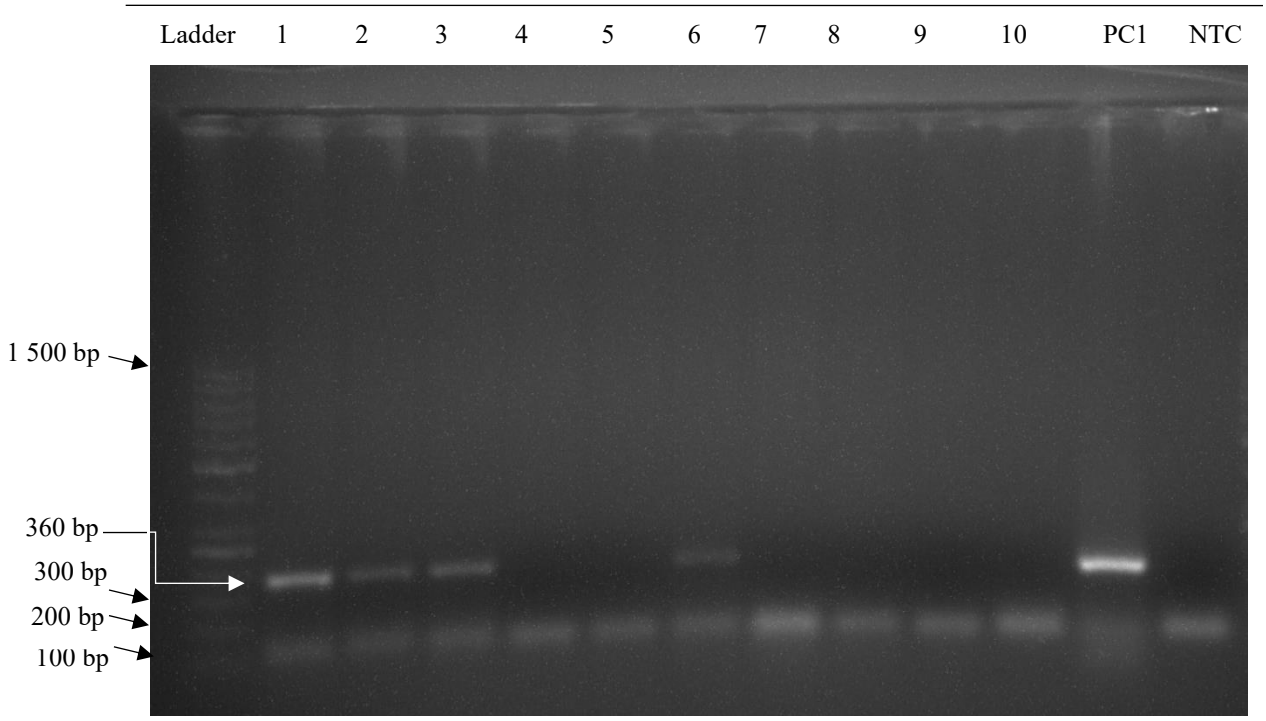

Figure 3.13. A 2% (w/v) agarose gel showing amplification of the *Lt1* gene (360 bp) for ETEC identification from *E. coli* isolates. A 100 bp DNA molecular weight marker (Promega) was used to size the PCR products (Lane 1). Samples 1, 2, 3, and 6 were positive for the *Lt1* gene. These were *E. coli* Buccleuch July 2023, *E. coli* Buccleuch October 2023, Buccleuch November 2023, and Marlboro October 2023, respectively. Samples 4, 5, 7, 8, 9, and 10 were negative for the *Lt1* gene. These included *E. coli* Dainfern February 2023, *E. coli* Dainfern April 2023, Dainfern July 2023, Bruma February 2023, and Bruma July 2023, respectively. PC is the ETEC positive control and lane 13 is the NTC.

3.4.2. *Escherichia coli* typing using WGS Data: Serotypes, FimType, MLST, and cgMLST

All 27 *E. coli* were confirmed as *E. coli* using the SpeciesFinder pipeline on the Centre for Genomic Epidemiology bioinformatics tool (Appendix G), the genome sizes ranged between 4.23Mb to 4.43Mb which is the expected genome size for *E. coli*. Furthermore, the 27 *E. coli* are human pathogens according to the PathogenFinder pipeline (Appendix G). The 27 *E. coli* were further divided into seven different serotypes and 59% of the isolates (n=18) belong to the O16:H48 serotype, followed by O16:H25 and O16 serotypes at 11% respectively (Figure 3.14).

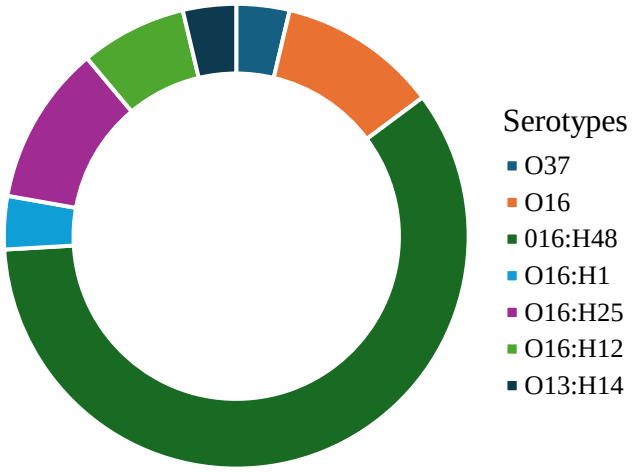

Figure 3.14. Pie chart showing the serotypes of *E. coli* isolates. The *E. coli* isolates were determined using the SerotypeFinder pipeline on the Centre for Genomic Epidemiology bioinformatics tool.

Amongst the 27 *E. coli* isolates, eight FimTypes were detected using the FimTyper pipeline (Appendix G). Most isolates had fimH27 (55%), followed by fimH34 and fimH305 at 11% (Figure 3.15).

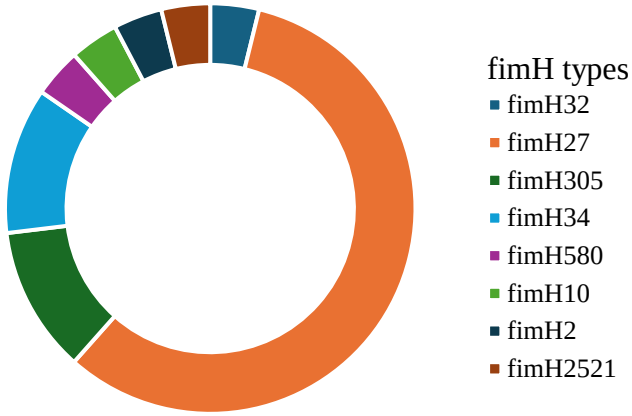

Figure 3.15. Pie chart of the fimH types detected in the 27 *E. coli* isolates. The *E. coli* serotypes were identified using the FimTyper pipeline on the Centre for Genomic Epidemiology bioinformatics tool.

The *E. coli* isolates collected from the Jukskei River are very diverse and belong to 23 different sequence types, with the most abundant being ST1946 (Figure 3.16). Additionally, the core genome MLST (cgMLST) profile was determined for all the *E. coli* isolates, and analyses classified these isolates into 14 distinct cgMLST types (Figure 3.17). The most abundant being cgMLST1597 (30%; n= 10), followed by cgMLST10176 and cgMLST12392 at (11%; n= 3), respectively, lastly cgMLST1112 and cgMLST149 at (7%; n= 2), respectively.

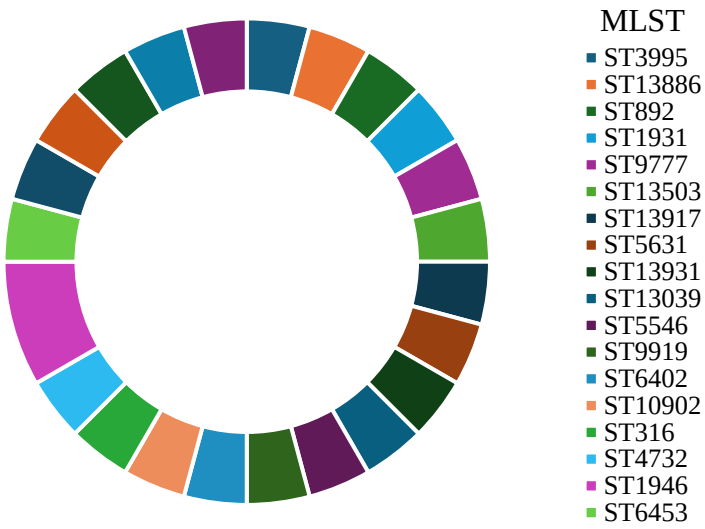

Figure 3.16. Pie chart of the sequence types of the 27 *E. coli* isolates. The *E. coli* strains were determined using the MLST pipeline on the Centre for Genomic Epidemiology bioinformatics tool which is based on the 7 loci MLST scheme.

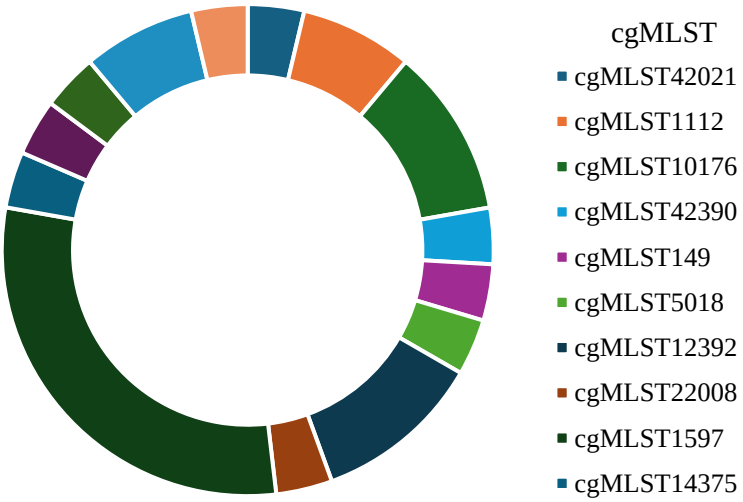

Figure 3.17. Pie chart of the cgMLST of the 27 *E. coli* isolates. The *E. coli* serotypes were determined using the cgMLSTFinder pipeline on the Centre for Genomic Epidemiology Bioinformatics tool which is based on the 2,513 loci scheme.

3.4.3. Virulence genes and antimicrobial resistance profiles

The following virulent genes were found across all isolates; *csgA, fimH, gad, hlyE, iss, nlpl, ompT, yehA, yehB, yehC,* and *yehD* (Table 3.4). Other virulent genes such as *AslA*, *terC*, *usp*, *fdeC*, and *ipfA*, among other genes, were unique to some *E. coli* strains. Eighteen *E. coli* isolates harbored both *AslA* and *terC* genes. Interestingly, one *E. coli* from Marlboro harbored *ipaH*, *ipaH9.8*, *ShiA*, *SigA*, *sitA*, and other unique virulent genes compared to the rest of the isolates. This isolate is the enteroinvasive *E. coli* (EIEC). The antibiotic resistance to quinolone, aminoglycosides, and macrolides was found in two *E. coli* isolates collected from Bruma and Marlboro respectively. The *E. coli* from Marlboro was resistant to aminoglycosides and macrolides. Moreover, the folate pathway antagonist profile was identified in one isolate from Dainfein (Table 3.4). The majority of the isolates did not harbor any antibiotic resistance profiles.

Table 3.4. Virulence and antibiotic resistance profile of 27 *E. coli* isolated from the Jukskei River

Isolate	Virulence Genes	Antibiotic Resistance
***E. coli* Marlboro November 2023**	*AslA, csgA, fdeC, fimH, gad, hlyE, iss, nlpl, ompT, terC, yehA, yehB, yehC, yehD*	None
***E. coli* Bruma July 2023**	*AslA, csgA, fimH, gad, hlyE, iss, nlpl, ompT, terC, yehA, yehB, yehC, yehD*	Quinolone
***E. coli* Bruma November 2023**	*AslA, csgA, fimH, gad, hlyE, iss, nlpl, ompT, terC, yehA, yehB, yehC, yehD*	None
***E. coli* Buccleuch November 2023**	*csgA, fimH, gad, hlyE, iss, nlpl, ompT, yehA, yehB, yehC, yehD*	None

***E. coli* Bruma October 2023**	*AslA, csgA, fimH, gad, hlyE, iss, nlpl, ompT, terC, yehA, yehB, yehC, yehD*	None
***E. coli* Buccleuch April 2023**	*AslA, csgA, fimH, gad, hlyE, iss, nlpl, ompT, terC, yehA, yehB, yehC, yehD*	None
***E. coli* Dainfern November 2023**	*AslA, csgA, fimH, gad, hlyE, iss, nlpl, ompT, terC, yehA, yehB, yehC, yehD*	None
***E. coli* Dainfern July 2023**	*AslA, csgA, fimH, gad, hlyE, iss, nlpl, ompT, terC, yehA, yehB, yehC, yehD*	None
***E. coli* Buccleuch July 2023**	*AslA, csgA, fimH, gad, hlyE, iss, nlpl, ompT, terC, yehA, yehB, yehC, yehD*	None
***E. coli* Giloollys November 2023**	*AslA, chuA, csgA, fimH, gad, hlyE, iss, ompT, terC, usp, yehA, yehB, yehC, yehD, yfcV*	None
***E. coli* Giloollys October 2023**	*AslA, csgA, fimH, gad, iss, nlpl, ompT, terC, usp, yehA, yehB, yehC, yehD, yfcV*	None
***E. coli* Bruma April 2023**	*csgA, fimH, gad, hlyE, iss, nlpl, ompT, terC, yehA, yehB, yehC, yehD*	None
***E. coli* Dainfern October 2023**	*csgA, fimH, gad, hlyE, iss, nlpl, ompT, terC, yehA, yehB, yehC, yehD*	Folate pathway antagonist
***E. coli* Buccleuch October 2023**	*csgA, fimH, gad, hlyE, iss, nlpl, ompT, yehA, yehB, yehC, yehD*	None
***E. coli* Marlboro July 2023**	*AslA, csgA, fimH, gad, hlyE, iss, ompT, terC, yehA, yehB, yehC, yehD*	None

***E. coli* Giloollys April 2023**	*csgA, fimH, fdeC, gad, ipfA, nlpl, terC, yehB*	None
***E. coli* Marlboro April 2023**	*csgA, fimH, gad, hlyE, ipaH, ipaH9.8, iucC, iutA, ipfA, nlpl, pic, shiA, sigA, sitA, terC, yehA, yehB, yehC, yehD*	Aminoglycosides, macrolides
***E. coli* Giloollys July 2023**	*AslA, fimH, gad, iss, ompT, terC, yehA, yehB, yehC, yehD*	None
***E. coli* Dainfern April 2023**	*AslA, csgA, fimH, gad, hlyE, iss, nlpl, ompT, terC, yehA, yehB, yehC, yehD*	None
***E. coli* Bruma February 2023**	*AslA, csgA, fimH, gad, hlyE, iss, nlpl, ompT, terC, yehA, yehB, yehC, yehD*	None
***E. coli* Giloollys February 2023**	*AslA, csgA, fimH, gad, hlyE, iss, nlpl, ompT, terC, yehA, yehB, yehC, yehD*	None
***E. coli* Marlboro February 2023**	*AslA, csgA, fimH, gad, hlyE, iss, nlpl, ompT, terC, yehA, yehB, yehC, yehD*	None
***E. coli* Buccleuch February 2023**	*AslA, csgA, fimH, gad, hlyE, iss, nlpl, ompT, terC, yehA, yehB, yehC, yehD*	None
***E. coli* Dainfern Februaury 2023**	*csgA, fimH, gad, hlyE, iss, nlpl, ompT, yehA, yehB, yehC, yehD*	None
***E. coli* Marlboro May 2023**	*csgA, fimH, gad, hlyE, iss, nlpl, ompT, yehA, yehB, yehC, yehD*	None
***E. coli* Buccleuch May 2023**	*AslA, csgA, fimH, gad, hlyE, iss, nlpl, ompT, terC, yehA, yehB, yehC, yehD*	None

E. coli **Giloollys May 2023**	*AslA, csgA, fimH, gad, hlyE, iss, nlpl, ompT, terC, yehA, yehB, yehC, yehD*	None

*Virulence genes of interest are in red

3.4.4. Phylogenetic analysis

The evolutionary relationship between the nucleotide sequences of the 27 *E. coli* isolates collected from Jukskei River and ten genome sequences downloaded from enterobase - which were sequenced from clinical samples, were evaluated through a neighbor-joining tree (Figure 3.18). The isolates were grouped into 6 clusters (A to F) on the phylogenetic tree. The *E. coli* isolates from Jukskei were also clustered into three groups (Cluster A, B, and C). Cluster A consisted of one *E. coli* isolate from Giloollys sourced in April 2023. Cluster B has two isolates from Buccleuch and Dainfern, sourced from November 2023 and February 2023, respectively. These isolates are closely related with a bootstrap value of 100%. Cluster B has a paraphyletic relationship with Cluster C, the largest cluster on the tree. Cluster C consists of a polyphyletic grouping of 24 *E. coli* isolates with a bootstrap value of 100%. This cluster consisted of many isolates collected in different locations along the Jukskei River. The *E. coli* isolates from clinical samples clustered into three distinct groups (Cluster D, E, and F) and is genetically distinct from our environmental isolates. Cluster D consists of 6 *E. coli* isolates from NICD with a bootstrap of 82%. Cluster E consists of a single isolate sourced from a clinical specimen in 2020 from NICD. Cluster F consists of a paraphyletic grouping of 3 *E. coli* isolates from NICD, supported by a bootstrap of 98%. These isolates were collected from clinical specimens in 2021.

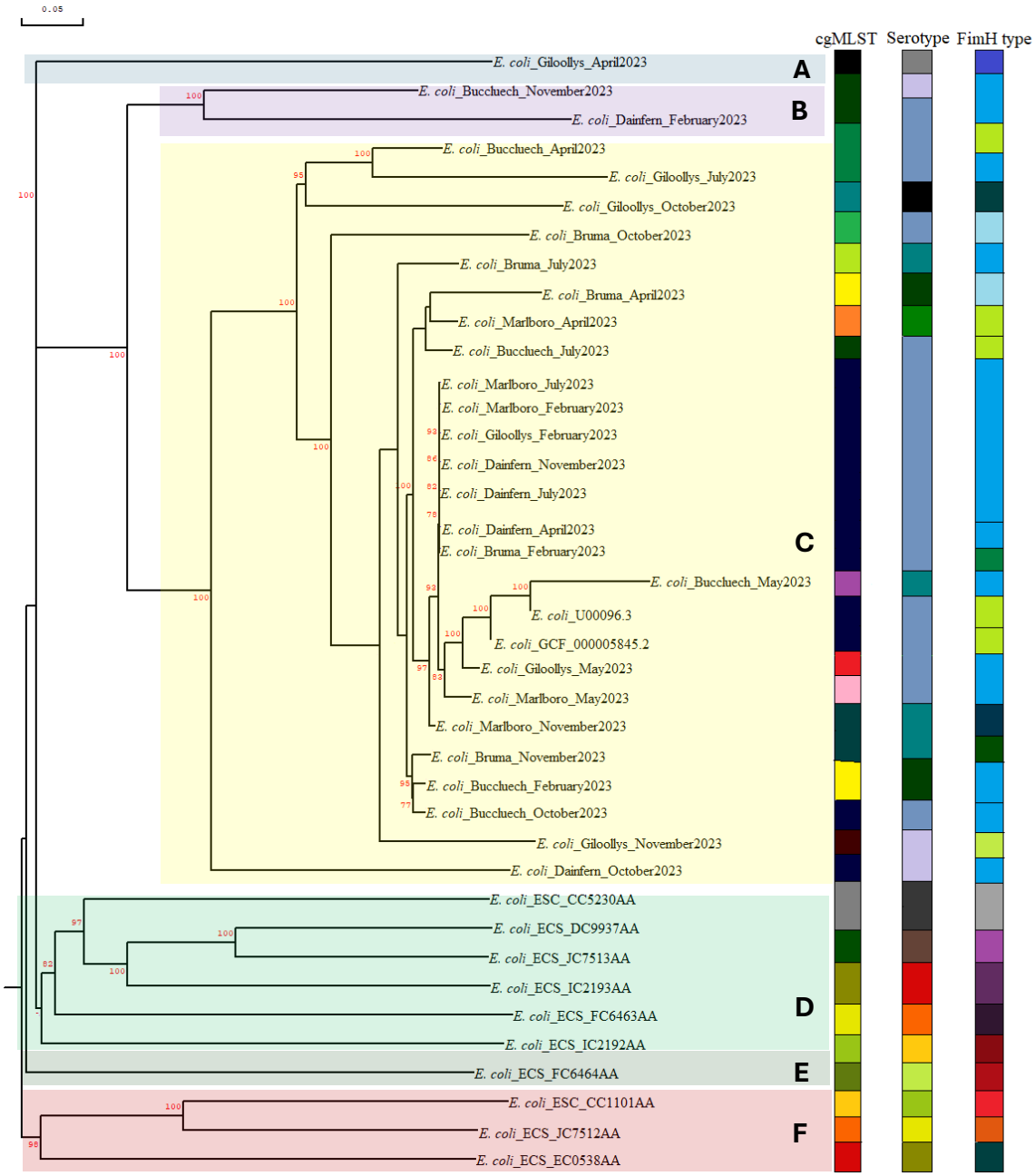

Figure 3.18. Neighbor-joining tree constructed using DNAMAN version 4.0 with a bootstrapping stringency of 1000. The nucleotide sequences of our 27 *E. coli* isolates as well as 10 genome sequences from NICD were aligned using a multiple sequence alignment tool (DNAMAN version 4.03. The branch lengths indicate the genetic distance and are depicted by the bar (0.05). Bootstrap values (percentages of 1000 replications) are indicated at the internodes of the tree. The cgMLST, serotype, and fimH type profiles are also included in the phylogenetic tree to show the diversity of the isolates.

3.5. Characterization of the *Shigella* isolates from the Jukskei River using WGS Data

3.5.1 *Shigella* species

All 14 *Shigella* isolates were confirmed as *Shigella* species using the SpeciesFinder pipeline on the Centre for Genomic Epidemiology bioinformatics tool. The genome sizes ranged between 4 and 4.48 Mb, the expected genome size for *Shigella*. 86% of the isolates were *Shigella flexneri* (n = 12), 7% were *Shigella boydii* (n= 1) and *Shigella sonnei* (n=1), respectively (Figure 3.19).

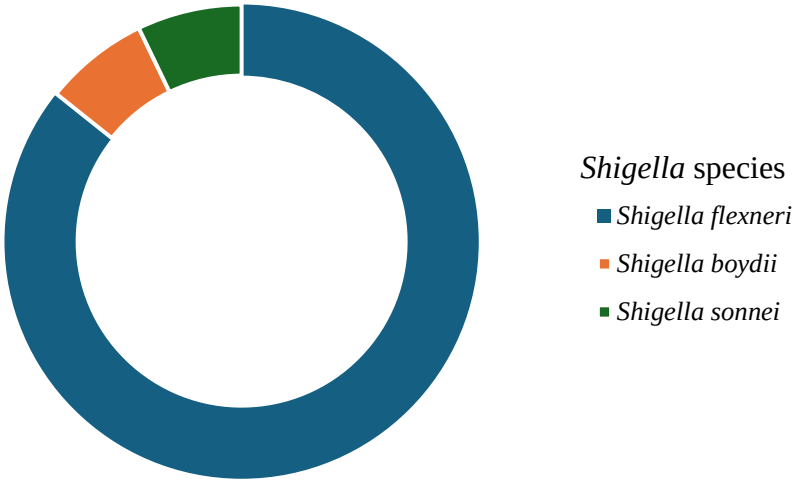

Figure 3.19. Pie chart of the 14 *Shigella* species. The *Shigella* species were determined using the SpeciesFinder pipeline on the Centre for Genomic Epidemiology bioinformatics tool which is based on the 16s rRNA-based species identification.

3.5.2. *Shigella* typing: serotypes, MLST, and cgMLST

The 14 *Shigella* species were identified as type 2a serotypes and separated further into 14 distinct sequence types based on the 7 *loci* MLST scheme (Figure 3.20). Additionally, the core genome MLST (cgMLST) profile of 2,513 loci was only determined for 14 isolates, and analyses classified

these isolates into 2 distinct cgMLST types namely, cgMLST1472 (80%; n= 11), and cgMLST5907 (14%; n=2). The remaining isolate had an unknown cgMLST profile (Figure 3.21).

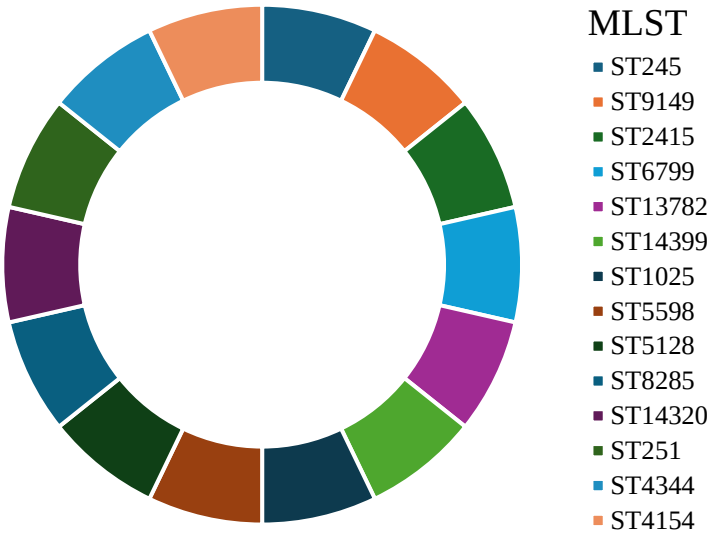

Figure 3.20. Pie chart of the MLST of the 14 *Shigella* isolates. The serotypes were determined using the MLSTFinder pipeline on the Centre for Genomic Epidemiology bioinformatics tool based on the 7 loci MLST scheme.

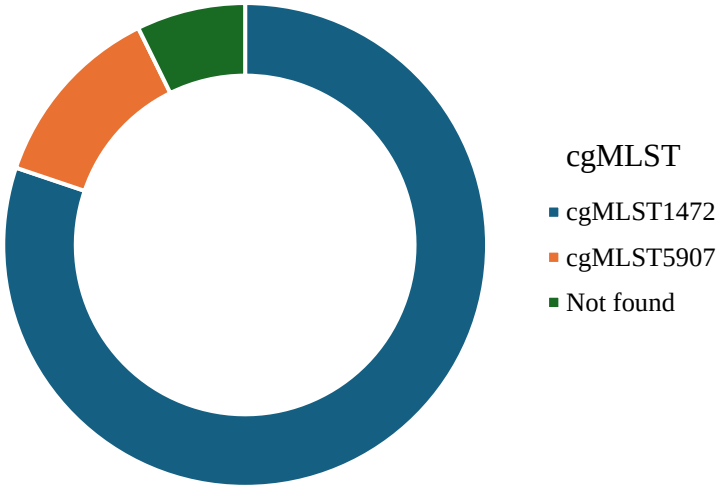

Figure 3.21. Pie chart of the cgMLST of the 14 *Shigella* isolates. The *Shigella* serotypes were determined using the cgMLSTFInder Finder pipeline on the Centre for Genomic Epidemiology bioinformatics tool based on the 2,513 loci scheme.

3.5.3. Virulence and antibiotic resistance genes

There were 21 virulence genes found in varying frequency across the 14 *Shigella* isolates. These genes include *csgA, fimH, gad, hlyE, ipaH, ipaH, ipaH9.8, iucC, iutA, ipfA, nlpl, pic, shiA, shiB, terC, sitA, yehA, yehB, yehC, yehD,* and *yfcv.* One isolate, a *S. sonnei* from Buccleuch, harbored the *ycfv* gene. Only one *Shigella* isolate, a *S. flexneri* isolated from Giloollys in May, harbored antibiotic-resistant genes, namely *gyrA*, *parC*, and *varG*. The rest of the isolates did not harbor any antibiotic-resistance genes (Table 3.5).

Table 3.5. Virulence and antibiotic resistance profile of the confirmed 14 *Shigella* genomes.

Isolate	Virulence Genes	Antibiotic Resistance
***S. flexneri* Buccleuch October 2023**	*csgA, fimH, gad, hlyE, ipaH, ipaH9.8, iucC, iutA, ipfA, nIpI, pic, shiA, sig, sigA, sitA, terC, yehA, yehB, yehC, yehD*	None
***S. flexneri* Dainfern July 2023**	*csgA, fimH, gad, hlyE, ipaH, ipaH9.8, iucC, iutA, ipfA, pic, shiA, shiB, sigA, terC, yehA, yehB, yehC, yehD*	None
***S. flexneri* Dainfern November 2023**	*fimH, gad, hlyE, ipaH, ipaH9.8, iucC, iutA, ipfA, nIpI, pic, shiA, shiB, sigA, sitA, terC, yehA, yehB, yehC, yehD*	None
***S. flexneri* Dainfern October 2023**	*csgA, fimH, gad, ipaH, ipaH9.8, iutC, ipfA, nIpI, pic, shiA, shiB, sigA, sitA, terC, yehA, yehB, yehC, yehD*	None
***S. flexneri* Giloollys July 2023**	*csgA, fimH, gad, hlyE, ipaH, ipaH9.8, iucC, iutA, ipfA, pic, shiA, shiB, sigA, sitA, terC, yehA, yehB, yehC, yehD*	None
***S. flexneri* Giloollys May 2023**	*csgA, fimH, gad ipaH, ipaH9.8, iucC, iutA, ipfA, nIpI, pic, shiA, shiB, sigA, terC*	*gyrA, parC, varG*
***S. flexneri* Giloollys November 2023**	*csgA, fimH, gad, hlyE, ipaH, ipaH9.8, iucC, iutA, ipfA, nIpI, pic, shiA, shiB, sigA, sitA, yehA, yehB, yehD*	None
***S. flexneri* Giloollys October 2023**	*csgA, fimH, gad, hlyE, ipaH, ipaH9.8, iucC, iutA, ipfA, nIpI, pic, shiA, shiB, sigA, sitA, yehA, yehB, yehD*	None
***S. flexneri* Marlboro November 2023**	*csgA, fimH, gad, hlyE, ipaH, ipaH9.8, iucC, iutA, ipfA, pic, shiA, shiB, sigA, sitA, terC, yehC, yehD*	None

***S. flexneri* Marlboro October 2023**	*csgA, gad, hlyE, ipaH, ipaH9.8, iucC, iutA, ipfA, nIpI, pic, shiA, shiB, sigA, sitA, terC, yehA, yehB, yehC, yehD*	None
***S. boydii* Buccleuch October 2023**	*csgA, fimH, gad, hlyE, iha, ipaH, ipaH9.8, iucC, iutA, yerc, terC, yehA, yehB, yehC, yehD*	None
***S. flexneri* Buccleuch July 2023**	*csgA, fdeC, fimH, gad, hlyE, ipfA, nIpI, terC, yehA, yehB, yehC, yehD*	None
***S. flexneri* Dainfern May 2023**	*csgA, fimH, gad, hlyE, ipaH, ipaH9.8, iucC, iutA, ipfA, nIpI, pic, shiA, shiB, sitA, terC, yehA, yehB, yehC, yehD*	None
***S. sonnei* Buccleuch April 2023**	*csgA, fimH, hlyE, nIpI, ompT, terC, yehA, yehB, yehC, yehD, yfcV*	None

*Virulence genes of interest are in red

3.5.4. Phylogenetic analysis

The evolutionary relationship between the nucleotide sequences of the 14 *Shigella* isolates collected from Jukskei River and seven nucleotide sequences from clinical specimens was evaluated through a neighbor-joining tree (Figure 3.22). The isolates were grouped into 4 distinct clusters (A to D) on the phylogenetic tree. Cluster A consists of two *S. flexneri* isolates from NCID, sourced from clinical specimens. These isolates are closely related to each other with a bootstrap value of 80%. Cluster B, the second and largest cluster, consists of a polyphyletic grouping of 12 *S. flexneri* isolates with a bootstrap value of 99%. The isolates in this cluster were collected in different locations along the Jukskei River. Cluster C has a monophyletic grouping of three *S. flexneri* isolates and one *S. sonnei* from NICD. These isolates were collected from clinical specimens in 2020, 2023, and 2024. The *S. flexneri* and *S. sonnei* are closely related, supported by a bootstrap value of 99%. Cluster D is comprised of a paraphyletic grouping of *S. flexneri* from

NICD, *S. boydii reference genome* (CP068090.1), and an *S. boydii* isolate from Buccleuch. This was supported by a bootstrap value of 80%. Interestingly, one *S. flexneri* isolates collected from Buccleuch clustered, within *S. sonnei* isolate as well as a *S. sonnei* reference genome (CP05592.1) in cluster E with a 100% bootstrap value. This isolate is likely to have been misidentified as a *S. flexneri* and may warrant further investigation.

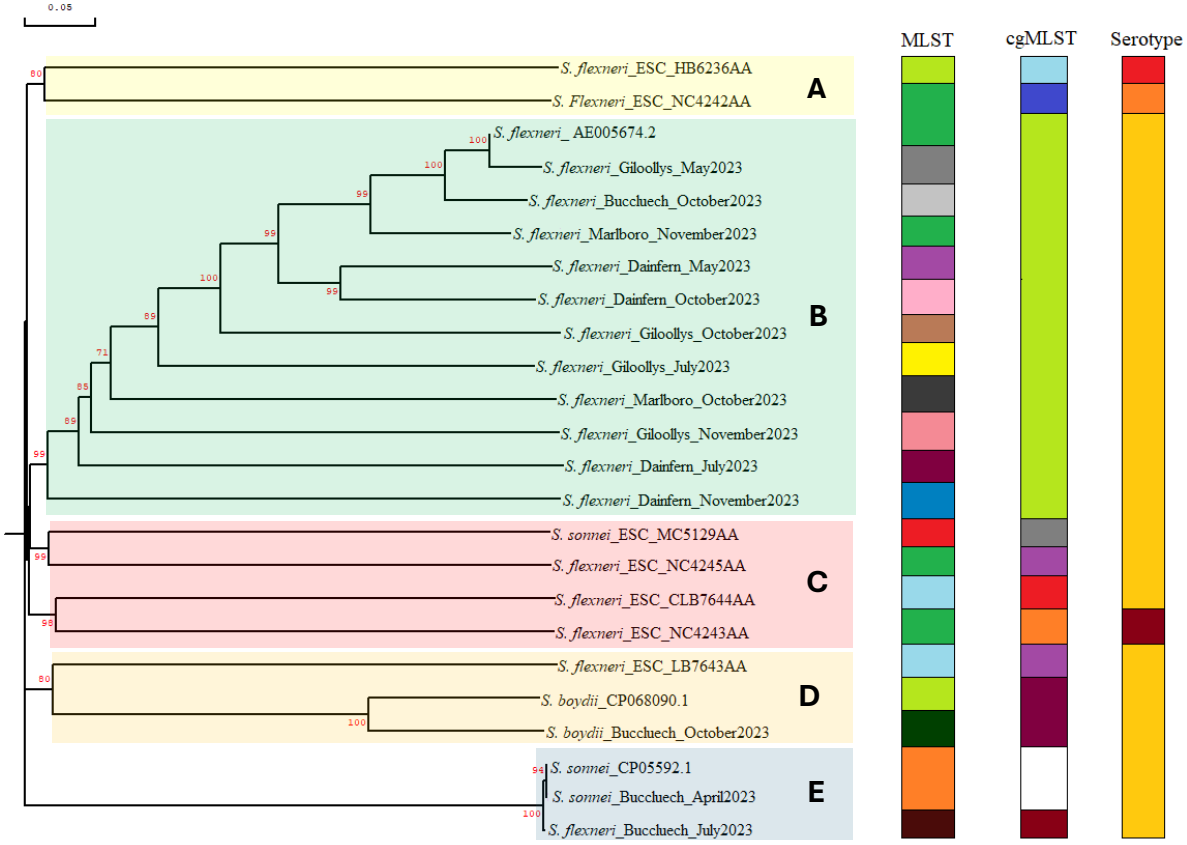

Figure 3.22. Neighbor-joining tree constructed using DNAMAN version 4.0 with a bootstrapping stringency of 1000. The nucleotide sequences of our 14 *Shigella* isolates as well as 10 *Shigella* genome sequences from NICD were aligned using a multiple sequence alignment tool (DNAMAN version 4.03. The branch lengths indicate the genetic distance and are depicted by the bar (0.05). Bootstrap values (percentages of 1000 replications) are indicated at the internodes of the tree. The cgMLST, serotype, and MLST profiles are also included in the phylogenetic tree to show the diversity of the isolates.

3.6. Characterization of the *V. cholerae* isolates collected from the Jukskei River

3.6.1 Serotyping *V. cholerae* using agglutination antisera and WGS data

The 24 *V. cholerae* isolates were negative for the Ogawa, Inaba, and O139 (Table 3.6.) and were classified as non-O1/ O139 serotypes. The results mirror WGS data as none of the 24 *V. cholerae* isolates had the serogroup-specific *rfbV* and *wbfZ* genes for O1 and O139, respectively (Appendix G). Furthermore, none of the 24 isolates harbored the *El Tor* biotype gene, however, all the isolates were positive for the species-specific *ompW* gene (Appendix G). Therefore, the 24 isolates collected from the Jukskei River were *V. cholerae* non-O1/O139.

Table 3.6. Agglutination reactions of *V. cholerae* isolates per antiserum.

	Agglutination reactions		
Isolate	Ogawa antisera	Inaba antisera	O139 (Bengal) antisera
***V. cholerae* Bruma April 2023**	-	-	-
***V. cholerae* Bruma May 2023**	-	-	-
***V. cholerae* Bruma July 2023**	-	-	-
***V. cholerae* Bruma October 2023**	-	-	-
***V. cholerae* Bruma November 2023**	-	-	-
***V. cholerae* Giloollys April 2023**	-	-	-
***V. cholerae* Giloollys May 2023**	-	-	-
***V cholerae* Giloollys October 2023**	-	-	-
***V. cholerae* Giloollys November 2023**	-	-	-
***V. cholerae* Giloollys February 2023**	-	-	-
***V. cholerae* Giloollys April 2023**	-	-	-
***V. cholerae* Giloollys July 2023**	-	-	-
***V. cholerae* Giloollys October 2023**	-	-	-

***V. cholerae* Giloollys November 2023**	-	-	-
***V. cholerae* Buccleuch February 2023**	-	-	-
***V. cholerae* Buccleuch April 2023**	-	-	-
***V. cholerae* Buccleuch May 2023**	-	-	-
***V. cholerae* Buccleuch July 2023**	-	-	-
***V. cholerae* Buccleuch October 2023**	-	-	-
***V. cholerae* Buccleuch November 2023**	-	-	-
***V. cholerae* Dainfern April 2023**	-	-	-
***V. cholerae* Dainfern May 2023**	-	-	-
***V. cholerae* Dainfern July 2023**	-	-	-
***V. cholerae* Dainfern November 2023**	-	-	-

3.6.2. *V. cholerae* typing: MLST and cgMLST

There was no diversity observed amongst the 24 *V. cholerae* isolates from Jukskei River as all 24 isolates were ST741 and cgMLST1309 (Figure 3.23).

3.6.3. Virulence, pathogenic islands, and antimicrobial resistance genes

Nine virulent genes were detected in the 24 *V. cholerae* isolates from the Jukskei River (Table 3.7). Of the 24 *V. cholerae* isolates, 23 had the *toxR*, *vspD*, *vsN2*, *als*, *hlyA*, *vcsC2*, *makA*, *rtxA*, and *vcsV2* virulent genes. The one isolate collected in Giloollys in February 2023 harbored only the *hlyA*, *makA*, *vscN2*, *vspD*, and *vcsC2* virulent genes (Table 3.7). Only two types of pathogenic islands were found amongst all 24 *V. cholera* isolates namely; VP12_VC1758, and VP12_VC1776 (Table 3.7), all 24 isolates harbored both the pathogenic islands. Antibiotic resistance genes were detected in 20 of the 24 *V. cholera* isolates. The antimicrobial resistance genes identified were *parC*, *varG*, and *gyrA* (Table 3.7). Three isolates namely *V. cholerae* Giloollys April 2023, *V.*

c*holerae* Buccleuch April 2023, and *V. cholerae* November 2023 harbored all three antibiotic-resistant genes and the remaining 17 isolates harbored two antibiotic-resistance genes (*parC, varG*).

Table 3.7. Virulence and antibiotic resistance profile of the confirmed 24 *V. cholerae* isolates collected from the Jukskei River.

Isolate	Virulence Genes	Pathogenic Island	Antibiotic Resistance
***V. cholerae* Bruma April 2023**	*toxR, vspD, vcsN2, als, hlyA, vcsC2, makA, rtxA, vcsV2*	VPI2_VC1758, VPI2_VC1776	*parC, varG*
***V. cholerae* Bruma May 2023**	*rtxA, vcsN2, vcsC2, vcsV2, toxR, hlyA, makA, als, vspD*	VPI2_VC1758, VPI2_VC1776	*gyrA, varG*
***V. cholerae* Bruma July 2023**	*als, vcsV2, rtxA, vcsN2, vcsC2, toxR, makA, vspD, hlyA*	VPI2_VC1776, VPI2_VC1758	*parC, varG*
***V. cholerae* Bruma October 2023**	*hlyA, vcsN2, rtxA, vcsC2, vcsV2, vspD, makA, als, toxR*	VPI2_VC1776, VPI2_VC1758	*parC, varG*
***V. cholerae* Bruma November 2023**	*als, vcsC2, toxR, vspD, makA, vcsV2, vcsN2, rtxA, hlyA*	VPI2_VC1758, VPI2_VC1776	*None*
***V. cholerae* Giloollys April 2023**	*hlyA, toxR, vspD, als, vcsC2, vcsV2, makA, rtxA, vcsN2*	VPI2_VC1776, VPI2_VC1758	*gyrA, parC, varG*
***V. cholerae* Giloollys May 2023**	*hlyA, als, toxR, makA, rtxA, vcsV2, vspD, vcsN2, vcsC2*	VPI2_VC1758, VPI2_VC1776	*gyrA, varG*
***V cholerae* Giloollys October 2023**	*rtxA, vspD, vcsV2, hlyA, als, toxR, vcsN2, makA, vcsC2*	VPI2_VC1776, VPI2_VC1758	*parC,varG*
***V. cholerae* Giloollys November 2023**	*toxR, als, vcsV2, vcsN2, hlyA, makA, vcsC2, vspD, rtxA*	VPI2_VC1776, VPI2_VC1758	*parC, varG*

***V. cholerae* Giloollys February 2023**	*hlyA, makA, vcsN2, vcsV2, vspD, vcsC2*	VPI2_VC1776, VPI2_VC1758	*gyrA, varG*
***V. cholerae* Giloollys April 2023**	*toxR, makA, hlyA, rtxA, vcsC2, vcsN2, als, vspD, vcsV2*	VPI2_VC1758, VPI2_VC1776	*gyrA, varG*
***V. cholerae* Giloollys July 2023**	*toxR, vspD, hlyA, vcsN2, rtxA, makA, vcsV2, vcsC2, als*	VPI2_VC1758, VPI2_VC1776	*None*
***V. cholerae* Giloollys October 2023**	*toxR, makA, vcsV2, rtxA, vcsC2, als, hlyA, vspD, vcsN2*	VPI2_VC1776, VPI2_VC1758	*parC, varG*
***V. cholerae* Giloollys November 2023**	*vcsV2, vcsC2, toxR, vspD, makA, rtxA, vcsN2, als, hlyA*	VPI2_VC1776, VPI2_VC1758	*parC, varG*
***V. cholerae* Buccleuch February 2023**	*makA, vspD, rtxA, vcsC2, als, toxR, hlyA, vcsN2, vcsV2*	VPI2_VC1776, VPI2_VC1758	*parC, varG*
***V. cholerae* Buccleuch April 2023**	*vcsN2, hlyA, vspD, toxR, als, vcsV2, vcsC2, rtxA, makA*	VPI2_VC1776, VPI2_VC1758	*parC, gyrA, varG*
***V. cholerae* Buccleuch May 2023**	*toxR, vcsN2, vcsC2, vcsV2, als, makA, rtxA, hlyA, vspD*	VPI2_VC1758, VPI2_VC1776	*parC, varG*
***V. cholerae* Buccleuch July 2023**	*vcsV2, toxR, rtxA, vcsC2, vcsN2, makA, hlyA, vspD, als*	VPI2_VC1776, VPI2_VC1758	*parC, varG*
***V. cholerae* Buccleuch October 2023**	*vspD, hlyA, toxR, vcsN2, vcsV2, rtxA, vcsC2, makA, als*	VPI2_VC1776, VPI2_VC1758	None

***V. cholerae* Buccleuch November 2023**	*rtxA, vcsC2, vcsV2, als, toxR, vspD, vcsN2, makA, hlyA*	VPI2_VC1758, VPI2_VC1776	*parC, gyrA, varG*
***V. cholerae* Dainfern April 2023**	*hlyA, vcsV2, rtxA, als, makA, toxR, vspD, vcsN2, vcsC2*	VPI2_VC1758, VPI2_VC1776	*parC, varG*
***V. cholerae* Dainfern May 2023**	*als, rtxA, vcsN2, vspD, toxR, makA, vcsC2, hlyA, vcsV2*	VPI2_VC1758, VPI2_VC1776	None
***V. cholerae* Dainfern July 2023**	*toxR, vcsN2, als, rtxA, vcsV2, vspD, makA, vcsC2, hlyA*	VPI2_VC1776, VPI2_VC1758	*parC, varG*
***V. cholerae* Dainfern November 2023**	*als, vspD, hlyA, vcsV2, toxR, vcsC2, vcsN2, rtxA, makA*	VPI2_VC1776, VPI2_VC1758	*gyrA, varG*

3.6.4. Phylogenetic analysis

The evolutionary relationship between the nucleotide sequences of the 24 *V. cholerae* isolates collected from Jukskei River and 16 nucleotide sequences (from clinical specimens, food, and environmental samples) was evaluated through a neighbor-joining tree (Figure 3.23). The isolates were grouped into seven distinct clusters (A to G) on the phylogenetic tree. Cluster A consists of two isolates collected from the Jukskei River at Marlboro and Buccleuch, these isolates are closely related and are supported by a 100% bootstrap value (Figure 3.23). Cluster A is paraphyletic to cluster B with a bootstrap value of 100%, moreover, cluster B is the largest cluster consisting of 22 *V. cholerae* isolates all collected from the different collection sites along the Jukskei River. Interestingly, one *V. cholerae* isolate in cluster C (*V. cholerae*_EA0364AA) from NICD collected from an environmental sample in 2020 is closely related to *V. cholerae* isolates collected from the

Jukskei River, as seen with a paraphyletic relation with isolates in Cluster B, this relationship is supported by a 100% bootstrap value (Figure 3.23). Cluster D showed a polyphyletic grouping of 6 *V. cholerae* isolates from environmental, food, and clinical samples. A basal taxon was observed in cluster E of a *V. cholerae*_EA6213AA sourced from a clinical specimen. Cluster F showed a monophyletic grouping of 6 *V. cholerae* all from clinical specimens with a bootstrap of 100%. A monophyletic grouping of 3 *V. cholerae* from clinical samples was observed in Cluster G.

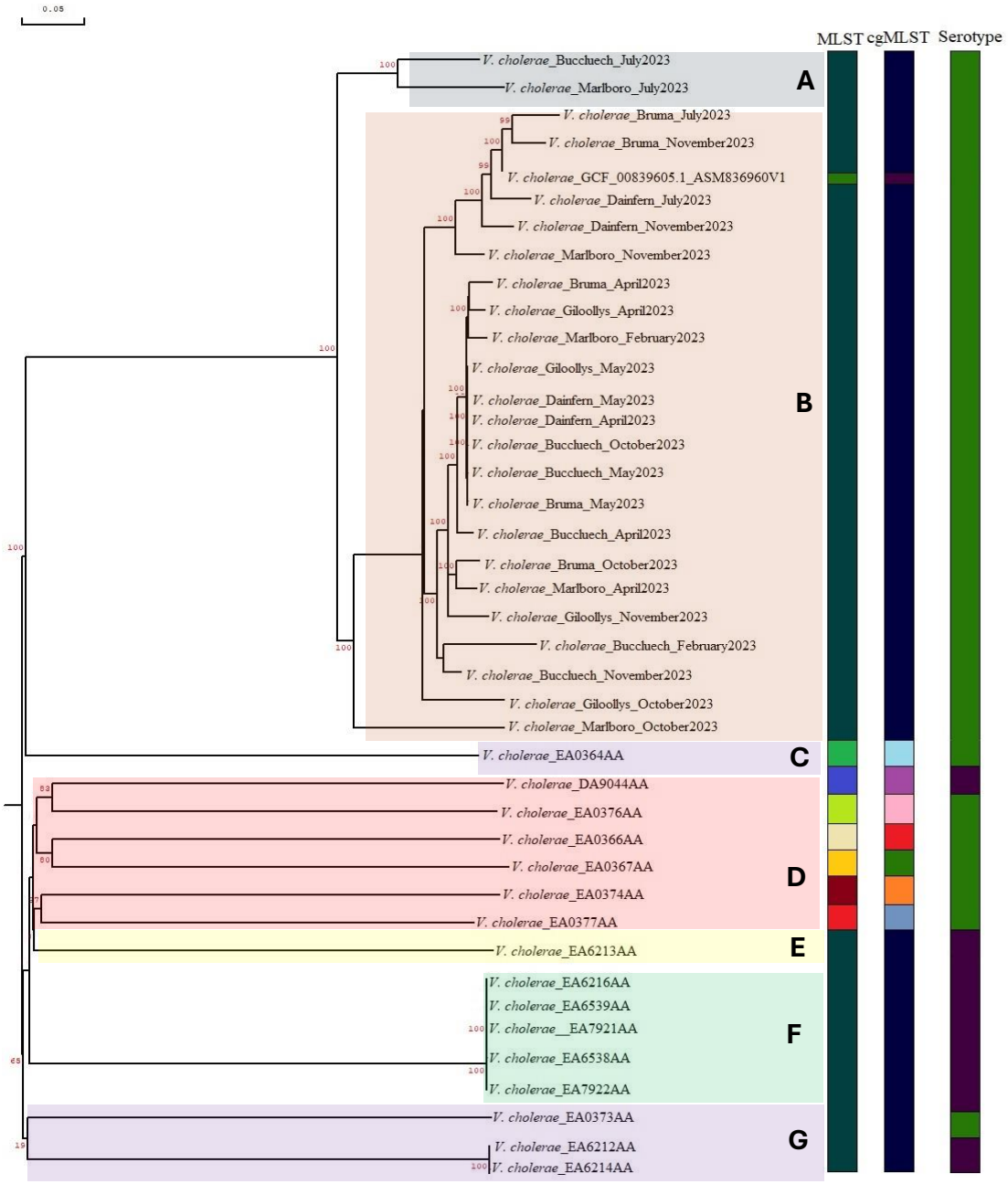

Figure 3.23. Neighbor-joining tree constructed using DNAMAN version 4.0 with a bootstrapping stringency of 1000. The nucleotide sequences of our 24 *V. cholerae* isolates as well as 10 *V. cholerae* genome sequences from NICD were aligned using a multiple sequence alignment tool (DNAMAN version 4.03. The branch lengths indicate the genetic distance and are depicted by the bar (0.05). Bootstrap values (percentages of 1000 replications) are indicated at the internodes of the tree. The cgMLST, serotype, and MLST profiles are also included in the phylogenetic tree to show the diversity of the isolates.

CHAPTER 4: DISCUSSION

4.1 Water quality of the Jukskei River

The water quality of the Jukskei River has been monitored since 1955 by organizations such as the Department of Water Affairs and Forestry (DWAF) (Department of Water Affairs and Forestry, 1996a, 1996b; DWAF, 1996), Water Research Commission (WRC) (Dube *et al.*, 2017), and the City of Johannesburg (Burke and Bokako, 2004). The physio-chemical properties; turbidity, TDS, pH, conductivity, as well as nutrients, are measured monthly by the City of Johannesburg to determine the extent of industrial effluent discharges, anthropogenic activities, and sewage pollution in the river (Neswiswi, 2014). In our study, we determined the water quality of the Jukskei River by measuring temperature, pH, ORP, DO saturation, DO concentration, EC, resistivity, TDS, and salinity.

The temperature of water in aquatic ecosystems is an important parameter as it affects the rates of chemical reactions and thus metabolic rates of organisms (Department of Water Affairs and Forestry, 1996a). In the current study, the mean water temperature of the Jukskei River ranged from 19 to 23°C. Although the temperature is within the acceptable range of 5 to 30°C for most inland waters in South Africa (Department of Water Affairs and Forestry, 1996a), previous studies of the Jukskei River have found ranges of 8.3 to 27.9°C in 2009 (Matowanyika, 2010) and ranges from 13.07 to 19°C in 2021 (Hoorzook *et al.*, 2021). The minimum temperature range of the Jukskei River in our study was higher than in previous studies, suggesting the Jukskei River is getting warmer indicative of the effects of climate change. A study by (Adeola *et al.*, 2022), observed a similar trend in the increase of temperatures of the Olifants River Catchment, a

Limpopo River basin extending through four African countries (South Africa, Mozambique, Zimbabwe, and Botswana).

The pH of the Jukskei River ranged from 6.93 to 7.4 across the five sites. This pH was within the acceptable range of 6 to 8 for most fresh waters in South Africa (Department of Water Affairs and Forestry, 1996a). The Jukskei River is bordered by many communities especially inhabitants of the Alexandra township that lack adequate water and sanitation. These communities use the Jukskei River for domestic water needs, irrigation, and religious ceremonies. Thus, we looked at the target water quality range for pH for domestic and agricultural water use. The pH was within both target water quality ranges of 6.0 to 9.0 (DWAF, 1996) and 6.5 to 8.4 (Department of Water Affairs and Forestry, 1996b), respectively. The pH of the Jukskei River was consistent with a 2021 study by (Hoorzook *et al.*, 2021).

The oxidation-reduction potential (ORP) ranged from 239.7 to 263.7 mV across all sites, which is within the acceptable range of +200 and +600 mV and suggests that the river can break down waste products, such as contaminants and dead plants and animals. The Dissolved Oxygen (DO) saturation across all sites was below the target range by the DWAF in aquatic ecosystems of between 80 to 100% (Department of Water Affairs and Forestry, 1996a). In our study, DO saturation was at an alarming 3 to 4.7% compared to the 2009 study of 19 to 73% (Matowanyika, 2010). The reduction in DO can be attributed to several factors such as flooding, waste discharges, and the amount of suspended material in the water (Department of Water Affairs and Forestry, 1996a). Continued exposure to a DO of less than 80% is very harmful and can cause physiological and behavioral stress in aquatic biota (Department of Water Affairs and Forestry, 1996a).

The electrical conductivity (EC) ranged from 14 to 40 mS/m across all sites and fell under the stipulated target range for domestic use by the DWAF (DWAF, 1996). The Total Dissolved Solids (TDS) across the five sites of the Jukskei River ranged from 70 to 193.2 mg/L. These values fell within the quality standards of the WHO (WHO, 1996) and the DWAF (DWAF, 1996). While the TDS in our study is acceptable, the values increased from a 2010 study that ranged from 10 to 104 mg/L (Matowanyika, 2010). In a 2021 study by (Hoorzook *et al.*, 2021), TDS ranged from 262 to 302 mg/L. These values were extremely high compared to our study, suggesting the variability of TDS values and the importance of determining local conditions for site-specific TDS concentrations (Department of Water Affairs and Forestry, 1996a). The DWAF further states that the TDS in rivers should not differ more than 15% in any season. A similar study by (Teffo *et al*., 2023), had the highest TDS of 349 mg/L. This deviated from our study and the stipulated 15% change by the DWAF suggesting huge discrepancies in TDS values. While (Teffo *et al*., 2023) measured the TDS on-site using a portable pH/EC/TDS meter, we measured our TDS in the laboratory further accounting for the discrepancies.

4.2. Microbial contamination and contamination levels in Juskei River

The Jukskei River, among other rivers in South Africa and the developing world, is heavily polluted due to the anthropogenic activities of the growing population, and industrial and sewage discharge on account of ailing infrastructure (or lack thereof). As a result, the river is a breeding ground for waterborne pathogens that cause infections that range from diarrhea to more severe typhoid and can lead to fatalities. The Jukskei River passes along geographically distinct sites characterized by developments, industries, and varying population densities before it reaches the Hartbeespoort dam. In our study, we selected five different sites to describe the extent of microbial contamination (*E. coli*, *Shigella* spp., and *V. cholerae*) of the Jukskei River.

The pathogen, *E. coli* is an indicator microorganism that signifies recent fecal contamination of rivers (Uprety *et al.*, 2020). Fecal contamination is a major source of waterborne pathogens and thus diseases (Devane *et al.*, 2020). In our study, the mean *E. coli* count ranged from 4.94 log_{10}CFU/mL for Dainfern to 6.52 log_{10}CFU/mL for Marlboro. The presence of *E. coli* in the Jukskei River indicates the presence of bacterial pathogens such as *Salmonella*, *Shigella, Vibrio cholerae*, *Campylobacter coli*, *Campylobacter jejuni*, *Yersinia enterocolitica* and pathogenic *E. coli* (Tandlich *et al.*, 2012). Moreover, *E. coli* is only found in human and animal intestines, and thus presence in Jukskie river water indicates faecal contamination from human origin. Indeed, the *E. coli* counts were highest in Marlboro and Buccleuch possibly attributed to the activities of the neighbouring Alexandra township compared to other sites. Anthropogenic activities (such as pollution) and population density have an effect on the health of the nearby water bodies. Microbial contamination, more specifically by *E. coli* resulting from informal settlements along riverbanks has been well documented in various studies (Opisa *et al.*, 2012; Gqomfa *et al*., 2022; Karimi *et al.*, 2023). In contrast to Dainfern Golf Estate, a more affluent residential area and lower levels of *E. coli*.

The mean *Shigella* count ranged from 5.32 log_{10}CFU/mL for Giloolly's to 6.12 log_{10}CFU/mL for Marlboro. These results followed the same trend as the *E. coli* counts where the counts fluctuated from Bruma to Giloolly's, followed by a sharp increase in counts in Marlboro and a slight decrease in Buccleuch, ending with an even lower count in Dainfern. There was statistical significance across all sites and between sites (Bruma-Marlboro; Giloolly's-Marlboro; Marlboro-Dainfern). Finally, the mean *V. cholerae* counts were lower than those of *E. coli* and *Shigella*. The *V. cholerae* counts ranged from 2.02 log_{10}CFU/mL for Giloolly's to 4.4 log_{10}CFU/mL for Marlboro and followed the mean *E. coli* count trend. Moreover, there was statistical significance across all sites.

This means that the Jukskei River is a potential breeding ground for *E. coli*, *Shigella*, and *V. cholerae* infection, especially for the impoverished communities around the river. There is evidence that, the microbial contamination is carried from Malboro downstream of the Jukskei River. This was evidenced by the high *E. coli* counts in Marlboro which gradually decreased as the water flows downstream to Buccleuch and Dainfern.

4.3. Characterization of the *E. coli* , *Shigella*, and *V. cholerae* collected from the Jukskei River

4.3.1. *E. coli*

A total of 80 water samples were collected for our study and 43 presumptive *E. coli* were isolated on selective media. Additional biochemical tests were performed where *E. coli* is oxidase negative and indole positive (Lennox *et al*., 2020), from which 27 *E. coli* were confirmed. Molecular tests were further performed through conventional PCR using the *UidA* gene and the 27 *E. coli* were indeed confirmed. Multiple identification procedures are essential to prevent misidentification (Janda and Abbott, 2002). Environmental samples are usually limited to culture-based methods and phenotypic characteristics in the identification of microorganisms (Osińska *et al.*, 2023), therefore, molecular tests allow for the high sensitivity and high-throughput identification of microorganisms in a short time. Species-specific genes for the 27 *E. coli* isolates were targeted through a series of conventional multiplex PCR to identify diarrheagenic *E. coli* (DEC). Thirteen (13) DEC were confirmed, among these 44.4% were enterotoxigenic *E. coli* (ETEC) and 3.7% was atypical enteropathogenic *E. coli* (aEPEC). Noticeably, EPEC is the predominant pathotype in many rivers in South Africa (Bolukaoto *et al.*, 2021). (Omar and Barnard, 2014) found aEPEC across a variety of river water sources at 18% and 27%, respectively. Interestingly, the most prevalent strain in rivers of the Vhembe district in Limpopo province (Potgieter *et al.*, 2020), and in the Jukskei River (Hoorzook *et al.*, 2021) was the aEPEC. In the 2021 study, (Hoorzook *et al.*,

2021) did not isolate any ETEC in the river. This suggests a shift in the microbial profile of the Jukskei River to resemble that of other developing countries. The Alexandra township is home to several foreign nationals from the Southern African Developing Communities (SADC) as well as other African countries that often seek asylum and better opportunities (Ekanade and Molapo, 2017). Therefore, the ETEC cases are likely imported. In the African continent, ETEC is predominant in the surface waters in Nigeria (Titilawo, Obi and Okoh, 2015) and Côte d'Ivoire (Kambire *et al.*, 2017). In India and Bolivia, ETEC was the most prevalent strain found in surface waters and rivers (Begum *et al.*, 2005; Guzman-Otazo *et al.*, 2019).

WGS analysis confirmed the 27 isolated strains as *E. coli*. An additional identification procedure is serotyping which assists in the identification of pathogenic strains of *E. coli* based on the lipopolysaccharide O- antigen and the flagellar H- antigen (Beutin *et al.*, 2005). Serotype analysis confirmed 7 serotypes within the 27 *E. coli* strains. The predominant serotype was O16:H48 (59%), followed by O16:25 (11%) and O16 (11%). *E. coli* O16:H48 is a subset of the non-pathogenic *E. coli* K12 MG1655 strain that is widely used in laboratories and is a commensal of the human gut (Lang *et al.*, 2018; Lee *et al.*, 2021). Interestingly, a study by (Bergeron *et al.*, 2012) isolated extraintestinal pathogenic *E. coli* strains containing the O16:H48 serotype in abattoirs in 2005 and 2007 in Canada. In 2005, the strain was susceptible to the common antimicrobials whereas in 2007 it was resistant to streptomycin, sulfisoxazole, and tetracycline. This further highlights *E. coli* K12 as a continuously evolving human gut commensal (Bhat *et al.*, 2019). Among the multilocus sequence types (MLST), ST1946 was predominant and the rest of the STs were unique. ST1946 has been linked to the zoonotic transmission of *E. coli* from animals to humans, however, a study by (Hamame *et al.*, 2022) found ST1946 isolated from a human source with no zoonotic origin. For cgMLST, ST1597 had the highest prevalence at 30%. FimH type

analysis confirmed 8 fimH types among the 27 *E. coli* strains. The fimH 27 was detected in 55% of isolates and was the highest fimH type followed by fimH 34 and fimH 305 at 11%, respectively. The fimH 27 was the predominant fimH type in a variety of sources in different studies. (Chen *et al.*, 2021) had a prevalence of fimH 27 at 25.4% on *E. coli* isolated from patients with community-onset bloodstream infections in China.

Among the 27 *E. coli* strains, eleven virulence genes were present in all strains. These genes are not serotype-specific as they are found in almost all *E. coli* serotypes, with studies reporting some of these genes in non-pathogenic *E. coli*. Five virulence genes of interest detected in our study are *AslA*, *terC*, *usp*, *fdeC*, *ipfA*, and *yfcV*. The *AslA* gene was detected in 74% of isolates and is responsible for the invasion of the brain by crossing the blood-brain barrier, a mechanism specific to neonatal meningitis *E. coli* (Kim, 2001; Hammad *et al.*, 2022). The *terC* gene was present in 85% of *E. coli* isolates and is a part of the *ter* operon that confers tellurite resistance (Valková *et al.*, 2007). The virulence genes *usp*, *fdeC*, and *ipfA*, were at 7% across all isolates. The *usp* gene is a precursor of the protein responsible for genotoxin active in mammalian cells causing bacteremia (*Rihtar et al*., 2020). The *fdeC* gene is a nonfimbrial intimin-like adhesin (Aleksandrowicz *et al.*, 2021). The *ipfA* gene is essential for adherence and colonization of the intestine by encoding a long fimbriae (Mahazu *et al.*, 2021). The y*fcV* gene was present in 3% of the isolates and encodes a major subunit of chaperone-usher fimbriae (Mahazu *et al.*, 2021).

Interestingly, an *E. coli* isolated in Marlboro harbored the *ipaH*, *ipaH*9.8, *ShiA*, *SigA*, and *sitA* genes, respectively. The *ipaH* genes are essential for the successful invasion of the pathogen to a host, specifically *Shigella* and EIEC (Zhang *et al.*, 2021). The *ShiA* gene is found predominantly in the pathogenicity island *Shigella* island 2 (SHI-2) and facilitates iron uptake and immune response evasion by encoding systems involved in these processes (Pasqua *et al.*, 2017). The *SigA*

gene encodes cytotoxins responsible for the accumulation of intestinal fluid in infected hosts (Pakbin *et al*., 2021). The *sitA* gene facilitates iron uptake in *Shigella* (Runyen-Janecky *et al.*, 2003). These virulence genes of the *E. coli* isolate are that of EIEC, as they are also found in *Shigella* from the successful acquisition of the pINV and horizontal gene transfer of others (*ShiA*, *sigA*, and *sitA*). EIEC was not detected previously through the multiplex PCR due to several factors. The first is the chosen target (*ial* gene), which was not detected even with WGS analysis. While it is an important virulence gene responsible for host invasion and cell-to-cell dissemination (Farajzadeh-Sheikh *et al.*, 2020), it is not the only pathotype-defining gene. Additionally, there could be a mutation in the primer binding site of the target gene resulting in false negative results.

The majority of the isolates did not harbor antimicrobial resistance genes. This means that treatment will not be a challenge. Antimicrobial resistance was detected in (n=3, 11%) of the isolates. These isolates were resistant to quinolones, folate pathway antagonist, aminoglycosides, and macrolides.

Lastly, the evolutionary relationship of the 27 *E. coli* isolates collected from the Jukskei River and 10 isolates from clinical and food isolates from NICD was studied through phylogenetics. There was no genetic diversity between the collection date and collection site as evident from the polyphyletic cluster B with a 100% bootstrap. There was genetic diversity between the isolates from the Jukskei River and the NICD isolates collected from clinical and food samples as they formed distinct clusters within the phylogenetic tree.

4.3.2. *Shigella*

In this study, 14 *Shigella* strains were isolated from the water collected at the Jukskei River. The phenotypic and molecular characterization targeting the *ipaH* gene, encoding the invasion plasmid

antigen H, were all positive for this gene. The *ipaH* gene is commonly used in the molecular identification of *Shigella* spp. through PCR assays (Karimi-Yazdi *et al.*, 2020). Interestingly, *Shigella* was detected in all the water samples using real-time PCR. However, only 14 *Shigella* isolates were successfully isolated from the water samples. This is attributed to the inability of PCR to distinguish between DNA from live or dead cells, but detecting DNA from all cells in a sample (Àlvarez *et al.*, 2013). Whereas, culture-based methods only detect viable cells, therefore, *Shigella* present as viable but non-culturable or dead cells cannot be detected on culture (Tandlich *et al.*, 2012).

Encouragingly, WGS analysis confirmed the 14 *Shigella* spp. three distinct species were found: *S. flexneri* (86%), *S. boydii* (7%), and *S. sonnei* (7%). Similarly, (Ekwanzala *et al.*, 2017) isolated a majority of *S. flexneri* compared to other *Shigella* spp. in the riverbed and water samples collected in the Apies River in Gauteng, South Africa. However, a 2010 study by (Kinge and Mbewe, 2010) involving five river catchments in the North West province in South Africa found *S. boydii* as the dominant species isolated within the river catchments. This may suggest the geographical distribution of *Shigella* in South Africa. A study by (Stenhouse *et al.*, 2023) looked at the genomic epidemiology of the common serotypes, *S. flexneri* 2a and *S. sonnei*. In their study, the authors found both isolates in varying frequencies across all provinces in South Africa. Interestingly, in a 2021 study of the Jukskei River that focused on the extent of bacterial contamination of the river, no *Shigella* spp. were identified (Hoorzook *et al.*, 2021).

All MLSTs were unique amongst the 14 *Shigella* strains. The cgMLST profile of the *Shigella* isolates included the cgMLST 1472 (n=11, 80%) and cgMLST 5907 (n=2, 14%).

Among the 14 *Shigella* isolates, 20 virulent genes were detected in varying frequencies. Some of these genes are also found in other bacteria such as *E. coli* and *Salmonella* spp. *FimH* was found in all isolates and encodes a type 1 pilus adhesin (Poole *et al.*, 2017). This gene has also been reported in *E. coli* and *Salmonella* spp. (Poole *et al.*, 2017; Uchiya *et al.*, 2019).

Antimicrobial resistance genes were found in only one isolate amongst the 14 isolates collected in the Jukskei River. Specifically, *S. flexneri*, collected in Giloolly's in May had the resistance genes; *gyrA*, *parC*, and *varG*. The resistance genes *gyrA* (encodes a DNA gyrase) and *parC* (encodes a topoisomerase IV) form part of the quinolone resistance-determining regions (QRDR). Point mutations in this gene region result in quinolone resistance (Dutta *et al.*, 2005). The *varG* gene is part of the putative antibiotic resistance regulon encoding resistance in β-lactamase in most *V. cholerae* strains (Lin *et al.*, 2017). Different studies have displayed *Shigella* resistance to β-lactamase antibiotics (Pakbin *et al.*, 2021; Salleh *et al.*, 2022). Interestingly, none of the studies have linked the *varG* gene in conferring resistance to these β-lactamase antibiotics. *S. flexneri*, like other species of *Enterobacteriacea*e experiences bacterial transformation (Porter and Dorman, 1997) and is actively involved in horizontal and vertical gene transfer (Skippington and Ragan, 2012; Baker *et al.*, 2018). These phenomena allow for the uptake of genes such as the resistance *varG* gene, within the same gene pool available in the environment. Transmission of resistance genes can also occur in biofilm communities, where a large number of microbial pathogens exist in close proximity, thus facilitating the acquisition of resistance genes.

Our study also sought to understand the evolutionary relationship of the 14 *Shigella* isolates collected from the Jukskei River with 7 clinical isolates from NICD. The phylogenetic tree displayed no genetic diversity amongst the 12 *S. flexneri* isolates from the Jukskei River as they formed a polyphyletic relationship within cluster B and were supported by a 99% bootstrap. There

was no genetic diversity between sites and collection dates. Interestingly, two clusters formed distinct relationships between different species. Cluster D formed a paraphyletic relationship between *S. boydii* from the Jukskei River and *S. flexneri* from a clinical sample from NICD, suggesting that the two are genetically related. This was also supported by a bootstrap of 80%. An *S. flexneri* isolate from the Jukskei River and an *S. sonnei* from a clinical sample from NICD formed cluster E and was supported by a bootstrap of 100%. This is a peculiar grouping and warrants further investigation.

4.3.3. *Vibrio cholerae*

The microbial agent *V. cholerae* has been implicated in several outbreak cases reported in the past two years with *V. cholerae* O1 and O139 being the primary cause of infection (Yuan *et al.*, 2022). The Jukskei River has been implicated in some cholera illnesses in South Africa (NICD,2023).

In our study, a total of 24 *V. cholerae* were isolated from the Jukskei River in this study. The results from phenotypic and molecular characterization targeting the *toxR* were all in concordance with the *in silico* genotypic results targeting the species-specific gene *ompW* and serogroup-specific *rfbV* and *wbfZ* genes for *V. cholerae* O1 and O139 strains. The *toxR* and *ompW* genes have been used in several studies to detect *V. cholerae* from different sample types (Kumar *et al.*, 2009; Kahler *et al.*, 2015; Ramamurthy *et al.*, 2020; Pal *et al.*, 2023; Preeprem *et al.*, 2023). Moreover, serogroup-specific genes have also been used in the literature to classify *V. cholerae* strains (Siriphap *et al.*, 2017; Li *et al.*, 2019; Baddam *et al.*, 2020).

Cholera toxin produced by *V. cholerae* O1 and O139 are responsible for acute diarrheal disease and are known etiological agents of cholera epidemics and pandemics (Chowdhury *et al.*, 2017). Whereas *V. cholerae* non-O1/non-O139, also known as non-agglutinable *Vibrios* are not associated

with epidemic cholera as the majority of these genes do not harbor the *ctxA* gene responsible for CTX (Vezzulli *et al.*, 2020). However, they can still cause mild to life-threatening illnesses (Takahashi *et al.*, 2021). In our study, all the *V. cholerae* isolated from the Jukskei River were *V. cholerae* non-O1/non-O139 as they were non-agglutinable and lacked the serogroup-specific *rfbV* and *wbfZ* genes for O1 and O139, respectively. Moreover, these isolates lack the *ctxA* gene that encodes cholera toxin. Our results are in line with other studies in the literature which detected a high prevalence of *V. cholerae* non-O1/non-O139 from environmental samples such as River water (Sharma and Chaturvedi, 2006; Li *et al.*, 2020; Luo *et al.*, 2021). Furthermore, serogroups O1, O139, and cholera toxin in environmental strains are rare. A study in the Eastern Cape province, South Africa, by (Abioye *et al.*, 2023) reported 84% of non-O1/ non-O139 *V. cholerae* in freshwater sources. Surprisingly and in contrast to our results, a study by (Jiang *et al*., 2003) reported the occurrence of the *ctxA* gene in 10% of *V. cholerae* non-O1/non-O139 environmental strains in India, a country endemic for cholera. In their study, the authors reported that the cholera toxin gene in environmental samples could be attributed to human waste from diseases in the population (Jiang *et al*., 2003). Another study conducted in Brazil reported the presence of the *ctxA* gene amongst other genes located in the Cholera Toxin genetic elements (*zot* and *ace*) in *V. cholerae* non-O1/non-O139 strains collected from different environmental sources (Theophilo *et al.*, 2006). A more recent study in Kolka India isolated three *V. cholerae* non-O1/non-O139 strains possessing the *ctxA* gene from ponds (Takahashi et al., 2021). In addition to the cholera toxin gene, these isolates also harbored the *tcpA* gene, a major subunit of TCP which plays an important role in bacterial pathogenesis (Takahashi *et al.*, 2021). The authors further highlighted that although neglected, *V. cholerae* non-O1/non-O139 infections are on the rise.

Although the *V. cholerae* non-O1/non-O139 strains lack the cholera toxin gene, *ctxA,* they still harbor virulent genes. Some of the virulent genes detected in our *V. cholerae* non-O1/non-O139 strains are *toxR, vspD, als, hlyA, vcsC2, makA, rtxA, vscV2,* and *vsN2.* Out of the following genes, only a few are associated with disease, these are the *toxR, makA*, and *rtxA* genes, respectively. The *toxR* is an important factor in the secretion of the cholera toxin by activating the toxT promoter that triggers a regulatory cascade thus, causing infection (Canals *et al.*, 2023). Moreover, *makA* is a major factor in the secretion of cytotoxic activity of *V. cholerae* (Nadeem *et al.*, 2021). The *rtxA* gene is responsible for encoding the MARTX family of toxins that are produced in humans, animals, and insect pathogens (Dolores and Satchell, 2013).

There are currently two types of *V. cholerae* pathogenicity islands (VPI), these are the VPI-1 and VPI-2. The VPI-1 is crucial in the pathogenicity of *V. cholerae* and has been known to be the responsible cause for the majority of the 6th and 7th cholera pandemic (Kumar *et al*., 2020). VPI-2 encodes sialidase as well as a sialic acid catabolism gene cluster to illicit pathogenicity in *V. cholerae* (Almagro-Moreno *et al*., 2010). A single pathogenicity island (VPI-2), with two gene identities, was detected in our *V. cholerae* non-O1/non-O139 strains from the Jukskei River. These pathogenic islands are VPI2_VC1758 and VPI2_VC1776. The VC1758 is the gene identity of an *intV2* gene, an integrase responsible for the excision of *Vibrio* seventh pandemic II (Almagro-Moreno *et al*., 2010). The VC1776 encodes a putative N-acetylneuraminate lyase to confer pathogenicity to *V. cholerae* (Dziejman *et al.*, 2002; Almagro-Moreno and Boyd, 2009).

Antimicrobial resistance is a growing concern worldwide and resistance to recommended antibiotics for the treatment of *V. cholerae* has increased (Wang *et al.*, 2012; Das *et al.*, 2020; Bhandari *et al.*, 2021). Aquatic environments are a reservoir not only for *V. cholerae* but multi-drug resistant *V. cholerae* and antimicrobial-resistant genes (ARG) (Yu *et al.*, 2022) that can be

easily acquired by *V. cholerae* through transformation (Jiang *et al.*, 2022). Bacterial transformation is a process where bacteria take up extracellular or free DNA present in the environment. *V. cholerae* are naturally competent (Sun *et al.*, 2013) for transformation and can, therefore, easily acquire ARGs present in river water. The presence of resistant genes in the *V. cholerae* non-O1/non-O139 isolated from Jukskei River was also determined using genomic data. These genes are the *parC*, *gyrA*, and *varG*. The genes, *parC,* and *gyrA* encode for the quinolone-resistance determining region and infer resistance to the widely used quinolones in treating cholera (De, 2021). The *varG* gene is a class of metallo-β-lactamases that encode resistance to β-lactamase antimicrobials (penicillin, cephalosporin, carbapenem, meropenem) (Lin *et al.*, 2017).

Lastly, our study sought to understand the diversity and the evolutionary relationship among the *V. cholerae* isolates collected from the Jukskei River. In addition, the evolutionary relationship between *V. cholerae* isolated in our study and those collected from clinical, environmental, and food samples was investigated. Our study revealed no genetic diversity in the *V. cholerae* non-O1/non-O139 isolates from the Jukskei River as all 24 *V. cholerae* non-O1/non-O139 were ST741 and belonged to the cgMLST1309. There is limited literature surrounding ST741, the earliest is a 1961 *V. cholerae* strain from the Philippines isolated during an outbreak (Wallace *et al.*, 1964; Arteaga *et al.*, 2020). Therefore, ST741 is not a common sequence type of *V. cholerae*. This was also observed in the phylogenetic tree where 22 of the *V. cholerae* non-O1/non-O139 isolates clustered together in a monophyletic relationship in cluster B, Figure 3.23 with 100% bootstrap support. No diversity was observed for the different collection sites or collection dates. There was a clear genetic diversity between *V. cholerae* isolates collected from Jukskei River with those collected from clinical, food, and environment as these isolates formed distinct clusters on the phylogenetic tree. Interestingly, one *V. cholerae* non-O1 isolate in cluster C (*V.*

*cholerae*_EA0364AA) from NICD collected from an environmental sample in 2020 was closely related to *V. cholera* non-O1/ non-O139 isolates collected from the Jukskei River, as seen with a paraphyletic relation with isolates in Cluster B, this relationship is supported by a 100% bootstrap value (Figure 3.23). This suggests that the Jukskei River is a vehicle for the transmission of infectious enteric pathogens.

CHAPTER 5: CONCLUSION

This study aimed at isolating and characterizing enteric bacterial pathogens, specifically *E. coli*, *Shigella*, and *V. cholerae* from the Jukskei River. The presence of these pathogens in the river is a public health concern, especially for the vulnerable communities along the riverbanks. While most of our measured physio-chemical properties fall within the stipulated guidelines set by the DWAF for drinking, recreational, and water sources (i.e. rivers), enteric pathogens (some with antimicrobial resistance) were detected. The temperature and TDS of the Jukskei River have increased drastically from previous studies but still fall within DWAF guidelines. However, DO saturation and concentration were below the guidelines, and have dire consequences for aquatic biota. The results from quantification of the extent of microbial contamination of the Jukskei River is of great concern and accentuate the need for rehabilitation. Contamination levels were higher in Marlboro and Buccleuch compared to the other sites. This could be due to the close proximity of these collection sites to the overpopulated and underdeveloped Alexandra township compared to the more developed and affluent Dainfern site. Thus, improvements in infrastructure, proper sewage systems, and flood prevention strategies within impoverished communities are essential to ensure the adequate sanitation of the Jukskei River and the surrounding communities. Results from this study, suggest that the Jukskei River is a breeding ground for enteric pathogens and carries with it pathogens that cause severe diseases. Two DEC pathotypes (ETEC and aEPEC) were

detected from the Jukskei River through multiplex PCR reactions, and one (EIEC) was detected from WGS analysis. Our results highlight the importance of WGS analysis in characterization, particularly on environmental samples. The presence of the *ipaH* gene among other virulence genes on one *E. coli* isolate confirmed the isolate as EIEC, previously not detected through multiplex PCR. In previous studies of the Jukskei River, aEPEC was the prevalent pathotype while ETEC was predominant in our study. These pathotypes cause diarrheal diseases affecting millions annually and are notifiable in South Africa by the NICD. Therefore, it is a public health concern causing potential outbreaks as recorded from previous studies. WGS analysis revealed three *Shigella* spp. in the river causing infections ranging from invasive shigellosis to bacillary dysentery. *S. flexneri* was the dominant species, is a notifiable pathogen by GERM-SA requiring a low infective dose to cause infection, and has caused outbreaks in developed countries. While *S. sonnei* has been detected at a low 7% in the Jukskie River, it is the most frequently isolated strain in clinical samples in South Africa. *S. boydii* has also been detected at 7% in the Jukskei River and causes shigellosis, HUS, and reactive arthritis. This pathogen is the least isolated among *Shigella* but has caused foodborne outbreaks in the past. Previous studies of the Jukskei River have not detected *Shigella* species. *V. cholerae* non-O1/non-O139 was detected in all isolates from the Jukskei River. The absence of the cholera toxin in non-O1/non-O139 *V. cholerae* strains should not be overlooked, as non-O1/non-O139 *V. cholerae* can still cause mild to life-threatening illnesses. Interestingly, the cholera toxin was detected in the DNA extracted from 80 water samples and amplified using real-time PCR. Suggesting the presence of the gene in the Jukskei River. According to several studies, the *ctx* gene can also be taken up by non-O1/non-O139 through horizontal gene transfer. This is concerning, as most rivers harbor *V. cholerae* non-O1/non-O139. Additionally, an outbreak occurred involving *V. cholerae* O1 isolated in the Jukskei River in 2023.

Indeed, contaminated water is a breeding ground for enteric pathogen dominance and transmission from environments. Improvements in the ecological health of rivers will directly improve the health of the nearby population. Part of this is the establishment of the rehabilitation strategies of rivers. In our study, we found that the water quality of the Jukskei River was 'good' regarding the DWAF guidelines when looking at the physio-chemical properties, but 'bad' regarding the extent of bacterial contamination. Establishing the cause of the problem ensures proper rehabilitation strategies are followed. The Jukskei River, as previously documented, suffers from sewage pollution from domestic waste and livestock. This was evidenced in the high enteric pathogen counts in Marlboro and Buccleuch, sites closest to the Alexandra township. Rehabilitation spotlights the bigger issues causing the degradation in water quality. Urbanization, overpopulation, and reduced resources to match the growing population result in detrimental effects on the environment. For the Jukskei River to be fully rehabilitated, these issues need to be addressed. Other factors to improve water quality is the management of diffuse runoff, point and non-point source pollution management, and educating citizens about the general health of the river. The Jukskei River, among other urban rivers in South Africa, is monitored consistently but is limited to physio-chemical properties, the presence of *E. coli* coliforms, and other nutrients. Surveillance of enteric pathogens is also limited to clinical specimens. Complete surveillance of enteric pathogens from environmental samples, including rivers, is necessary to prevent the transmission of potentially dangerous pathogens from these environments to humans. Therefore, a 'One Health Approach' needs to be implemented.

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APPENDICES

Appendix A: Ethics waiver

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research & Innovation)

30/05/2022

Ref: W-CBP-220530-02

TO WHOM IT MAY CONCERN

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical)

Investigator: Mr L Mkhize, et al
Student No. (if appropriate): 1690192
Staff No. (if appropriate):

Supervisor: Ms ST Duze

School: Pathology
Department: Clinical Microbiology & Infectious Diseases
Division: Medical School
University

Project title: *Isolation and characterization of enteric pathogens from the Jukskei River*

Reason: In vitro laboratory study
No human participants will be involved in the study

pp

Dr CB Penny
Chairperson: Human Research Ethics Committee (Medical)

Research Office Secretariat:
Third Floor, Phillip Tobias Building, corner of St Andrews and York Roads, Parktown, Johannesburg 2193
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Office E-mail: HREC-Medical.ResearchOffice@wits.ac.za
Website:
https://www.wits.ac.za/research/researcher-support/research-ethics/ethics-committees/

Appendix B: Media and reagents

Preparation of media and reagents

1. Phosphate buffered saline (PBS) (Merck, Darmstadt, Germany)
   1 tablet of phosphate-buffered saline was added to 100 mL distilled water. The mixture was sterilized by autoclaving at 115°C for 10 minutes.

2. MacConkey-Sorbitol Agar (Merck, Darmstadt, Germany)
   51.5 g of MacConkey-Sorbitol powder was suspended in 1000 mL of distilled water. The mixture was brought to a boil and sterilized by autoclaving at 121°C for 15 minutes.

3. Thiosulfate citrate bile-salts sucrose (TCBS) Agar (Merck, Darmstadt, Germany)
   88 g of TCBS powder was suspended in 1000 mL of purified water. The mixture was heated with constant stirring until boiling. It was then poured into plates.

4. Xylose Lysine Deoxycholate (XLD) agar (Merck, Darmstadt, Germany)
   55.43 g of XLD powder was suspended in 1000 mL of distilled water. The mixture was heated with constant stirring until boiling. It was then poured into plates.

5. Buffered peptone water (BPW) (Merck, Darmstadt, Germany)
   20 g of BPW powder was dissolved in 1000 mL of distilled water. The mixture was mixed well and sterilized by autoclaving at 121°C for 15 minutes.

6. Alkaline peptone water (APW) (Merck, Darmstadt, Germany)
   20 g of APW powder was dissolved in 1000 mL of distilled water. The mixture was mixed well and sterilized by autoclaving at 121°C for 15 minutes.

7. Nutrient agar (Merck, Darmstadt, Germany)
   28 g of nutrient agar was suspended in 1000 mL of distilled water and brought to a boil to dissolve completely. The mixture was sterilized by autoclaving at 121°C for 15 minutes.
8. Tris-EDTA (TE) buffer (Merck, Darmstadt, Germany)

0.5 M EDTA (pH 8)

100 mL: 18.61 g EDTA disodium dehydrate + 2g NaOH, autoclave.

1 M Tris (pH 8)

10 mL : 1.21g Tris + 0.42 mL HCL, autoclave

100 mL TE buffer: 1 mL Tris, 55 g boric acid, 40 mL 0.5 M EDTA, autoclave.

9. TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.3)

Appendix C: Formulae

1. Total Viable Counts:

   Colony Forming Units per mL (CFU/mL) = (Number of colonies x dilution factor) / volume on culture plate

   Log (CFU/mL) = **total viable counts**

Appendix D: P values of enumerated pathogenic bacteria

Table Analyzed	E coli		
Kruskal-Wallis test			
P value	0.0065		
Exact or approximate P value?	Gaussian Approximation		
P value summary	**		
Do the medians vary signif. (P < 0.05)	Yes		
Number of groups	5		
Kruskal-Wallis statistic	14.27		
Dunn's Multiple Comparison Test	Difference in rank sum	Significant? P < 0.05?	Summary
Bruma vs Gilollys	4.917	No	ns
Bruma vs Marlboro	-16.33	No	ns
Bruma vs Buccleuch	-9.333	No	ns
Bruma vs Dainfern	5.542	No	ns
Gilollys vs Marlboro	-21.25	Yes	*
Gilollys vs Buccleuch	-14.25	No	ns
Gilollys vs Dainfern	0.6250	No	ns
Marlboro vs Buccleuch	7.000	No	ns
Marlboro vs Dainfern	21.88	Yes	*
Buccleuch vs Dainfern	14.88	No	ns

Table Analyzed	Shigella		
Kruskal-Wallis test			
P value	0.0019		
Exact or approximate P value?	Gaussian Approximation		
P value summary	**		
Do the medians vary signif. (P < 0.05)	Yes		
Number of groups	5		
Kruskal-Wallis statistic	17.05		
Dunn's Multiple Comparison Test	Difference in rank sum	Significant? P < 0.05?	Summary
Bruma vs Giloollys	2.250	No	ns
Bruma vs Marlboro	-23.04	Yes	*
Bruma vs Buccleuch	-3.958	No	ns
Bruma vs Dainfern	0.3750	No	ns
Giloollys vs Marlboro	-25.29	Yes	**
Giloollys vs Buccleuch	-6.208	No	ns
Giloollys vs Dainfern	-1.875	No	ns
Marlboro vs Buccleuch	19.08	No	ns
Marlboro vs Dainfern	23.42	Yes	*
Buccleuch vs Dainfern	4.333	No	ns

Table Analyzed	V. cholerae		
Kruskal-Wallis test			
P value	0.0489		
Exact or approximate P value?	Gaussian Approximation		
P value summary	*		
Do the medians vary signif. (P < 0.05)	Yes		
Number of groups	5		
Kruskal-Wallis statistic	9.543		
Dunn's Multiple Comparison Test	Difference in rank sum	Significant? P < 0.05?	Summary
Bruma vs Giloollys	2.042	No	ns
Bruma vs Marlboro	-14.63	No	ns
Bruma vs Buccleuch	-10.42	No	ns
Bruma vs Dainfern	1.125	No	ns
Giloollys vs Marlboro	-16.67	No	ns
Giloollys vs Buccleuch	-12.46	No	ns
Giloollys vs Dainfern	-0.9167	No	ns
Marlboro vs Buccleuch	4.208	No	ns
Marlboro vs Dainfern	15.75	No	ns
Buccleuch vs Dainfern	11.54	No	ns

Appendix E: Qualitative analysis of water samples

Results of Real-time PCR amplification of the *uidA*, *ipaH*, *toxR*, and *ctxA* genes for the detection of *E. coli*, *Shigella* isolates, and *V. cholerae* respectively from water samples collected in the five collection sites from January to November 2023.

	E. coli	***Shigella***	***V. cholerae***	
Months	***UidA***	***ipaH***	***toxR***	***ctxA***
January*				
February	+	+	+	+
March*				
April	+	+	+	+
May	+	+	+	+
June	+	+	+	+
July	+	+	+	+
August*				
September*				
October	+	+	+	+
November	+	+	+	+

+ is indicative of the presence of a particular gene.

* indicates months of no real-time PCR performed due to samples not being collected.

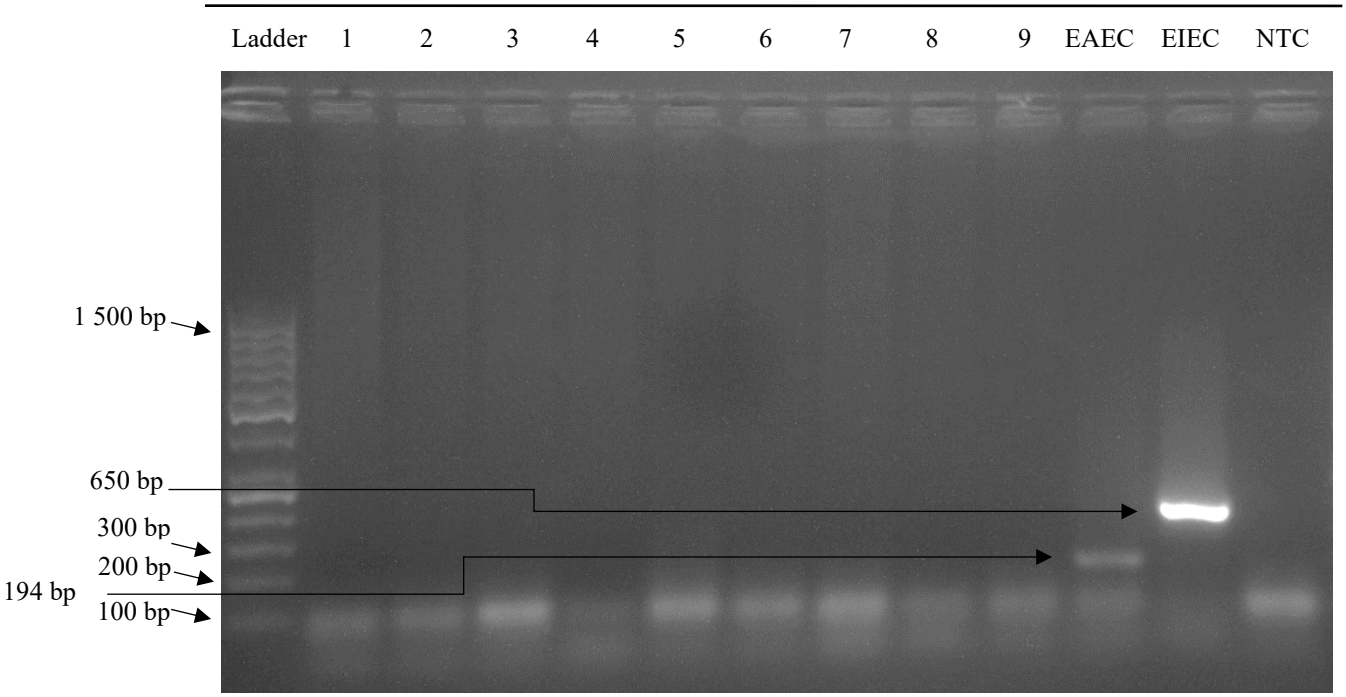


Figure 4. The 2% (w/v) agarose of a multiplex amplification of the *eagg* gene (194 bp), *ial* gene (650 bp) for the identification of EAEC and EIEC from *E. coli* isolates. A 100 bp DNA molecular weight marker (Promega) was used to size the PCR products (Lane 1 and 13). All samples were negative for EAEC and EIEC. The Positive controls amplified the target genes. Lane 13 is the NTC.

Appendix F: Cp values from real-time PCR amplification

Pos	Name	Type	CP
C1	Bruma Nov23	Unknown	24.48
C2	Giloollys Nov23	Unknown	31.79
C3	Marlboro Nov23	Unknown	20.36
C4	Buccleuch Nov23	Unknown	27.72
C5	Dainfern Nov23	Unknown	32.62
C6	Positive	Unknown	19.17
C7	NTC	Unknown	

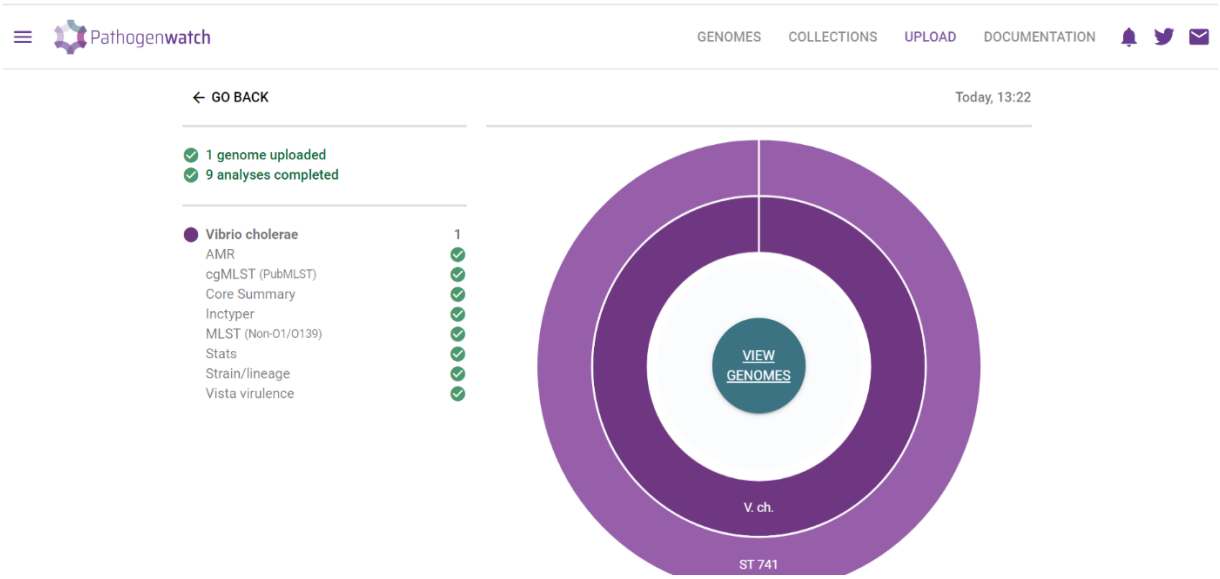
Pos	Name	Type	CP
E1	Bruma	Unknown	26.91
E2	Giloollys	Unknown	30.43
E3	Marlboro	Unknown	30.58
E4	Buccluech	Unknown	32.35
E5	Dainfern River Water	Unknown	31.20
E6	Dainfern Irrigation Water	Unknown	31.16
E7	Positive	Unknown	16.75
E8	NTC	Unknown	

Pos	Name	Type	CP
F1	Bruma Oct23 P2	Unknown	28.82
F2	Giloollys Oct23 P2	Unknown	30.04
F3	Marlboro Oct23 P2	Unknown	29.34
F4	Buccleuch Oct23 P2	Unknown	28.56
F5	Dainfern Oct23 P2	Unknown	27.82
F6	Bruma Oct23 S2	Unknown	28.84
F7	Giloollys Oct23 S2	Unknown	28.29
F8	Marlboro Oct23 S2	Unknown	27.99
F9	Buccleuch Oct23 S2	Unknown	27.99
F10	Dainfern Oct23 S2	Unknown	27.65
F11	Positive	Unknown	27.70
F12	NTC	Unknown	

Pos	Name	Type	CP
E1	Feb23 L1S1	Unknown	22.08
E2	Feb23 L2S1	Unknown	26.22
E3	Feb23 L3S2	Unknown	24.86
E4	Feb23 L4S1	Unknown	27.17
E5	Feb23 L5S2	Unknown	27.92
E6	Feb23 IW	Unknown	30.75
E7	Positive	Unknown	28.63
E8	NTC	Unknown	

Appendix G: Bioinformatics analysis software

The following results are from the different bioinformatics platforms used in our WGS analysis. Pathogenwatch was used to confirm the species, antimicrobial resistance genes, cgMLST as well as MLST. SpeciesFinder was also used to confirm the species even further. CholeraeFinder was specifically for the *V. cholerae* isolates and displayed organisms, serogroup, species-specific genes, virulence genes, pathogenicity islands, and antibiotic resistance genes. MLST and cgMLST were also determined according to their respective platforms. SerotypeFinder was used to find the H- and O- gene determinants for *E. coli* serotypes. Resfinder was used to determine the antimicrobial resistance genes. Lastly, fimtyper was used to determine the *E. coli* fimH types.



SpeciesFinder-2.0Server - Results

Species	Match	Confidence of result
Escherichia coli str. K-12 substr. MC4100	HG738867	PASS

Input Files: *Escherichia-coli_Bruma_April2023.fasta*

CholeraeFinder-1.0 Server - Results

Organism: *Vibrio Cholerae*

Serogroup: *No O1 / No O139*

Biotype: *Not able to determine*

Wave: *Not able to determine*

Pandemic (7th pandemic strain): *Not able to determine*

Genes found	
Relevance	**Gene(s)**
Specie specific gene	ompW
Serogroup specific	None
Biotype specific	None
7th Pandemic	None
Virulence	toxR, vspD, vcsN2, als, hlyA, vcsC2, makA, rtxA, vcsV2
Mobile Genetic Elements	None
Phage sceptibility	None
Pathogenicity Islands	VPI2_VC1758, VPI2_VC1776
Antibiotic Resistance	parC

Antibiotic resistance genes						
Vibrio Cholerae Gene	**Identity**	**Query / Template length**	**Contig**	**Position in contig**	**Predicted phenotype**	**Accession number**
gyrA	98.23	453 / 453	CP010812.1 basecall_model=dna_r10.4.1_e8.2_400bps_sup@v4.2.0	2064172..2064624	Mutated DNA gyrase subunit A. Resistance to Fluoroquinolone	GQ502315
parC	99.05	210 / 210	CP010812.1 basecall_model=dna_r10.4.1_e8.2_400bps_sup@v4.2.0	3329131..3329340	Mutated Topoisomerase IV subunit A, resistance to Quinolone	KJ596550

Cholerae Gene	Identity	Template length	Contig	Position in contig	Predicted phenotype	Accession number
als	99.12	1704 / 1704	CP010812.1 basecall_model=dna_r10.4.1_e8.2_400bps_sup@v4.2.0	2419823..2421526	Als operon involved in glucose metabolism	vc1590
hlyA	99.54	217 / 217	CP010812.1 basecall_model=dna_r10.4.1_e8.2_400bps_sup@v4.2.0	881414..881630	Vibrio Cholerae hemolysin, causes cytolysis by forming heptameric pores	HQ452880
makA	99.82	1110 / 1110	CP010812.1 basecall_model=dna_r10.4.1_e8.2_400bps_sup@v4.2.0	339954..341063	Flagella-associated cytotoxin	VCA0883
rtxA	99.01	203 / 203	CP010812.1 basecall_model=dna_r10.4.1_e8.2_400bps_sup@v4.2.0	2300674..2300876	Multifuntional repeats-in-toxin	AY101181
toxR	98.47	721 / 720	CP010812.1 basecall_model=dna_r10.4.1_e8.2_400bps_sup@v4.2.0	1779635..1780355	Transcriptional regulator of ctx	HM042639
vcsC2	98.56	1458 / 1458	CP010812.1 basecall_model=dna_r10.4.1_e8.2_400bps_sup@v4.2.0	2624701..2626158	Type II/III secretion system (TTSS) protein	DQ124262
vcsN2	99.21	1263 / 1263	CP010812.1 basecall_model=dna_r10.4.1_e8.2_400bps_sup@v4.2.0	2623439..2624701	Type III secretion system (TTSS) cytoplasmic ATPase	DQ124262
vcsV2	99.84	609 / 609	CP010812.1 basecall_model=dna_r10.4.1_e8.2_400bps_sup@v4.2.0	2635698..2636306	Type III secretion system (TTSS) protein	DQ124262
vspD	99.24	1050 / 1050	CP010812.1 basecall_model=dna_r10.4.1_e8.2_400bps_sup@v4.2.0	2643009..2644058	Putative secreted protein (non-O1/non-O139)	DQ124262

Uploaded file: barcode_6.medaka.fasta

7 exact matches found.

Locus	Allele	Length	Contig	Start position	End position
adk	111	416	CP010812.1_basecall_model=dna_r10.4.1_e8.2_400bps_sup@v4.2.0	1782768	1783183
gyrB	125	431	CP010812.1_basecall_model=dna_r10.4.1_e8.2_400bps_sup@v4.2.0	4288	4718
mdh	14	421	CP010812.1_basecall_model=dna_r10.4.1_e8.2_400bps_sup@v4.2.0	3689323	3689743
metE	1	591	CP010812.1_basecall_model=dna_r10.4.1_e8.2_400bps_sup@v4.2.0	2560475	2561065
pntA	19	431	CP010812.1_basecall_model=dna_r10.4.1_e8.2_400bps_sup@v4.2.0	672147	672577
purM	1	476	CP010812.1_basecall_model=dna_r10.4.1_e8.2_400bps_sup@v4.2.0	3105442	3105917
pyrC	59	449	CP010812.1_basecall_model=dna_r10.4.1_e8.2_400bps_sup@v4.2.0	1312544	1312992

Only exact matches are shown above. If a locus does not have an exact match, try querying specifically against that locus to find the closest match.

Text Excel

MLST

Matching profile

ST: 741

Sample name	Total number of loci	Number of called alleles	% Called alleles	cgST	Allele matches in cgST	% Allele matches
Vibrio-cholerae_Buccleuch_May2023	1128	895	79.34	2547	895	79.34
Vibrio-cholerae_Bruma_April2023	1128	872	77.3	2547	872	77.3
Vibrio-cholerae_Giloollys_May2023	1128	883	78.28	2547	883	78.28
Vibrio-cholerae_Giloollys_November2023	1128	893	79.17	2547	893	79.17
Vibrio-cholerae_Buccleuch_April2023	1128	888	78.72	2547	888	78.72
Vibrio-cholerae_Marlboro_July2023	1128	782	69.33	2547	782	69.33
Vibrio-cholerae_Bruma_October2023	1128	872	77.3	2547	872	77.3
Vibrio-cholerae_Dainfern_May2023	1128	869	77.04	2547	869	77.04
Vibrio-cholerae_Dainfern_November2023	1128	867	76.86	2547	867	76.86
Vibrio-cholerae_Dainfern_April2023	1128	857	75.98	2547	857	75.98

Sample name	Total number of loci	Number of called alleles	% Called alleles	cgST	Allele matches in cgST	% Allele matches
Shigella-flexneri_ESC_HB6236AA	2513	2287	91.01	123999	2285	90.93
Shigella-flexneri_ESC_LB7644AA	2513	2319	92.28	140408	2314	92.08
Shigella-flexneri_ESC_NC4245AA	2513	2324	92.48	193394	2302	91.6
Shigella-flexneri_ESC_NC4242AA	2513	2289	91.09	58168	2281	90.77
Shigella-flexneri_ESC_NC4243AA	2513	2305	91.72	66326	2293	91.25
Shigella-flexneri_ESC_LB7643AA	2513	2319	92.28	140408	2314	92.08
Shigella-sonnei_ESC_MC5129AA	2513	2344	93.27	11269	2336	92.96

Database for H type genes						
Gene	Serotype	Identity	Template / HSP length	Contig	Position in contig	Accession number
fliC	H48	100	1497 / 1497	U00096.3 basecall_model=dna_r10.4.1_e8.2_400bps_sup@v4.2.0	2002407..2003903	AY250025

Database for O type genes						
Gene	Serotype	Identity	Template / HSP length	Contig	Position in contig	Accession number
wzx	O16	100	1248 / 1248	U00096.3 basecall_model=dna_r10.4.1_e8.2_400bps_sup@v4.2.0	2108615..2109862	AB811601
wzy	O16	100	1167 / 1167	U00096.3 basecall_model=dna_r10.4.1_e8.2_400bps_sup@v4.2.0	2106338..2107504	AB811601

Results

ResFinder-4.4.3

Phenotypes

Hide

escherichia coli | All

Antimicrobial	Class	WGS-predicted phenotype	Genetic background
gentamicin	aminoglycoside	No resistance	
tobramycin	aminoglycoside	No resistance	
amikacin	aminoglycoside	No resistance	
ciprofloxacin	quinolone	Resistant	
nalidixic acid	quinolone	Resistant	
ampicillin	beta-lactam	No resistance	
ampicillin+clavulanic acid	beta-lactam	No resistance	
cefepime	beta-lactam	No resistance	
cefotaxime	beta-lactam	No resistance	

FimTyper-1.0 Server - Results

FimH type: *fimH10*

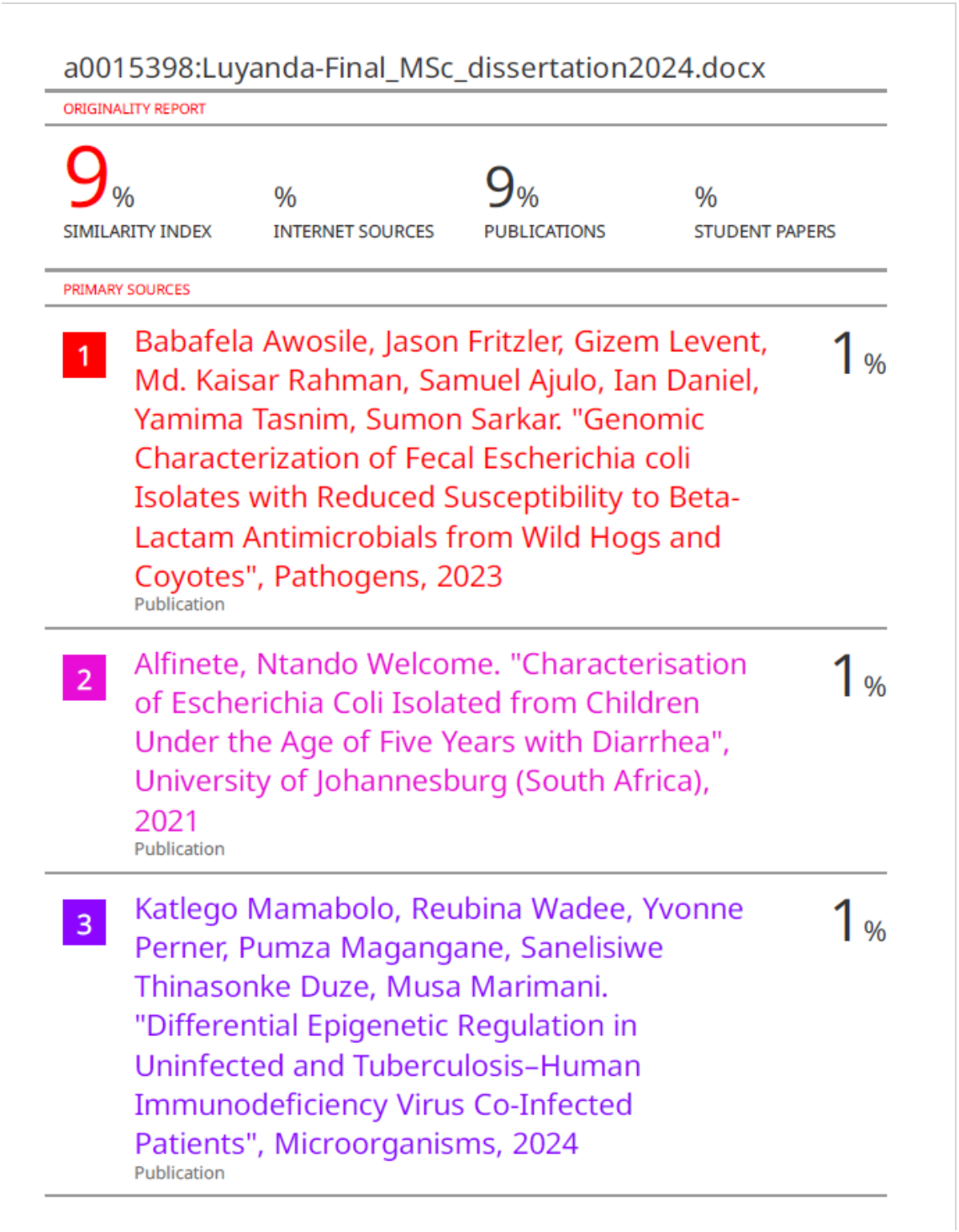
FimH type - E.coli					
Fimtype	%Identity	Query/HSP length	Contig	Position in contig	Accession number
fimH10	100.00	489 / 489	ESC_IC2192AA_AS_NODE_10_length_214148_cov_8.957780	13723..14211	

Appendix H: Drinking water standards

Table 12. Acceptable contamination levels in drinking water for each pathogen.

Pathogen	Acceptable Standards	Reference
Escherichia coli	0 CFU/mL	(DWAF, 1996)
Shigella	0 CFU/mL	(WHO, 2022)
Vibrio cholerae	0 CFU/mL	(WHO, 2022)

Appendix I: Similarity Index



a0015398:Luyanda-Final_MSc_dissertation2024.docx

ORIGINALITY REPORT

9%	%	9%	%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Babafela Awosile, Jason Fritzler, Gizem Levent, Md. Kaisar Rahman, Samuel Ajulo, Ian Daniel, Yamima Tasnim, Sumon Sarkar. "Genomic Characterization of Fecal Escherichia coli Isolates with Reduced Susceptibility to Beta-Lactam Antimicrobials from Wild Hogs and Coyotes", Pathogens, 2023 Publication	1%
2	Alfinete, Ntando Welcome. "Characterisation of Escherichia Coli Isolated from Children Under the Age of Five Years with Diarrhea", University of Johannesburg (South Africa), 2021 Publication	1%
3	Katlego Mamabolo, Reubina Wadee, Yvonne Perner, Pumza Magangane, Sanelisiwe Thinasonke Duze, Musa Marimani. "Differential Epigenetic Regulation in Uninfected and Tuberculosis–Human Immunodeficiency Virus Co-Infected Patients", Microorganisms, 2024 Publication	1%