INVESTIGATION OF THE OCCURRENCE AND RISK OF INFECTION OF PATHOGENIC AND ANTIBIOTIC RESISTANT *CAMPYLOBACTER* SPECIES IN SELECTED SOURCE WATERS WITHIN THE KOWIE CATCHMENT, EASTERN CAPE, SOUTH AFRICA

Report to the
Water Research Commission

by

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EXECUTIVE SUMMARY

BACKGROUND

Locally in South Africa, the annual prevalence of *Campylobacter* has remained steady since 2010 without declining. In fact, it has been suspected that *Campylobacter* infection could be endemic in the region. Despite these, the disease has not received much research attention compared to other gastroenteritis infections, such *as Shigella* and *Salmonella*. But worldwide, *Campylobacter* species are increasingly being recognised as leading agents of gastroenteritis that needs to be monitored. Surface water is continually implicated in the spreading of the bacteria in humans, including the antibiotic resistant ones. Direct contact and consumption of faecal contaminated water are principal risk factors for Campylobacteriosis. South Africa is a water-scarce country that relies heavily on surface water resources for irrigation, domestic, recreational and industrial purposes. One continuing problem is an incapacity to precisely predict and identify potential for an outbreak and identify source of spread. This study explores this surveillance gap using both culture-dependent and culture-independent approaches (molecular-based approaches).

AIMS

The following were the aims of the project:

1. To review existing literature on the prevalence, source and modes of transmission of pathogenic and antibiotic resistant *Campylobacter* spp. in water and associated risks to human and environmental health.
2. To screen for multiple virulence genes and antibiotic resistant genes of *Campylobacter* species origin in the metagenome of a selected source water within the Kowie Catchment in the Eastern Cape.
3. To assess the microbiological risks to human health occasioned by *Campylobacter* species contamination of source waters.
4. To identify the leading source of *Campylobacter* contamination in source waters within the Kowie Catchment.
5. To link local cases of human *Campylobacter* infections to identified sources and thence develop an indicator pathway for *Campylobacter* infections.

STUDY AREA AND METHODOLOGY

The Bloukrans, a tributary of Kowie River was selected as a study site because it represents all risk factors necessary for *Campylobacter pollution* and human exposure. The river flows through Makhanda which is a small university town in Makana Local Municipality, Eastern Cape. It is urbanised but not industrialised. The local people keep animals, and they use this stream as water for their animals, for leisure and spiritual purposes, as well as swimming. However, the river receives pollution inputs from different anthropogenic sources, including, free grazing and unrestricted movement of animals such as cattle and donkeys, inadequately treated effluent from dysfunctional and dilapidated Wastewater Treatment Works (WWTW), and nearby informal settlements. Preliminary testing was used to ascertain optimal sampling and method to recover *Campylobacter* cells from the river water samples. Then this study measured physicochemical quality of the river water, the occurrence of *Campylobacter* and its antibiotic resistant and virulence genes from January 2020 to December, 2021 (seasonally). The extent of subtype sharing between *Campylobacter* strains from Bloukrans River and human clinical isolates was measured, and the origin of bacterial contamination of the river tracked.

RESULTS AND DISCUSSION

The main outcomes are that:

- The detection rate of *Campylobacter* spp in the Bloukrans River is 66.67%.
- The result from this study does not show marked seasonality in Campylobacter detection in the river of study (r= -0.089366).
- Between 9.30E+03 × 101-1.75E+05 gene copies/1 ml of *Campylobacter* 16S rRNA gene are measurable in the Bloukrans River.
- About 1.24E-02-9.71E-03 gene copies/1 ml and 7.80E+01-2.77E+04 gene copies/1 ml of *Campylobacter* antibiotic resistance genes, *tetO* and *cmeB*, respectively are measurable in the river.
- Using data from all sites, *Campylobacter* antibiotic resistance genes *catB2*, *catQ*, *erm(B)*, *floR*, *mcr-7.1*, *tet(W)*, *tet(X)*, and *tetA (P)* conferring resistance to several important antibiotic classes are present in the river
- Users of the river are at risk of *Campylobacter* infection due to the widespread presence of *Campylobacter* spp in the sampled water if river water is used without appropriate treatment.
- The critical discriminative biomarkers as an indicator of a *Campylobacter* unhealthy environment are *flgD, fliP, glf, and pseB*. These four genes associate with *Campylobacter* unhealthy river water samples, and predictive models can combine their occurrence in river water to develop a diagnostic tool for water bodies risk assessment, a preventive strategy against *Campylobacter* outbreak.
- Animals are the main contributors of bacterial contamination of the Bloukrans River.
- An identical genetic profile was found between *E. coli* ST624 species in the Bloukrans River and human clinical isolates.
- It has not been possible to detect STs of *Campylobacter* spp. in the Bloukrans River samples.

CONCLUSIONS

The result from this study suggests the level of occurrence of *Campylobacter* and presence of antibiotic resistance genes in the river system presents public health concern. *Campylobacter* infection is endemic in South Africa. Livestock grazing around the river are the most bacterial pollution contributor to the Bloukrans River. The outcome of this study suggests that potential transmission of pathogenic strains from the river to humans may occur through direct or indirect contact. Relying on culture-based approaches for surveillance may underestimate *Campylobacter* prevalence. A metagenomic approach to evaluating water samples is more efficient and can be useful for source tracking and the surveillance of pathogens such as *Campylobacter* spp, as well as for monitoring virulence factors and antibiotic resistance genes. Therefore, this approach should be pursued

RECOMMENDATIONS

Campylobacter should be included in the list of microbial agents subjected to regulation in local Freshwater systems. This study has identified a set of discriminative biomarkers that may be useful indicators of risk of *Campylobacter* infection upon exposure to rivers, and this warrants further exploration. Further investigation is required in order to confirm the role of rivers in transmission of *Campylobacter* strains to humans. Research is needed to identify best livestock management strategies to reduce livestock presence around the river and improve the knowledge base to support policies, to reduce diffuse pollution from agricultural systems.

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CHAPTER 1: BACKGROUND

1.1 INTRODUCTION

Campylobacter specie is a gram-negative bacterium, either spiral, curved or rod shaped. Of the causes of *Campylobacteriosis*, *Campylobacter jejuni* is a widespread zoonotic multi-host pathogen and accounts for 90% of human infections in most parts of the world (Griekspoor et al., 2013; Marder et al., 2018; An et al., 2018). *Campylobacter coli* is responsible for only 5-10% of infections in human (Makiw et al., 2012). These infections are a leading cause of gastrointestinal disease globally, and their occurrence rates are higher than those caused by *Salmonella* and *Shigella* in developed countries (Kaakoush et al., 2015). The symptoms of the disease can vary from watery, non-inflammatory, non-bloody diarrhoea to acute inflammatory diarrhoea with ensuing fever and abdominal pain (Chen et al., 2011). Locally, diarrhoeal diseases pose a major public health problem in South Africa and are the third leading cause of death in the country (Chola et al., 2015). Apart from gastrointestinal infections *Campylobacter* spp., especially *C. jejuni*, has also been associated with other intestinal infections, such as, hepatitis, myocarditis myelitis, meningitis, haemolytic-uraemia syndrome, pancreatitis, as well as secondary complications, such as Guillain-Barr syndrome (Shobo et al., 2016). To complicate issues, high prevalence of resistance in clinical isolates of *C. jejuni* and *C. coli* to first line antibiotics (fluoroquinolones, macrolides and tetracycline) that are used for treatment used have been reported both developed and developing countries (Uaboi-Egbenni et al., 2012; Shobo et al., 2016). *Campylobacter* spp, apply several mechanisms to resist these clinically important antibiotics, such mutations in target genes, including gyrA mutations to fluoroquinolones (Jafari Sales, 2017; Tang et al., 2017) and 23S rRNA mutations to macrolides (Payot et al., 2006; Han et al., 2012), multidrug efflux pump *CmeABC* that extruding compounds and antimicrobials of different structures (Lin et al., 2002), horizontally acquired antibiotic resistance genes, including *tet(O)* and *aphA-3* (Crespo et al., 2016) as well as a ribosomal RNA methylase gene erm(B) conferring high-level resistance to macrolide (Qin et al., 2014; Wang et al., 2014). Worse still, a vaccine by way of a preventative measure is not yet available (Wagenaar et al., 2013). The impact cannot be overlooked particularly for South Africa with a high population of vulnerable individuals due to several reasons, including the HIV pandemic.

While there are no documented waterborne outbreaks of *Campylobacter* gastroenteritis in South Africa, small scale local studies demonstrate that the rates of *Campylobacter* infections might be underestimated in South Africa. In a study by Mason et al., 2013, out of 225 samples, C. jejuni (12%) and *C. coli* (7.5%) were present in stool specimens in children and adults suffering from diarrhoea in South Africa. In Venda, Limpopo province, out of 322 stool samples *C. jejuni* was more prevalent (10.2%), followed by *C. coli* 6.5% (Samie et al., 2007). From a Rotavirus surveillance project, 13% of isolates in stool specimens of children (<5 years of age) in South Africa were *Campylobacter* with acute diarrhoea (NICD annual review, 2014). In Cape Town, South Africa, 5 443 strains of *Campylobacter* were isolated from stools of children suffering from diarrhoea (Lastovica, 2009). Additionally, 801/848 of isolates that were submitted by Group for Enteric, Respiratory and Meningeal Surveillance in South Africa (GERMS-SA) laboratories from different Provinces in South Africa for a *Campylobacter* Surveillance Program in South Africa were positive for species of *Campylobacter* (Thobela, 2017).

This information and statistics are from clinical surveillance programs but have no linkage with the environment. Environmental surveillance of antibiotic resistant *Campylobacter* spp is still lacking. Yet, the primary reservoir of these pathogens are animals, faecal contaminated water or the environment.

Consumption of faecal contaminated water is a principal risk factor for Campylobacteriosis (Ravel et al., 2016)., South Africa is among water-scarce countries and depends directly on available freshwater systems including rivers for irrigation, domestic purposes as well as recreational activities. Reports have shown that the faecal contamination levels for most local rivers, surpass acceptable limits (<1 000 *E. coli*/100 mL and 100 cfu/ml) for South African Department of Water and Sanitation (DWS) and WHO (Alegbeleye et al., 2016). This is expectable because South Africa faces a sewage treatment crisis and high number of leaks that occur in pipelines that can convey faeces borne pathogens into rivers. Also, the close proximity to livestock farms and possibilities of contamination by manure from livestock are very high. The presence of faecal pollution demonstrates the presence of *E. coli* and other possible pathogens (Abia et al., 2016). More so, a study funded by the Water Research commission (WRC) reported the presence of sub therapeutic levels (in the range of ng/l) of antibiotics as well as antibiotic resistant bacteria (ARB) in source waters, distribution systems and final waters of three local drinking water production facilities (Bezuidenhout et al., 2016). it is widely acknowledgeable that environmental bacteria are reservoir of antibiotic resistance bacteria and antibiotic resistance genes that can potentially be incorporated into human as well as pathogens with time. A pathogen that is passing through a surface water system can be in contact with a resistant strain for a period that is long enough to permit horizontal gene transfer to take place (Alm et al., 2014). These resistance genes pool is amplified by the inflow of antibiotic resistance genes from human waste and livestock into water environment (Yang et al., 2017). In addition, antibiotics residues enter the environment from pharmaceutical industries, hospitals, and livestock farming that disturb the water microflora as well as exerting selection pressure for resistance development (Gullberg et al., 2011). Therefore, surveillance programs for antibiotic resistant *Campylobacter* spp that include river environment is critical

A pressing problem is the difficulty to detect *Campylobacter* in an environmental sample. A lack of appropriate diagnostic tools has hindered environmental surveillance, prediction and identification of potential outbreak. It is against this background that this project was initiated. To explore this surveillance gap, both culture dependent and culture-independent approaches (molecular-based approaches employing river water samples were taken. Furthermore, the relationship between humans and environmental isolates is investigated in order to ascertain an interplay of strains between human and the environmental reservoirs (Runcharoen et al., 2017; Jørgensen et al., 2017; Fagerströ et al., 2019).

Finally, the rifeness of the diseases warrants that options of intervention have to be prioritized. Numerous possible intervention measures targeting pollution of the environment have been recommended to lessen the disease burden of human infection (Opsteegh et al., 2015). However, in South Africa, intervention measures that target the environmental pollution sources are generally unavailable. Identification of the leading source of *Campylobacter* contamination is vital for intervention. Source attribution is used to estimate the impact of the individual primary source to the observed concentrations of a pollutant.

1.2 PROJECT AIMS

The following were the aims of the project:

1. To review existing literature on the prevalence, source and modes of transmission of pathogenic and antibiotic resistant *Campylobacter* spp in water and associated risks to human and environmental health

2. To screen for multiple virulence genes and antibiotic resistant genes of *Campylobacter* species origin in the metagenome of a selected source water within the Kowie catchment in the Eastern Cape
3. To assess the microbiological risks to human health occasioned by *Campylobacter* species contamination of source waters
4. To identify the leading source of *Campylobacter* contamination in source waters within the Kowie catchment
5. To link local cases of human *Campylobacter* infections to identified sources, and thence develop an indicator pathway for *Campylobacter* infections

1.3 STUDY APPROACH

The first phase of the project involved review of existing literature. The review of literature provided background information on the academic theories applied in this study. A study site, Bloukrans, (a tributary of Kowie River), which represents all risk factors necessary of *Campylobacter* pollution and potential for human exposure was selected. Preliminary testing was used to ascertain optimal sampling and method to recover *Campylobacter* cells from the river water samples. This was then followed by measurements physicochemical quality of the river water, occurrence of *Campylobacter* and its antibiotic resistant and virulence genes from January 2020 to December, 2021 (seasonally). The occurrence data was used to develop a set of discriminative biomarkers that may be useful indicators of risk of *Campylobacter* infection upon exposure to rivers. Lastly, we attempted to track the origin of bacterial contamination of the river, and potential of the river as a pathway for Campylobacteriosis transmission.

CHAPTER 2: LITERATURE REVIEW

2.1 DESCRIPTION OF *CAMPYLOBACTER*

Campylobacter is a gram-negative bacteria that is s-shaped and either has two or one flagellum, (depending on the species), which it uses for motility (Crushell et al., 2004). Some species do not have flagellum (Man, 2011; Kaakoush et al., 2015). The movement of *Campylobacter* is often termed as "corkscrew". According to Kaakoush et al. (2015), *Campylobacter* ranges from a size of 0.2-0.8 µm and do not form any spores. *Campylobacter* uses amino acids or tricarboxylic acid cycle intermediates as an energy source (Kaakoush et al., 2015). Their ideal atmospheric conditions for growth include 5% O_2 85% N_2 and 10% CO_2, and so they are microaerophilic. All *Campylobacter* species grow at 37°C. However, culturing at 42°C is a differential characteristic as only *C. lari*, *C. jejuni*, *C. upsaliensis* and *C. coli* can grow at this temperature. Therefore, these species are termed thermotolerant. In water, depending on the age and physiological state, *Campylobacter* cells exhibit two different morphological forms, such as vibriod or coccoid. The vibriod shape that have a flagella is the most commonly observed form, whereas the coccoid form occurs as a result of *Campylobacter* resistance during stress conditions or degeneration phase (Kinana et al., 2007).

The genus *Campylobacter* falls under the family *Campylobacter* aceae, order *Campylobacter* ales, class Epsilonproteobacteria and phylum Proteobacteria (Kaakoush et al., 2015). The genome of *C. jejuni* NCTC 11168 has been completely sequenced (Parkhill et al., 2000). *C. jejuni* NCTC 11168 has a circular chromosome of 1.64 Mb (30.6% GC), that encodes 1654 proteins. Introduction of insertion sequences or sequences of phage origin is uncommon. Alternatively, the hypervariable sequences commonly occur in genes that encode enzymes for biosynthesis, or enzymes that are involved in the modification of surface structures, and at the level of genes whose function is yet to be identified (Parkhill et al., 2000). There are 26 species and 12 subspecies of *Campylobacter,* and these species include human and animal pathogens namely *C. coli*, *C. lari, C. concisus*, *C. fetus*, *C. helveticus*, *C. upsaliensis*, *C. jejuni*, *C. mucosalis*, *C. hominis C. rectus*, *C. hyointestinalis*, *C. gracilis*, *C. curvus C. showae*, *C. sputorum*, *C. insulaenigrae* and *C. ureolyticus*. The dominating *Campylobacter* species in humans are *C. jejuni*, *C. coli* and *C. lari* (Khan et al., 2014; Tresse et al., 2017) (Table1). In humans, *Campylobacter* disease is mainly caused by *Campylobacter jejuni* (accountable for 95% of infections), *Campylobacter coli* (accountable for 4% of infections) *C. upsaliensis* or *C. lari* (1%)

Table 2-1 Overview of selected *Campylobacter* species

Campylobacter spp.	Description
C. jejuni	*C. jejuni* is the most common gastrointestinal pathogen that causes diarrheal diseases in humans (Teunis et al., 2018)
C. coli	*C. coli* is the second most common gastrointestinal pathogen in humans. It requires a high carbon dioxide content to grow (Silva et al., 2011).
C. upsaliensis	*C. upsaliensis* is thermotolerant and it has been reported that these species are intolerant to cultivation conditions optimized for *C. jejuni* and *C. coli*. These bacteria are ingested through contaminated water or food. *C. upsaliensis* is known as the leading cause of bacteremia (Nakamura et al., 2015).
C. showae	*Campylobacter showae* is linked with oral cavity in human and associated with periodontitis and gingivitis (Hsu et al., 2019)
C. helveticus	*C. helveticus* is a thermotolerant bacterium and is a catalase negative. It has been isolated from cats and dogs. There are reported cases of human disease connected with *C. helveticus* even though it is similar to *C. upsaliensis* (Miller et al., 2017).
C. concisus	*C. concisus* is suspected to be the causative agent of acute gastrointestinal infections in humans. This *Campylobacter* occurs in patients suffering with Crohn's disease (Huq et al., 2017).
C. fetus	*C. fetus* can cause severe systemic infections and bowel disease. These infections most likely occur in people that work with infected animals. People that are immunocompromised are at a higher risk. Sheep and cattle are the main reservoir, and *C. fetus* can grow at 25°C-37°C. It comprises of two subspecies, *C. fetus* sps. *venerealis* and *C. fetus* sps. *Fetus* (Schulze et al., 2006).
C. hyointestinalis	*C. hyointestinalis* hosts include sheep, deer and cattle. This specie is not thermotolerant. Members comprise of two subspecies *C. hyointestinalis* subsp. *lawsonii* found in pigs only, and *C. hyointestinalis* subsp. *hyointestinalis* that can be found in several mammalian hosts. *C. hyointestinalis* is reported to be present in up to 30 countries globally (Wilkinson et al., 2018).
C. gracilis	*Campylobacter gracilis* requires fumarate and formate for its metabolism. The majority of infections by *C. gracilis* are on the head and neck. Additionally, *C. gracilis* is associated with gingivitis or root canal infections (Siqueira and Rôças, 2003).
C. lari	*C. lari* belongs to thermotolerant group of *Campylobacter,* and has characteristics that are similar to *C. jejuni*, *C. coli*, *C. upsaliensis*, and *C. helveticus*. This *Campylobacter* is mostly found in an area near the coast and water bodies (Meinersmann et al., 2015).
C. rectus	*C. rectus* inhabits oral cavity and leads to chronic periodontitis, causes premature foetal development and labour in mothers (Veyrine et al., 2019).
C. sputorum	*C. sputorum* is thermotolerant and mainly isolated from sheep and litter. It includes 3 subspecies: *C. sputorum* sps. *faecalis*, *C. sputorum* sps. *paraureolyticus*, and *C. sputorum* sps *sputorum*. These species are distinguished by catalase and urease production. *C. sputorum* sps. *paraureolyticus* is positive for the urease production and negative for catalase production, whereas *C. sputorum* sps. *faecalis* is positive for catalase production and negative for urease production. Only *C. sputorum* sps. *sputorum* and *C. sputorum* sps. *paraureolyticus* has been described by humans (Miller et al., 2017).
C. ureolyticus	*C. ureolyticus* has been isolated mainly from children suffering from Crohn's disease. It is not clear whether *C. ureolyticus* plays a role in intestinal disease or a member of gastrointestinal tract normal flora (Burgos-Portugal et al., 2012).

2.2 EPIDEMIOLOGY OF *CAMPYLOBACTER* INFECTIONS IN HUMANS

This section discusses the epidemiology of *Campylobacter* infections, including incidence, transmission pathways and infectious dose. These factors are important when reporting diagnosis of sporadic or outbreak *Campylobacter* infections

2.2.1 Incidence

Campylobacteriosis is mainly a sporadic disease. The global burden of *Campylobacter* infection is yet to be fully understood (Platts-Mills and Kosek, 2014; Kaakoush et al., 2015). This is complicated by lack of improved and more consistently applied assays that can be applied to understand the epidemiology of various *Campylobacter* species. In the European Union (EU), *Campylobacteriosis* is a regularly reported zoonosis. In Germany it is a frequently notified bacterial gastrointestinal disease (Schielke et al., 2014). In the recent years, in many European countries the number of notified *Campylobacteriosis* cases has been increasing yearly compared to other enteric pathogens such as *Salmonella* (Nylen et al., 2002; CDC, 2010; Schielke et al., 2014; Thobela, 2017). The case is not very different with the United States of America (USA) where *Campylobacter* infection is the second most common cause of bacterial enteritis following salmonellosis. Up to 2.4 million cases have been estimated yearly (CDC, 2010). Milder but prolonged diarrhoea episodes associated with *C. concisus* were reported in children in Belgium (Vandenberg et al., 2013), and cases of children with gastroenteritis associated with *C. jejuni* were reported in Denmark (Nielsen et al., 2013).

Campylobacter infection is endemic in developing countries, including Africa, Middle East and Asia, and it is a foremost enteric bacterial pathogen (Kaakoush et al., 2015). Recurrent infection occurs as a result of lack of or poor sanitation, poor hygiene and living in a vicinity that is close to animals. Population based surveillance in the developing world is restricted because of frequent asymptomatic gastroenteritis. For instance *C. jejuni* is commonly isolated from the stools of healthy children and the infection rate between asymptomatic and symptomatic cases are often similar (Neitenbach et al., 2019). The frequency of asymptomatic infections is so high that to determine a statistically significant relationship between disease and detection is almost impossible. Therefore, in developing countries it has been very difficult to estimate the burden of *Campylobacter* (Platts-Mills and Kosek, 2014).

C. jejuni diarrhoea is major burden in developing countries, such as Bangladesh, Mirzapur, Pakistan, and Karachi. In Bangladesh, *C. jejuni* diarrhoea is about the fourth highest disease in the first year of life, whereas in Karachi and Pakistan, it is about the fifth highest disease burden in the first year of life. Other species of non-*jejuni*/*coli* species of *Campylobacter* , such as or *C. troglodytis* and *C. hyointestinalis* subsp. *lawsonii* have been identified in the Bangladesh, Tanzania and Peru, comprising about one-third of detections (Platts-Mills et al., 2014a).

In Africa, of all pathogens, *Campylobacter* has the highest related burden of diarrhoea in children (Platts-Mills and Kosek, 2014; Asuming-Bediako et al., 2019). In a study conducted in Burkina Faso, by using stool specimens and sociodemographic data from 1 246 patients suffering from enteritis, a *Campylobacter* isolation rate of 2.3% was reported, including *C. jejuni* (51.8%), *C. coli* (13.8%), and *C. upsaliensis* (3.5%) were detected (Sangaré et al., 2012). Furthermore, *Campylobacter jejuni*/*coli* were detected in 21% of diarrheal episodes in Malawi. *C. coli* was comprised more than one-third of bacteria detected in the diarrhoea samples (Mason et al., 2013). In 2012, 16.7% of 227 *Campylobacter* was detected in stools of diarrhoeal children under the age of five years in Ethiopia (Tafa et al., 2014). Another study detected a prevalence of 15.4% in 285 diarrhoeal children undergoing treatment at the University of Gondar Hospital in northwest Ethiopia (Lengerh et al., 2013). In the two studies, it was observed that occurrence of *Campylobacter* was higher in malnourished children and children from

households without access to a source of clean water as well as with direct contact with domestic animals, especially hens. Also, *Campylobacter* have been detected in 11.4% of stool samples sampled from 1195 individuals in Morogoro, Tanzania (Komba et al., 2015). The result suggested that symptomatic infections with young individuals are more prevalent than asymptomatic and adult individuals. Also in Tanzania, a cross-sectional study of 300 children that have acute watery diarrhoea indicated that 9.7% of the stool samples are *Campylobacter* positive (Deogratias et al., 2014). Additionally, up to 90% *C. jejuni* isolation rate have been recorded from human stool samples in yet another study in Tanzania (Mdegela et al., 2006). Similar to the studies from Tanzania, a 9.3% isolation rate of *Campylobacter* in 226 stool samples from diarrhoeal children have been recorded in Kampala, Uganda, *and C. jejuni* was the most detected species (80.9%) (Mshana et al., 2009).

In Egypt, from 1995 to 2003, *Campylobacter* was isolated from 9.37% of 6562 stools sampled from 1057 children (ElGendy et al., 2018). Also in another study in Gharbia, Egypt, an incidence of 2.8% and 12.3% for *C. coli* and *C. jejuni*, respectively, in 106 children was reported (ElGendy et al., 2018). In the eastern part of the Nile Delta, a *Campylobacter* prevalence of 2.7% in 110 stool samples sourced from hospital patients between the years 2012 and 2014 was reported (Awadallah et al., 2014). *Campylobacter* prevalence that is even higher (16.66% in 48 human faecal samples) has also been reported in Egypt in another study (Hassanain, 2011). The faecal samples were collected from people in contact with food-producing animals. Also, in another study, out of 3477 episodes of diarrhoea that occurred 1995 to 1998 in Egypt, 366 (10.5%) were linked with *Campylobacter* infection (Rao et al., 2001).

In Nigeria, examination of 292 stool samples that were collected from patients in hospitals in a north-western state (Sokoto) identified the presence of *Campylobacter* in 55% of the samples (Nwankwo et al., 2016). In the same state, 43% of 57 women that are not pregnant and 70% of 23 pregnant women were observed to be stool-positive for *Campylobacter* (Okwundu Nwankwo et al., 2016). The authors attributed the exposure to *Campylobacter* to poor environmental conditions (Okwundu Nwankwo et al., 2016). In the South eastern Nigeria, a lower isolation rate (8.3%) was observed in 514 children younger than five years, and 93% of the positive isolates are *C. jejuni* (Ohanu and Offune, 2009). *Campylobacter* was detected in 8.2% of the stool samples from 306 diarrhoeal children under the age of 2 in Ilorin in the middle belt zone of Nigeria (Samuel et al., 2006). The exposure to *Campylobacter* was attributed to consumption of contaminated water, food as well as unclean environment.

Literature on the epidemiology of *Campylobacter* precisely for South Africa is scarce (Thobela, 2017). From the 2007 to 2022, a few studies have investigated *Campylobacte*r infection in diarrheal paediatrics in South Africa. Particularly for *C. jejuni*, the isolation rate reached to 10-90% (Samie et al., 2007, 2022; Mason et al., 2013; Thobela, 2017; Chukwu et al., 2019). In Venda, Limpopo province, out of 322 stool samples *C. jejuni* was more prevalent (10.2%), followed by *C. coli* (6.5%) (Samie et al., 2007). The study by Mason et al. (2013), revealed that out of 225 samples, *C. jejuni* (12%) and *C. coli* (7.5%) were present in stool specimens in children and adults suffering from diarrhoea in South Africa. Additionally, 801/848 of isolates that were submitted by GERMS-SA (Group for Enteric, Respiratory and Meningeal Surveillance in South Africa) laboratories from different Provinces in South Africa for a *Campylobacter* Surveillance Program in South Africa were positive for species of *Campylobacter* (Thobela, 2017). *C. jejuni* was the most predominant (80%) specie of *Campylobacter* followed by *C. coli* (6%). In another report, the possibility of potentially antibiotic resistant *Campylobacter* spp. circulating in the Northwest Province of South Africa have been suggested (Chukwu et al., 2019). Results from the study showed 16% *C. jejuni* and15% *C. coli* were isolated from diarrhoea stool samples. More recently, in Limpopo province *Campylobacter* was found in diarrheal stools (20.4%), and compared to non-diarrheal stools (12.4%), with *C. jejuni* as the most prevalent (90.3% (232/257)) compared to *C. coli* (25/257 (9.7%)) , stored well water, stored tap water and water samples collected from the Crocodile River. (Chukwu et

al., 2019). It seems the isolation rate is increasing. Besides, From a Rotavirus surveillance project, 13% of isolates in stool specimens of children with acute diarrhoea (<5 years of age) in South Africa were *Campylobacter* (NICD annual review, 2014). In Cape Town, South Africa, 5 443 strains of *Campylobacter* were isolated from stools of children suffering from diarrhoea (Lastovica, 2009). It is difficult to discern the burden of *Campylobacter* infections in the country because not all cases result to severe illness (Pitkänen, 2013a). In most cases, when the illness is mild, medical assistance may not be required. Therefore, there will not be hospital records for those cases, and the overall record for the prevalence within the community becomes underestimated (Pitkänen, 2013a)..

2.2.2 Seasonal occurrences of human *Campylobacter*iosis

Seasonality is noticeable for human *Campylobacter* infections. In most temperate countries, the peak of infection occurs in spring, whereas those areas with milder winter have peaks earlier in the year. Similarly, for South Africa, seasonal peaks is observed for *Campylobacter*, having a sharp rise in June and July. In South Africa, June and July are early winter months. A possible explanation to the sharp rise in Campylobacter infection during these periods is that milder winter temperatures possibly favour some transmission routes, and enhance the survival and multiplication of the bacteria (Samie et al., 2022). Furthermore, the study suggests that *Campylobacter* infection is endemic in South Africa because the prevalence remained almost at the same level for over 4 years period of their study.

2.2.3 Reservoir and transmission pathways

The transmission cycle begins with an animal reservoir such as cattle, waterfowl, poultry, and wildlife which contaminate water. Poultry is an important reservoir of *C. jejuni* (65-95%) compared to *C. coli.* Additional *Campylobacter* species, including *C. concisus, C. lari* and *C. upsaliensis* can also be harboured in poultry (Tresierra-Ayala et al., 1994; Kaakoush et al., 2014). *Campylobacter* species are found in abundance in environments, such as water and soil that are surrounded by poultry farms (Kaakoush et al., 2014). Wild birds have been associated with contamination of drinking water tanks with *Campylobacter* in Greensville, Florida, USA (Sacks et al., 1986), coastal bathing waters (Obiri-Danso and Jones, 1999, 2000; Obiri-Danso et al., 2001). The occurrence of *Campylobacter* s in migratory birds indicates how far of a distance that *Campylobacter* s can be transferred (Stelzer et al., 1988; Jones, 2001; Kwon et al., 2017). It then travels to humans in the form of drinking water, recreational water and irrigation water (Kaakoush et al., 2015). In addition, person-to-person transmission (faecal-oral route) is possible (Kaakoush et al., 2015).A link between contact to environmental water and *Campylobacter* infection is well established (Schönberg-Norio et al., 2004; Mughini Gras et al., 2012; Ravel et al., 2016), mainly in outbreak situations (Dale et al., 2010; Harder-Lauridsen et al., 2013). Both freshwater and sea water harbour *Campylobacter* spp. (Moore et al., 2001; Kovanen et al., 2016). In Denmark, up to 4% of sporadic infections was caused by recreational water (Gaardbo Kuhn et al., 2018). Humans get infected by the bacteria and then transfer it to each other. Furthermore humans may excrete the bacteria through faeces and then back into the environment in the form of sewage (Kaakoush et al., 2015; Pitkanen and Hanninen, 2017). It is notable that the contribution of each pathway to human *Campylobacter* infection in South Africa is still less understood (Thobela, 2017).

Campylobacter survival in water is critical for transmission to humans through the ingestion of contaminated water. There are several factors that influence *Campylobacter* survival in water including temperature, dissolved minerals, concentration of dissolved organic matters. A temperature of 4°C favours survival which can last up to 4 months (Murphy et al., 2006; Vadde et al., 2019) and *Campylobacter* can survive in water at a temperature of up to 10°C . The ability to survive in the

environment varies by strains, with strains from different sources having different survival potential (Bronowski et al., 2014; Trigui et al., 2015). *Campylobacter* have the ability to switch into viable but non-culturable (VBNC) state after protracted exposure to water (Bronowski et al., 2014). Following the temperature in water, ultraviolet radiation (UV) has a significant impact on *Campylobacter* survival in water. According to a study by Obiri-Danso et al. (2001), a UV dose of 200 mW m/s^2 which corresponds to the dose that is provided by solar radiation in the summer led to lack of culturability of 90% of the natural population of *Campylobacter* in river water, following 2 minutes of exposure. The effect of UV will depend on the depth that the *Campylobacter* occur in the river water because UV can only penetrate to a certain depth. The presence of organic matter in water impairs *Campylobacter* survival (Tatchou-Nyamsi-König et al., 2007). Other factors including salinity, pH, and microbial predation can also affect *Campylobacter* survival in water. Importantly, *Campylobacter* can exhibit a “non-culturable viable" state” during stressful conditions. Therefore, while the bacteria may not be culturable, the cells may still be viable. In addition, *Campylobacter* can form biofilms in water environments that protect them from atmospheric oxygen (Trachoo et al., 2002; Lehtola et al., 2006; Magajna and Schraft, 2015). Biofilm formation is an adaptive mechanism in organisms when exposed to stressful environmental conditions (Święciło and Zych-Wężyk, 2013).

2.2.4 Outbreak

Outbreaks have often been linked to contaminated raw (unpasteurized) milk, animal contact, water, and environmental exposures, such as sand and mud (Stuart et al., 2010; Harder-Lauridsen et al., 2013; Zeigler et al., 2014; CDC, 2017; Kuhn et al., 2017; Lahti et al., 2017; Gaardbo Kuhn et al., 2018). However, most outbreaks have been mainly attributed to drinking contaminated water (Pitkänen, 2013a; Chukwu et al., 2019). Waterborne *Campylobacter* outbreaks have been reported in many countries (Kuhn et al., 2017). This can affect a large number of the public especially if drinking water distribution networks are involved. The first reported large waterborne outbreak was in Vermont USA (1978), where almost 3,000 patients were killed (Vogt et al., 1982; Tissier, 2012). In the majority of water-borne outbreaks, various zoonotic pathogens were detected in water and in human faeces, and this is as a result of non-specific contamination. Non-specific contamination mostly occurs if the source of contamination is wastewater (Maurer and Stürchler, 2000; O’Reilly et al., 2007). Polluted surface water, well water or rain water are suggested waterborne *Campylobacter*iosis risk factors (Neimann et al., 2003; Kapperud et al., 2003; Evans et al., 2003; Schönberg-Norio et al., 2004; Michaud et al., 2004; Carrique-Mas et al., 2005; Tissier, 2012). Between 1998 and 2006, 10 waterborne outbreaks occurred in France, and *Campylobacter* have been implicated in all cases (Tissier, 2012). The first outbreak in 2000 was as a result of a problem with the chlorination of drinking water treatment system, and 5,100 people were affected. *C. coli* was amongst the microorganisms implicated (Gallay et al., 2006). The second outbreak that ensued in 2003, affected up to 35000 people who consumed contaminated river water. *C. jejuni* and *Cryptosporidium* spp. were responsible for this outbreak (Beaudeau et al., 2008). In 2017, an outbreak occurred in Nebraska as a result of the malfunctioning of a centre pivot irrigation system that pumps livestock wastewater from a neighbouring animal feeding operation onto a nearby farmland (Pedati et al., 2019). This led to excessive runoff of wastewater and subsequent collection at a road ditch that is close to wells that supplied water to the city. This indicates the role of agricultural runoff contamination in outbreaks of *Campylobacter* enteritis. There is no documented waterborne outbreaks of *Campylobacter* gastroenteritis in South Africa, but those small scale local studies in 2.2.1 suggests that the rates of *Campylobacter* infections might be underestimated in South Africa (Samie et al., 2007, 2022; Mason et al., 2013; Thobela, 2017; Chukwu et al., 2019).

2.2.5 Infectious dose

Infections caused by *Campylobacter* are as result of ingestion of a quantity of cells. The infectious dose of *Campylobacter* is low, and ranges from 500-800 organisms (Schielke et al., 2014; Thobela, 2017). For children under than 5 years old, smaller infectious dose are required because their immunity is not well developed. Transmission from one person to another (person-to-person), occurring through faecal-oral route is possible. If sufficient dose of organisms is ingested through the oral-gastric route one or more gastrointestinal and extra gastrointestinal manifestations can occur. While low doses enable infection, some infected patients may not show symptoms even with high doses. That is low dose exposure is not generally associated with acute illness (Teunis et al., 2018) However, *Campylobacter* spp. are sensitive to hydrochloric acid in the stomach, and so some of the inoculum is destroyed. The low infective dose implies that the organisms had the capacity to survive exposure to s gastric acidity (Mahmoud et al., 2015; Varsaki et al., 2015). The dose response relation for infection strongly depends on the strain.

2.2.6 Pathogenesis

Pathogenesis of *Campylobacter*iosis is a multifactorial process and commences with adherence. Then it must invade cells to produce a host response (Wassenaar and Blaser, 1999; Bolton, 2015a; Koolman et al., 2016). The bacteria invade cells of the distal small bowel. Thereafter, it circulates to the colon. The damage is contained at the tip of intestinal villi (Konkel et al., 2001). Cellular invasion is followed by production of toxin and protein to avoid lysosomal fusion to the vacuole that contains the bacterium. This could explain how the bacterium evades the immune system of the host (Konkel et al., 2001; Watson and Galán, 2008; Rose et al., 2012; Schnee et al., 2017). The bacterial virulence factors enable the bacteria to survive within the host, but simultaneously results in an immune response. For instance, *Campylobacter* production of cytolethal distending toxin (CDT) stimulates an inflammatory response through IL-8 and promotes evasion of the immune system by arresting cell cycle (Faïs et al., 2016). Simultaneously, Toll-like receptors on intestinal epithelial cells and dendritic cells of the gut are activated. This stimulates the innate and adaptive immune pathways, which allows inflammatory cells to be recruited that are probably the cause of the resultant diarrhoea, and the eventual clearance of the organism (Bourke, 2002; Hu and Hickey, 2005; Zilbauer et al., 2007; Rathinam et al., 2008, 2009; Zheng et al., 2008; Friis et al., 2009; Masanta et al., 2013).

2.2.7 Virulence factors

The factors critical for bacterial colonisation and pathogenicity are well understood, including adhesion, chemotaxis, motility, response to oxidative stress, cytolethal distensor toxin (CDT) production, regulation of iron uptake and invasion, lipopolysaccharide and capsular polysaccharide (Konkel et al., 2001; Koolman et al., 2016). These are discussed in the following section.

2.2.7.1 Adhesion

Numerous *Campylobacter* proteins used for adhesion have been detected (Rubinchik et al., 2012). *CadF* protein is used to bind fibronectin. Fibronectin is a glycoprotein that is found at sites of cell to cell contact on gastrointestinal tract epithelial cells. Binding of *CadF* to fibronectin triggers a signalling cascade, leading to GTPase Rac1 activation (Boehm et al., 2011). This promotes the internalisation of *Campylobacter* cells through actin-mediated phagocytosis. Also, lipopolysaccharide (LPS) and lipooligosacharride (LOS) contribute to bacterial adhesion (Preston et al., 1996).

2.2.7.2 Invasion

The flagellum is important in host cell invasion. It facilitates the secretion of non-flagellar proteins through its type three secretion system (T3SS) channel. The *FlaC* proteins and CiaB (*Campylobacter* invasion antigen B) are conveyed through T3SS to the cytoplasm of the host cell and are necessary for adhesion and invasion (Perera et al., 2007). Additional intracellular invasion and invasion survival factors include *CiaC*, *CiaI*, invasion-associated protein A (*IamA*) and a chaperon protein-HtrA (Saouaf et al., 2009; Bhunia, 2018). Binding of bacteria to the host cell triggers rearrangement of host cell cytoskeleton by activating microtubules and microfilaments that allow bacteria to be internalised. Internalisation could be by zipper or trigger mechanisms. The zipper mechanism occurs by binding of adhesins on the surface of bacteria to host cells that initiates a signalling cascade that leads to zippering of the cytoskeleton of host cell plasma membrane around the bacterium. Subsequently, the bacterium gets internalised into a vacuole. Alternatively, the trigger mechanism occurs by the bacterium injecting effector proteins using T3SS and initiating a signalling event that activates small Rho GTPases together with cytoskeletal reorganisation in order to encourage membrane ruffling (Bhunia, 2018). Subsequently, the bacterium becomes internalised into the membrane bound vacuole. *Campylobacter* cannot escape from the membrane bound vacuole. Therefore at least one cycle of replication occurs within the vacuole.

2.2.7.3 Motility

Flagella and a chemosensory system that pushes flagella movement depending on the conditions of the environment make up the motility system of *Campylobacter* (Konkel et al., 2001). Flagella in *Campylobacter* are essential for swimming through the mucus layer covering the epithelial lining of the intestine. This allows *Campylobacter* to reach its site of colonisation (i.e. inner mucus layer of the intestine).The motility system is made up of a number of proteins that perform different functions. Structurally, the flagella is made up of a hook-basal body that is made up of proteins such as *FliF*, the T3SS proteins (*FliR, FliQ, FliP, FliO, FLhB, and FLhA),* motor components (*MotB* and *MotA*), motor switch proteins *(FliG, FliM, FliN, FliY*), as well as minor hook components (*FlgI, FlgH, FlgE, FliK, FliM*). The extracellular filament is made of *FlaA* and *FlaB* proteins. Mutation in key genes, including *flaA*, *flaB*, and *flhA* and *flhB*, will prevent *FlaA* or *FlaB* production and eventually halt motility, invasion and pathogenesis (Bhunia, 2018).

2.2.7.4 Chemotaxis

Chemotaxis is a physiological response of the motile bacterium towards chemical cues (Konkel et al., 2001). There are two components of chemosensors: methyl-accepting chemotaxis proteins (MCPs) and histidine kinase-dependent signal transduction system (Chandrashekhar et al., 2017). The histidine kinase-dependent signal transduction system is well understood and is made up of chemotaxis proteins such as *CheZ, CheY, CheW, CheR*, and *CheB, CheA*. The flagellar proteins are controlled by the chemosensing proteins thereby controlling directional movement of the pathogen (Bhunia, 2018).

2.2.7.5 Lipopolysaccharide and Capsular Polysaccharide

Lipooligosacharride (LOS), O and N linked glycans, and capsular polysaccharides (CPS) are the different groups of carbohydrate structures that can be found on the cell surface of *Campylobacter* (Karlyshev et al., 2004; Day et al., 2012). The LOS molecule comprises core oligosaccharide and Lipid A, and is associated with different roles, including host cell adhesion, evasion host immune system, invasion as well as guard from complement mediated killing (García-Sánchez et al., 2018). Sialylation of LOS increases potential of invasion and decreases *C. jejuni* immunogenicity (Nachamkin et al., 1998). Sialylated *LOS* of *C jejuni* can mimic those human antigens that are involved in the appearance

of Miller Fisher syndrome and GBS (Louwen and Hays, 2013). The sialyltransferase c*stII* gene provides LOS with a protective barrier that enhances its invasion of the epithelial cells by mimicking the human ganglioside in the vertebrate nerve cells (Otigbu et al., 2018b). This causes the immune system of the host to self-destruct its ganglioside.

The N-linked glycosylation system of *C. jejuni* is involved in posttranslational modification of periplasmic proteins, such as flagellin. This function is encoded by *pgl* multigene locus that is found in *C. jejuni*. N-linked glycosylation of surface proteins mediates immune evasion and shield *C. jejuni* from gut proteases (Karlyshev et al., 2005). CPS has been linked with several functions, such as protecting the pathogen from hostile environmental conditions, such as biofilm formation, resistance to desiccation, and virulence in the gastrointestinal tract (GIT) (Karlyshev et al., 2005). In *C. jejuni* there is a significant diversity of genes that encode CPS as well as LOS (Table 2). The structural variation of LOS and CPS has been associated with the existence of multiple strategies for evading the host immune response by the pathogen.

2.2.7.6 Iron acquisition system

For several microorganisms, iron acquisition is necessary for colonisation and infection of host. Iron is a cofactor in several proteins that are intricate in basic cellular pathways and metabolism of pathogens. *C. jejuni* cannot synthesize siderophores that are essential for all bacteria to acquire iron. However, it possesses an iron uptake system that can use siderophores from competing species (Parkhill et al., 2000). Many iron uptake systems have been proposed for *Campylobacter,* and the enterobactin system is the most used. The enterobactin system is encoded by *CfrA* and *CfrB* genes. The proteins Fur (ferric uptake regulator) and *PerR* (peroxide stress regulator) regulate most of the genes involved in uptake of iron (Konkel et al., 2001; Bolton, 2015a; Bhunia, 2018).

2.2.7.7 Cytolethal Distending Toxin (CDT)

The Cytolethal Distending Toxin (CDT) is produced by a group of Gram negative bacteria such as *C. jejuni*, and it triggers apoptosis of host cell (CJ) (DeFraites et al., 2014; Otigbu et al., 2018b). CDT is important for the host mucosal inflammatory response for the release of interleukin-8 (IL-8) by intestinal cells (Perera et al., 2007; Otigbu et al., 2018b). Structurally, CDT comprises of an AB_2 tripartite structure (Samosornsuk et al., 2015). The tripartite connotation stems from the fact that CTD is made up of three similar sized molecular weight toxins, *cdtA, cdtB* and *cdtC. cdtB* is the main effector, whereas *cdtA* and *cdtC* are makeup units that are involved in cell membrane binding. The molecular masses of cdtA, *CdtB*, and cdtC proteins are 27 KDa, 29 KDa and 20 KDa, respectively (Samosornsuk et al., 2015). Subunit A is directly involved in DNA damage, whereas subunit B, a binding subunit, assists in the binding of the toxin to the specific target cells which inhibit cdc2. This leads to cellular distention and ultimately death. The DNase activity of CDT is fatal, and causes the breakage of DNA single strands at a lethal dose (LD) of approximately 50 pg/mL (Samosornsuk et al., 2015). As a result of the cell and DNA degradation, inflammatory diarrhoea with faecal leukocytes occurs. Additionally, the DNA degradation can result in lesions in fragmented DNA strands that can lead to cancer (World Health Organization, 2001; DeFraites et al., 2014). CDT is highly similar amongst cross species with *cdtB* having the highest interspecies similarity (Samosornsuk et al., 2015). However, it is notable that that *cdtB* can be absent in some species (Samosornsuk et al., 2015).

2.2.7.8 Stress response/survival

Campylobacter lacks most of the adaptive responses detected in other bacteria (Parkhill et al., 2000). However, the *spoT* gene encodes a stringent control of stress response in *C. jejuni*. Stress conditions, such as amino acid starvation results to the building up of uncharged tRNA. This stalls the ribosomes, and ribosome associated RelA or SpoT is activated, eventually. Furthermore, during the course of *C. jejuni* lifecycle, it is exposed to a wide variety of oxygen conditions. When the oxygen concentration is low, alkyl hydroperoxide reductase (AhpC) detoxifies hydrogen peroxide. Also, as a result of partial reduction of oxygen, through aerobic respiration reactive oxygen species are generated. Superoxide anion and hydrogen peroxide (H_2O_2) are the reactive oxygen species generated, and they can combine to produce the highly toxic hydroxyl radical (HO) (Bolton, 2015a). *C. jejuni* single catalase, KatA, converts hydrogen peroxide to water and oxygen at a high concentration of hydrogen peroxide in cytoplasm (Bingham-Ramos and Hendrixson, 2008; Atack and Kelly, 2009). The superoxide dismutase SOD proteins shield the bacteria from the superoxide anion (Palyada et al., 2004). *C. jejuni* bears a single SOD which is produced constitutively and not as a result of superoxide anion (Garénaux et al., 2008). Thiol peroxidise (Tpx) is another peroxiredoxins found in *C. jejuni.* Also, Rrc (Rbr/Rbo like protein) encoded by *Cj0012c* and the periplasmic *C. jejuni* protein Cj1371 defend *Campylobacter* against aerobic stress and reactive oxygen species (Garénaux et al., 2008; Bolton, 2015a). Additionally, a heat shock reaction has been observed in *Campylobacter* as means to enhance survival (Konkel et al., 1997; Mihaljevic et al., 2007). The *dnaJ* gene encodes a heat shock protein (Ziprin et al., 2001; Konkel et al., 2001).

2.3 ANTIMICROBIAL RESISTANCE

When treatment of human *Campylobacteriosis* is required, fluoroquinolones (ciprofloxacin predominantly), macrolides (erythromycin), and tetracyclines are the drugs of choice (Sifré et al., 2015; Asakura et al., 2019). However, increasing resistance to these antimicrobials among *Campylobacter* is observed (Zhang et al., 2016; Pergola et al., 2017; Vinueza-Burgos et al., 2017; Tang et al., 2017). Indiscriminate use of antibiotics as food additives in livestock for infection control and prevention as well as growth enhancement has led to the increase of microbial resistance to these antibiotics (Engberg et al., 2001; Koolman et al., 2015). In serious systemic infections, aminoglycosides such as gentamicin (Acheson and Allos, 2001; Ge et al., 2002; Luangtongkum et al., 2009) are administered.

2.3.1 Mechanism of resistance to quinolones

Quinolones inhibit bacterial DNA synthesis and thus cause cell death. They target two enzymes (DNA gyrase and topoisomerase IV) intricate in bacterial DNA replication, transcription, recombination and repair (Jacoby, 2005). Gyrase and topoisomerase consist of two types of subunits – GyrA, GyrB and ParC, ParE, respectively (Payot et al., 2004). Resistance to fluoroquinolones is as a result of the substitution of amino acids located in the quinolone resistance determining region (QRDR). QRDR is located on the surface of the enzyme. The *gyrA* gene in *Campylobacter* contains several modifications that are connected with fluoroquinolone resistance (Asp-90-Asn, Thr-86-Ile, Thr-86-Lys, Thr-86-Val, Thr-86-Alaand Asp-90-Tyr). The most commonly observed mutation in *Campylobacter* quinolones resistance involves the change in C257T on the *gyrA* gene, this causes Thr-86-Ile to be replaced (Payot et al., 2006). Ciprofloxacin resistance may be due to the Thr-86-Ile point mutation in the *gyrA* gene (Ge et al., 2005). Also, CmeABC is a principal efflux mechanism that gives rise to resistance to various antimicrobials, such as fluoroquinolones and macrolides. CmeABC encodes an operon that is made up of three genes, *cmeA*, *cmeB* and *cmeC*. These three genes encode the outer membrane protein, internal transport membrane transporter and periplasmic fusion protein respectively. g*yrA* mutations

are the most common mechanism of fluoroquinolone resistance in *Campylobacter* spp.(Pumbwe and Piddock, 2002). *mfd* (mutation frequency decline) is another important gene for the development of fluoroquinolone resistance in *Campylobacter* (Han et al., 2008). The *mfd* gene encodes a transcription-repair coupling factor intricate in strand-specific DNA repair. Expression of *mfd* is induced by ciprofloxacin. By mutating the *mfd* gene, the rate of spontaneous ciprofloxacin mutation resistance reduced up to 100-fold. Alternatively, overexpression of *mfd* increased the frequency of mutation. Loss of *mfd* in *C. jejuni* significantly lessened the development of fluoroquinolone-resistance in *Campylobacter* culture treated with fluoroquinolones (Han et al., 2008).

Table 2-2 Virulence factors involved in *Campylobacter* pathogenesis. Adapted from (Bolton, 2015a)

		Virulence factor(s)	Encoding gene(s)	References
***Campylobacter* factors**	**motility**	FlaA, the major flagellin protein	*flaA*	Nachamkin et al. (1993); Wassenaar et al. (1993); Lertsethtakarn et al. (2011)
		FlaB, the major flagellin protein	*flaB*	Nachamkin et al. (1993); Wassenaar et al. (1993); Lertsethtakarn et al. (2011)
		FliF, hook-basal body protein	*fliF*	Carrillo et al. (2004)
		FliM & FliY, flagellar motor proteins	*fliM & fliY*	Nachamkin et al. (1993); Wassenaar et al. (1993); Lertsethtakarn et al. (2011)
		FlgI, P-ring in the peptidoglycan	*flgI*	
		FlgH, L ring in the outer membrane	*flgH*	
		FlgE & FliK, minor hook components	*flgE & fliK*	
***Campylobacter* factors**	**motility**	σ28 promoter regulates *flaA* gene expression	*fliA*	
		σ54 promoter regulates *flaB* gene expression	*rpoN*	
		Proteins involved in flagellin O-linked glycosylation	*cj1321-cj1325/6*	
Chemotaxis factors		Chemotaxis proteins; Che A, B, R, V, W, & Z.	*cheA, cheB, cheR, cheV, cheW & che Z.*	
		Methyl-accepting chemotaxis proteins (MCPs) also called transducer-like proteins	*tlp1, tlp4, tlp 10*	

	CheY, response regulator controlling flagellar rotation	*cheY*	
	Campylobacter energy taxis system proteins CetA (Tlp9) and CetB (Aer2)	*cetA & cetB*	
Chemotaxis factors	AI-2 biosynthesis enzyme	*luxS*	
	AfcB, MCP protein required for persistence in the cecum	*acfB*	
***Campylobacter* adhesion factors**	CadF, outer membrane protein	*cadF*	
	CapA, *Campylobacter* adhesion protein A	*capA*	
	Phospholipase A	*pldA*	
	Lipoprotein	*jlpA*	
	Peb1, periplasmic binding protein	*peb1A*	
	Peb3, transport protein	*peb3*	
	Peb4, chaperone playing an important role in exporting proteins to the outer membrane	*peb4*	
	FlpA, fibronectin-like protein A	*flpA*	
***Campylobacter* adhesion factors**	Type IV secretion system possibly involved in adhesion	*virB11*	
	JlpA, 42-kDa lipoprotein involved in adhesion to Hep-2 cells	*jlpA*	
			References
***Campylobacter* invasion factors**	FlhA, FlhB, FliO, FliP, FliQ & FliR, components of the flagellar T3SS	*flhA, flhB, fliQ, fliP, fliO & fliR*	Carrillo et al. (2004)
	FlaC protein secreted into the host cells and essential for colonisation and invasion	*flaC*	Carrillo et al. (2004); Konkel et al. (2004)
	CiaB, 73-kDa protein involved in adhesion	*ciaB*	Konkel et al. (2004)

	CiaC, protein required for full invasion of INT-407 cells	*ciaC*	Christensen et al. (2009); Eucker and Konkel (2012)
	CiaI, reported role in intracellular survival	*ciaI*	Buelow et al. (2011)
	IamA, invasion associated protein	*iamA*	Carvalho et al. (2001); Rivera-Amill et al.(2001)
***Campylobacter* invasion factors.**	HtrA, chaperone involved in the proper folding of adhesins	*htrA*	
	VirK, may have a role in protection against antimicrobial proteins	*virK*	
	FspA, protein with a role in apoptosis	*fspA*	
***Campylobacter* invasion factors.**	HtrA, chaperone involved in the proper folding of adhesins	*htrA*	
	VirK, may have a role in protection against antimicrobial proteins	*virK*	
	FspA, protein with a role in apoptosis	*fspA*	
Toxin	Cytolethal distending toxin (CDT) subunits	*cdtA, cdtB& cdtC*	
	1,3 galactosyltransferases involved in lipopolysaccharide production	*cgtB & wlaN*	
Capsule	Capsular polysaccharide transport gene M	*kspM*	
	Capsule biosynthesis gene	*kspE*	
N-linked glycosylation system	N-linked glycosylation	*pgl*	
Iron uptake system	Outer membrane ferric enterobactin FeEnt receptors	*cfrA & cfrB*	
	CeuE, lipoprotein involved in iron acquisition	*ceuE*	
	Cj0178 putative transferring bound iron utilization outer membrane receptor	*cj0178*	
	Ferric uptake regulator	*fur*	
	Outer membrane receptor for hemin and haemoglobin	*chuA*	

Multidrug and bile resistance and stress response genes in *Campylobacter*	CME efflux pumps consisting of a periplasmic protein (CmeA), inner membrane efflux transporter (CmeB) and an outer membrane protein (CmeC)	*cmeA, cmeB & cmeC*	

Table 2-2 Virulence factors involved in *Campylobacter* pathogenesis continued. Adapted from (Bolton, 2015a)

	Virulence factor(s)	**Encoding gene(s)**	**References**
Multidrug and bile resistance and stress response genes in *Campylobacter*	CmeR, CME efflux pump transcriptional repressor	*cmeR*	Lin et al. (2002)
Stress response	Stringent control	*spoT*	Gaynor et al. (2005)
	Kat A, Catalase (convert hydrogen peroxide to water and oxygen)	*katA*	Bingham-Ramos and Hendrixson (2008); Atack and Kelly (2009)
	AhpC, alkyl hydroperoxide reductase	*ahpC*	Atack et al. (2008)
	Tpx, thiol peroxidase	*tpx*	
	Cytochrome c peroxidases		
	SOD proteins, antioxidant proteins	*sod*	Mihaljevic et al. (2007)
	Cj545c, an NADPH quinine reductase	*cj545c*	Wang and Maier (2004)
	Other proteins that protect against reactive oxygen species	*cj0012c & cj1371*	Garénaux et al. (2008)
	Heat shock protein	*dnaJ*	Konkel et al. (1998); Ziprin et al.(2001)

2.3.2 Mechanism of tetracycline resistance

Tetracycline enters into the periplasmic space using porins by binding with Mg 2^{+} cations. In the periplasmic space it is disconnects from Mg 2+ and travels into the cytoplasm where it binds to the 30S ribosomal subunit (Wieczorek and Osek, 2013). Inhibition of the placing of aminoacyl tRNA into the ribosome and inhibition of protein synthesis occurs. Tetracycline resistance in *Campylobacter* is due to the *tetO* gene. *tetO* genes encoding ribosomal protective proteins, which are plasmid borne, and they give rise to tremendously high levels of resistance to tetracycline (Connell et al., 2003).

2.3.3 Mechanism of resistance to macrolides

Erythromycin is the earliest isolated antimicrobial macrolide. Macrolides inhibit peptide chain extension by binding to the 23SRNA in the 50S subunit of bacterial ribosome (Wegener et al., 2008). Previous studies discovered that 23SrRNA nucleotide positions 2058 and 2059 as the main sites for macrolide binding, which results in changes in the structure of the ribosome. In *Campylobacter,* there are three copies of the 23S rRNA gene. Modification of the ribosome binding site through mutation of 23S rRNA or modifications in resulting proteins leads to macrolide resistance in *Campylobacter.* Also, in *Campylobacter,* resistance to macrolides antimicrobials may be as a result of a bacterial efflux pump. Interplay between mutations in the 23SrRNA gene and efflux activity has been suggested in literature, and this plays a major role in the high level of macrolide resistance in some *Campylobacter* isolates (Corcoran et al., 2006). A unique characteristic of macrolide resistance in *Campylobacter* is that it is slowly developed during antibiotic treatment and comprises a multistage process (Luangtongkum et al., 2009). This is unlike fluoroquinolone-resistance that develops rapidly during antibiotic treatments. In *Campylobacter* , macrolide as well as fluoroquinolone resistance is as a result of mutations in target genes (Table 3) (Mikysková, 2018).

2.3.4 Mechanism of resistance to aminoglycosides

Aminoglycoside resistance genes occur in several bacteria and generally encode proteins that regulate antimicrobial agents. The A-site (A for aminoacyl) of a ribosome is the site where charged t-RNA molecules bind during protein synthesis. Aminoglycosides (e.g. streptomycin, gentamycin, and kanamycin) bind to A- site of the bacterial ribosomal 30S subunits, thereby making the ribosome unavailable for translation (Jana and Deb, 2006). This leads to cell death (Kotra et al., 2000). Several aminoglycoside-modifying enzymes have been defined in *Campylobacter* spp., such as aminoglycoside-adenyltransferase, aminoglycoside phosphotransferases type I, III, IV and VII, and 6-aminoglycoside adenyltransferase (Mikysková, 2018). Each of these enzymes has their unique modifying site and substrates. The resistance to aminoglycosides is as a result of an enzymatic modification that reduces affinity of aminoglycosides to rRNA A-site. It is important to note that the *apha-7* gene encoding resistance to kanamycin is unique to the genus *Campylobacter* (Rao et al., 2001; Mikysková, 2018). Also, it is noteworthy that a plasmid can mediate kanamycin resistance and tetracycline resistance simultaneously. Therefore, *Campylobacter* spp. can be resistant to kanamycin and tetracycline, concurrently (Gibreel et al., 2004a).

2.3.5 *Campylobacter* mechanism of resistance to other antimicrobials

Chloramphenicol prevents peptide chain extension. It binds reversibly to the peptidyl transferase centre in 50S ribosomal subunit, thereby inhibiting bacterial protein synthesis. Chloramphenicol resistance is as a result of plasmid-carried- acetyl transferase gene that modifies chloramphenicol (Mikysková, 2018). Chloramphenicol resistance has been described in *C. coli*, although it is rare (Schwarz et al., 2004). Resistance to sulfonamide

is common in *C. jejuni* isolates. The target for resistance to sulfonamides is the enzyme dihydropteroate synthase. Resistance is as a result of substitution of four amino acid residues in dihydropteroate synthetase (DHPS), which results in reduction of the binding affinity for sulfonamides. This is the only mechanism that has been used to explain sulfonamide resistance in *Campylobacter* spp. (Wegener et al., 2008; Mikysková, 2018).

Mechanisms of *Campylobacter* resistance to beta-lactams, including cephalosporins and ampicillin varies and is not well understood. Usually, most *Campylobacter* are resistant to narrow spectrum beta-lactams, such as penicillins and cephalosporins (Wieczorek and Osek, 2013). Beta-lactams interrupt cell wall formation, resulting in cell death. Beta-lactam resistance may be due to changes in the membrane structure (Martin and Kaye, 2004). The majority of *C. coli* and *C. jejuni* are capable of producing beta-lactamases that inactivate the beta-lactam ring. Some *Campylobacter* species are predominantly susceptible to ampicillin and amoxicillin (Wieczorek and Osek, 2013). An overview of mechanism of resistance to antibiotics for *Campylobacter* spp. is presented in Table 3

Table 2-3 Summary of basic resistance mechanisms for *Campylobacter* spp (Wegener et al., 2008; Mikysková, 2018)

Antibiotics	Mechanism of resistance	Resistance genes or mutation
Aminoglycosides	Antibiotic modification by enzymes (AphA AadE, Sat)	Apha-3, Ant-6, Ant-3, Apha-7
β-lactams	Enzymatic inactivation of antibiotics with β-lactamases, Reduced membrane permeability due to MOMP Efflux via CmeABC	- ND *Cat*
Fluoroquinolones	Modification of DNA gyrase targets Efflux via CmeABC	*gyrA*; Ala-70's Thr; Thr-86's Ile, Lys, Ala, Wall; Asp90 to Ala, Asn, Tyr *Mfd*
Macrolides	Mutations at 23S rRNA site Mutation in ribosomal proteins L4 / L22 Efflux via CmeABC Reduced membrane permeability thanks to MOMP	23S rDNA; A to G at position 2.075; AND to C to position2.074
Tetracyclines	Modification of Target Ribosomal Site A by TetO binding Efflux via CmeABC	*TetO*

2.4 *CAMPYLOBACTER* ANTIBIOTIC RESISTANCE RATES IN SOUTH AFRICA: ASITUATIONAL ANALYSIS

An overview of the rates of antibiotic resistance in of *Campylobacter* from poultry human, cattle, and pigs in South Africa is presented in Figure 2-1. Locally, molecular characteristics and prevalence of antibiotic resistant thermophilic *Campylobacter* spp. in human, animal and environmental reservoirs have not been well researched and this could be due to lack of funding. A high rate of resistance against tetracycline is common in *Campylobacter* isolates from humans (82%), and poultry (100%). Almost half (43%) of *Campylobacter* from poultry are multidrug-resistant. (Bester and Essack, 2008). It is observed that the rate of resistance to tetracycline in *Campylobacter* spp. from poultry has fluctuated, but continues to be high (up to 100%) since 2008 and 2013 (Bester and Essack, 2008, 2013). There is a dearth of information on the occurrence of antibiotic resistant *Campylobacter* in human isolates, with a limited number of studies in this area (Shobo et al., 2016; Thobela, 2017) Existing studies show high antibiotic resistance rates for *Campylobacter* from human

diarrhoeal stools. Between 2016 and 2017, antibiotic resistance of *Campylobacter* spp. against tetracycline has fluctuated from high resistance (82%) to highly susceptible (15%) (Shobo et al., 2016; Moré et al., 2017). Also, the rate of resistance to azithromycin has reduced from 70 to 14%, between 2016 and 2017 (Shobo et al., 2016; Thobela, 2017). . A gradual increase in resistance of human isolates against ciprofloxacin (4%-53%) and erythromycin (35-53%) is observed from 2004-2017 (Obi et al., 2004; Thobela, 2017). There seem to be higher susceptibility of *Campylobacter* to newer fluoroquinolone antibiotics, i.e. gatifloxacin compared to ciprofloxacin. The study by Shobo et al. (2016) showed 23.6% resistance to ciprofloxacin and 8.3% to gatifloxacin. At specie level, *C. jejuni* shows high susceptibility to erythromycin (92%) and azithromycin (88%) and tetracycline (44%). On the other hand, *C. coli* shows high sensitivity to both the macrolides, accounting for erythromycin (82.5%) and azithromycin (77.5%) (Thobela, 2017). Besides it is alleged that the actual number of MDR *Campylobacter* cases in South Africa is likely to be underreported because of difficult culturing for antimicrobial testing.

According to a few local studies, *Campylobacter* isolates from cattle have shown reckonable resistance rates (Kambuyi, 2018; Karama et al., 2020). But the resistance rates for azithromycin, florfericol, gentamicin, and telithromycin are low. The rate of ciprofloxacin resistance in cattle has decreased between 2012 (≤56%) and 2020 (5.8%) (Uaboi-Egbenni et al., 2012; Kambuyi, 2018; Karama et al., 2020). Furthermore, multidrug resistance to clindamycin, nalidixic acid and tetracycline has been recorded in cow isolates. 32.5% of isolates from cattle are resistant to more than one antibiotic (MDR). Compared *to C. upsaliensis* (18.7%), significantly higher levels of MDR are found in *C. jejuni* (36.9%) and *C. coli* (33.3%) isolates. Multidrug resistance patterns detected are majorly nalidixic acid/clindamycin (17.8%) and tetracycline/clindamycin (14.2%) (Karama et al., 2020). Additionally, *C. coli* and *C. jejuni* isolates from pigs have shown resistance to ciprofloxacin (53.4% and 67.1%), nalidixic acid (27.7% and 26.8%), and much lower resistance to gentamycin (12%). In a recent study, all *Campylobacter* isolates from estuarine water samples have shown resistance against tetracycline (Otigbu et al., 2018b). Isolates from estuarine water showed multidrug resistance for fluoroquinolones, macrolides, and tetracyclines. Anthropogenic activities around the estuary included agriculture, extensive human development, and various industries. *Campylobacter* isolates from the river have shown increasing rates of resistance against Clarithromycin (95%) (Chukwu et al., 2019). It can be deduced that the rate of resistance against to azithromycin (92%) is higher in isolates that are found in water sources compared to human (70%), poultry (none recorded) and livestock (8.1%) isolates. A potential increase in resistance against azithromycin in human isolates is foreseen. It is possible that sharing of resistance may occur in the future between environmental and clinical isolates (Chlebicz and Śliżewska, 2018). Similarly, the rate of resistance against clindamycin is higher in isolates that are found in water sources compared to livestock (36%). Clindamycin resistance has not been recorded for isolates from humans and poultry, so far. The occurrence of antibiotic resistant isolates that are devoid in human and animal reservoirs in source water emphasize uncontrolled entry of the selection pressure (antibiotics) from sources independent of their application in animal and human health. This phenomenon supports that water resources present an environment where novel resistance in *Campylobacter* can emerge. In source water, the rate of *Campylobacter* that is resistant against azithromycin is higher and may potentially disseminate to human and animal isolates. Source water presents an environment where novel antibiotic resistance, such as clindamycin resistance can emerge in *Campylobacter.* Major facilitators of antibiotic resistance spread in South Africa are unsuitable prescribing behaviours, treatment based only on symptoms instead of diagnosis, lenient regulation of antibiotic use in animal health and a lack of monitoring antibiotics contaminants in WWTW effluents, which are subsequently discharged into source waters.

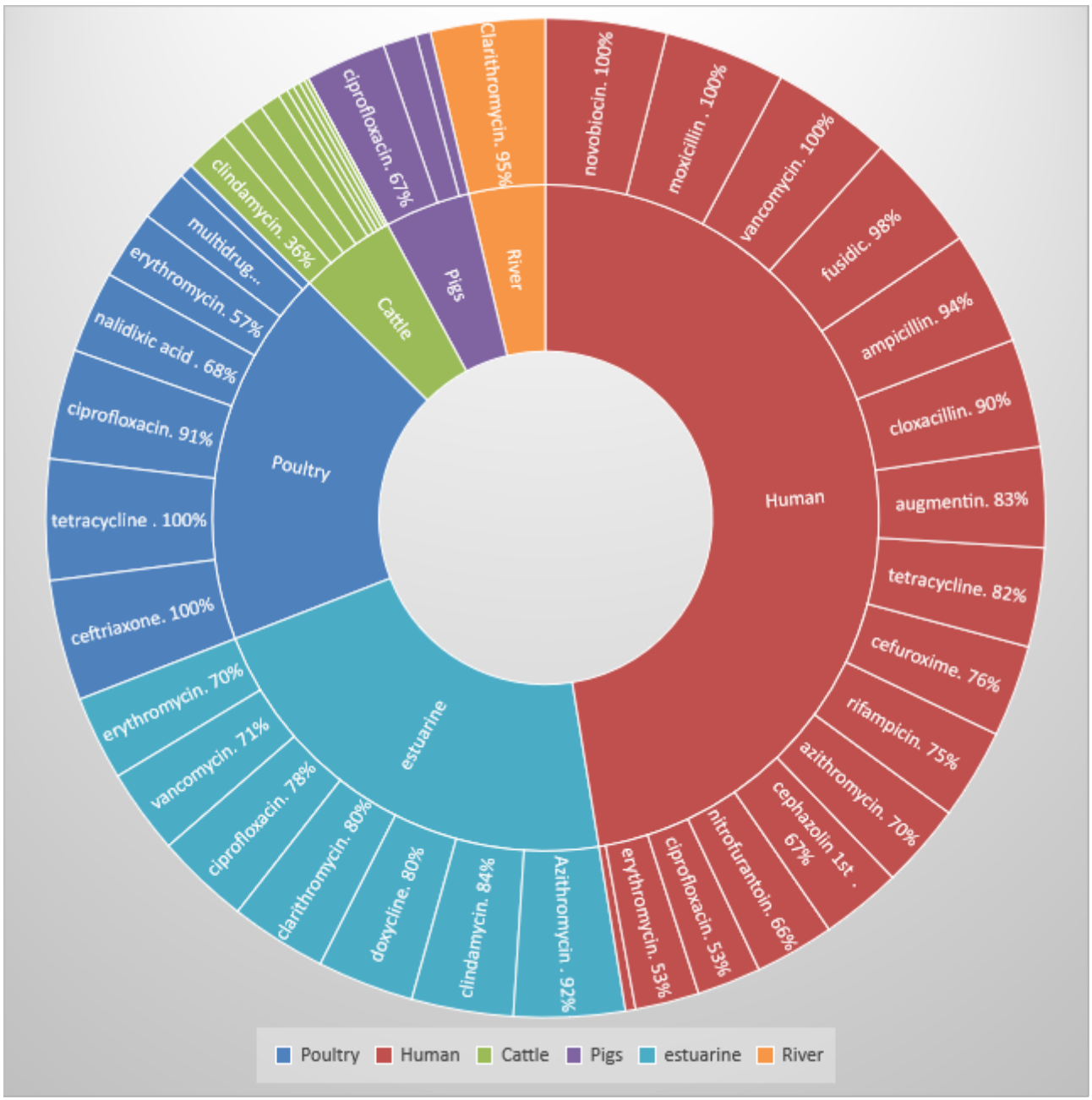

Figure 2-1 Summary of *Campylobacter* antibiotic resistance rates in poultry human, cattle, pigs and environmental waters in South Africa

2.5 METHODS USED TO STUDY *CAMPYLOBACTER,* THE TEMPORAL AND GEOGRAPHICAL DISTRIBUTION OF SPECIES, MODES OF TRANSMISSION TO HUMAN, AND THE SOURCE OF CONTAMINATION DURING OUTBREAK.

To date, the environmental surveillance for *Campylobacter* in South Africa is scarce. According the few previous local publications (Diergaardt et al., 2004a; Chukwu et al., 2019). *Campylobacte*r isolation rate for environmental water sources in South Africa is low. One of the studies investigated the presence of *Campylobacter* in water samples collected from the Crocodile River and could not detect *Campylobacter* contamination the samples (Chukwu et al., 2019). These observations might be because the detection method is inefficient. This is supposed because results from investigating clinical specimens suggest that

Campylobacter infection is endemic in South Africa, with high proportion of diarrheal patients. As long as a river is impacted by human activities *Campylobacter, it* is bound to be present (Chukwu et al., 2019). With the effective screening method, it will be possible to obtain the reliable result, and it will specifically benefit the Campylobacteroisis risk assessment plan in South Africa.

Current understanding of the diversity and transmission of pathogenic *Campylobacter* is based on the application of highly selective cultivation (Tissier, 2012; Ugarte-Ruiz et al., 2012; Di Giannatale et al., 2016).. However, not all viable *Campylobacter* species are culturable (Baffone et al., 2006; Silva et al., 2011; Bronowski et al., 2014). Moreover, *Campylobacter* that has been exposed to water for a prolonged period of time may enter a dormant phase and change their shape from s-shaped to coccal shaped making it hard to culture (Rollins and Colwell, 1986b; Jones et al., 1991; Ikeda and Karlyshev, 2012). Isolation of these bacteria is difficult because they are slow growing, and adaptation to in vitro conditions for growth is difficult (Acheson and Allos, 2001). Polymerase chain reaction (PCR) amplification using *Campylobacter*-specific primers has been effectively applied for sequencing of pathogens from known outbreaks and epidemics (Berenger et al.; Platts-Mills et al., 2014b, 2018; Liu et al., 2016; Liang et al., 2018; Shrestha et al., 2019; Pedati et al., 2019; Yu et al., 2020; Tzani et al., 2021). PCR techniques are advantageous because they are quick, with relatively high sensitivity and rapidity, and remove nearly all background organisms in the sample, amplifying only the target's genomic regions. However, in surveillance studies, (PCR) may fail because of genetic evolution of an existing pathogen, or poor assay design. A small number of mismatches between primers and the targeted genome could lead to failure to generate amplicons of interest (Maljkovic Berry et al., 2020). PCR techniques require different primer pairs for the detection of individual organism, and changes to conditions. Furthermore, Also, PCR requires extra validation when changing the detectable species. There are 27 possible Campylobacter species, and so it is impractical to design PCR specific primer for each one of them (Parker et al., 2022).

2.6 NEXT GENERATION SEQUENCING

The advent of next generation sequencing (NGS) has made it possible to sequence all the microorganisms within a sample to identify and track infectious aetiologies responsible for outbreak (Rasko et al., 2011). Specifically, metagenomics next generation sequencing applies massive parallel next generation sequencing (NGS) technologies to produce sequence data, which is used to profile hundreds of microorganisms that are within a sample. Precisely, the shotgun metagenomics approach provides classification at higher resolution, e.g. resolution of several Campylobacter species that are seldom culturable and for which exact probes have not been developed, as well as other pathogenic and non-pathogenic microorganisms present in a sample. Metagenomics circumvents the challenge of growing most fastidious microorganisms, which is encountered by using traditional culture-based techniques. Another advantage is that it generates a pathogen genome for source tracking or outbreak investigations without needing culturing. By assembling and analysing of pathogen genomes in a shotgun library it is possible to unravel pathogen spread, epidemics dynamics, possible sources, etc. (Maljkovic Berry et al., 2020). For example, it enabled the genetic complexity and polyclonality of bacterial species, such as *Campylobacter* in samples, necessary to understand transmission dynamics and achieving accurate source attribution (Parker et al., 2022).

Metagenomic sequencing has been used for epidemiologic investigation targeting *Campylobacter* (Parker et al., 2022). According to the study, using this approach it is possible to identify *Campylobacter* species with sequencing reads that is as low as 1,400 reads, but the possibility of identifying the *Campylobacter* species depends on the quantity of these organisms in the microbiome. A high level of Campylobacter many distinct reads to be mapped around a known *Campylobacter* genome, and samples with high read counts to a specific *Campylobacter* species enable remapping of reads to numerous variable regions of the species from multiple strains, allowing strain level characterisation. Nevertheless, samples that provide a few million reads is

advocated. Shotgun metagenomic sequencing requires only a single sequencing method and with the extensiveness of species in the Campylobacter genus, it presents comparatively an efficient approach to clear species identification.

However, most studies were performed using stool samples. Water, especially unprotected sources, such as rivers, are recognised reservoir of *Campylobacter* spp. and the prevalence can range from 60% to 79% of the total bacteria detected (Savill et al., 2001; Van Dyke et al., 2010; Chukwu et al., 2019). A literature study demonstrated the importance of considering exposure to untreated water sources as a potential cause for Campylobacter outbreaks. One possible explanation for the lack of up-take of this method, in water studies, is the large volume of water that needs to be filtered in order to detect low concentration microbes. It is important to investigate this risk factor, in order to expedite public health intervention (Pitkänen, 2013b; Pedati et al., 2019).

2.7 SHOTGUN METAGENOMICS RISK ASSESSMENT AND SOURCE ATTRIBUTION

The use of metagenomics to characterise complex microbial populations which are comprised by fastidious microbes, such as *Campylobacter*, makes it amenable not only for surveillance, but source attribution and risk assessment, as well. Regarding risk assessment, hazard identification is one of the different elements of microbiological risk assessment. Metagenomics provide the ability to potentially generate in some cases consensus draft genomes of the strains of interest, allowing a very rapid characterisation (virulence and AMR repertoire, among others) of the pathogenic strains present in a sample (Hendriksen et al., 2019b) and, in most times ARG occurrence in environmental waters mirror the clinical resistance type circulating amongst bacterial species in an area (P€arn€anen et al., 2019). Shotgun metagenomics renders data of the whole pool of microbial genes in a sample. Therefore, can be used to deduce the full repertoire of resistance determinants within such a sample, although depending on the approach, those determinants might not be assigned to particular taxa and it is still difficult to obtain information on the AMR determinants harboured by specific bacterial strains. The advantage of this approach is that it allows he estimation of their relative abundance, and in some cases, it is possible to get important information on the genetic background of the detected AMR determinants (microbial species or strain of origin and/or association with mobile genetic elements, such as plasmids, integrons, transposons, prophages, etc.) (Ravi et al., 2017). With recent advances in long read sequencing technologies, it can be possible to increase the resolution of ARGs, which will enable specific information on their location within mobile genetic elements of host strains to be obtained (Charalampous et al., 2019; Che et al., 2019). Similar approaches to those followed to study the resistome through shotgun metagenomics can be used to identify in a given sample virulence determinants linked to colonisation, cellular communication or pathogenicity functions, although this has been less frequently carried out. However, there are some examples available in the literature. For instance, the role of the rumen as a reservoir of virulence-associated genes has been monitored by (Singh et al., 2012) and (Auffret et al., 2017). The benefits (rapidity; simultaneous detection of a range of virulence genes; potential for obtaining information on the genetic background of the virulence determinants), limitations (results will depend on the quality of the databases; uncertainty on the agreement between virulence genes detection and phenotype), and opportunities (potential to re-analyse previously sequenced genomes and to link sequencing data with metadata from the samples) of using shotgun metagenomics with this aim are similar to those previously described for the resistome. In summary, the hazard identification step will help in providing a whole pool of microbial genes (pool of antibiotic resistance genes, mobile genetic elements, virulence genes and determinants) in a given sample. By using metagenomic shotgun sequencing, it is possible to couple multiple layers of risk assessment indices obtained concomitantly to make predictions at a very high level of confidence.

There have been growing support to incorporate shotgun metagenomic sequencing in microbial risk assessment (Cocolin et al., 2017; Fresia et al., 2018; Koutsoumanis et al., 2019; Ekwanzala et al., 2020;

Lindner et al., 2022). The most important step will be to link the variance in shotgun metagenomics sequence data between samples to difference in contamination/pollution state. In that regard, progress has been made in distinguishing pollution by pathogenic strains of bacteria and physiologically similar ecotypes using metagenomic shotgun sequencing. The most remarkable evidence of potential *Campylobacter* contamination of a sample is the abundance and type of virulence and antibiotic resistance genes associated with *Campylobacter* organisms. Identification features (virulence and antibiotic resistance genes associated with Campylobacter) by shotgun metagenomics that are reproducible across independent samples is crucial to establishing a robust and generalizable predictive model for *Campylobacter* pollution/contamination of sample, and they can become risk assessment indices. This will require experimentally modelled explanations of the big-data generated, as well as comparative analysis of the results (Mercer et al., 2020).

Significant progress has already been made regarding using features of microbiome as a diagnostic tool for disease (Truong et al., 2015; Pasolli et al., 2016a). Machine learning algorithm can handle the amount of data generated from shogun metagenomic sequencing. Machine learning algorithms comprise developing and applying computer algorithms in order to identify predictor combinations that will predict the risk outcome (Njage et al., 2018). MLA models depend on extracting “features” from sequence data (LaPierre et al., 2019). These features may signify various aspects of a microbiome, including, taxonomic composition or functional profiles. In clinical studies, Machine learning methods has been used to unravel potentially important disease state biomarkers o(Segata et al., 2011; Nagata et al., 2022). The most informative features provides insights into how the microbiome relates to the disease data (LaPierre et al., 2019).

Using predicted functional profiles and their abundances as features and applying numerous popular classical machine learning algorithms, it is possible to predict the safety of the water samples. For this study, the functional features will be virulence genes of *Campylobacter* origin within the metagenomic data. Various genes have been linked to *Campylobacter* virulence, including but not limited to *ciaB*, *cadF*, cdtB*, htrB* and *clpP* (Abu-Madi et al., 2016). Popular classical machine learning algorithms include Naïve Bayes, logistic regression, decision tree, Support Vector Machines (SVMs), Random Forests (RFs) and K-nearest neighbour that can be used to predict the safety status of water sample (Pasolli et al., 2016b).

A risk assessment approach applying shotgun metagenomics to produce distinct associations between informative features and *Campylobacter* safety status of any environmental samples as healthy and unhealthy from measured parameters is not available but is necessary. There is no established protocol, expertise, capacity and means for NGS microbial risk assessment for environmental water samples, yet. However, the behaviour of a specific hazard and its virulence potential within an environment can equally be used in hazard identification and characterization studies during risk assessment (Couto et al., 2018). There is need to investigate the potential of MLA to identify of relevant features in a complex shotgun metagenomics data set, which can facilitate robust and reliable predictions of *Campylobacter* safety of any environmental sample. The new approach will alleviate the problem of lack of dose-response models for ARB and difficulty to culture fastidious pathogens for concentration estimation, which are necessary for MRA.

For source attribution, Machine learning algorithms applied to metagenomes originating from humans and from different sources to identify clusters of source-specific genetic markers offer a promising approach. The potential of such a source-attribution approach has been recently demonstrated by Gupta et al. (2019), who could distinguish distinct aquatic environments based on their resistome profiles, by applying an extremely randomised tree algorithm to identify discriminatory resistance genes (Koutsoumanis et al., 2019). Metagenomic data from different reservoirs that potentially contribute to river pollution has become increasingly available (Baral et al., 2018; McGhee et al., 2020a), and therefore renders the prospects to develop and discover new source-attribution approaches for metagenomics. Particularly, shotgun metagenomics offers the possibility to perform source-attribution based on genetic source-indicators alternative to the established

standards (cgMLST, SNPs, etc.), eventually even based on a mix of taxa and/or functional genes (Koutsoumanis et al., 2019).

Besides, human disease burden of Campylobacteriosis can be partitioned to specific sources by source attribution. Different methods have been used to estimate the relative contribution of different sources of *Campylobacter* infections, such as epidemiological studies, microbiological studies, and evidence synthesizing approaches. Microbiological studies, which focus on cases only are most suitable for the purpose this study. Microbiological approaches are based on statistical modelling of subtyping data from human cases and pathogen occurrences in particular animal, environmental and food sources (Pires et al., 2009). It involves one-to-one matching the subtypes of the human isolates with the most likely subtypes of isolates from different sources. This matching method has been applied for *Campylobacter* (Mughini-Gras et al., 2016) and some other major foodborne pathogens, such as *Salmonella* (Hald et al., 2004), and *Escherichia coli* (Mughini-Gras et al., 2019). Therefore, It is possible to compare the strains from river water samples, which have been contributed by the different identified sources/ reservoirs, to MLST typed South African *Campylobacter* reference strains of human infection. Allelic profiles of MLST typed South African *Campylobacter* reference strains of human infection are publicly available in the MLST database http://pubmlst.org/*Campylobacter* /. Furthermore, the same principle can be applied to attribute *Campylobacter* that is present in the aquatic environment (river in this case) to different reservoirs. A recent study has applied molecular genotyping (multilocus sequence typing) in an attempt to inform transmission of *Campylobacter jejuni* from several potential sources to human hosts in South Africa (Thobela, 2017). The study sampled *Campylobacter jejuni* from human hosts and animal hosts (chicken, sheep, and bovine. A diversity of genotypes was obtained from Chicken, (ST257, ST1932, ST613, ST7997, ST227, St52), Human (ST6091, 475, 658, 475, 918, 22, 583, 4063, 45, 2109, 403 and 829,7997, 50, 883,52, 881, 51, 356, 4624, 1471, 354, 572, 1737, 474, 5809, 19, 1875) and Bovine (21, 381) *C. jejuni* isolates in the study. The outcome of this study forms a background knowledge for following studies, enabling the linking of different reservoirs and pathways to infection for human Campylobacteriosis, locally.

CHAPTER 3: SITE CHARACTERIZATION

The Bloukrans River flows through Makhanda and is a tributary of the Kowie River. Makhanda is a small university town in Makana Local Municipality that hosts Rhodes University. It is urbanised but not industrialised. Housing is high cost, medium cost, low cost and Informal settlements. Free grazing and unrestricted movement of animals such as cattle and donkeys are common, even in the town's Central Business District (CBD). Figure 3-1 shows the Map of the Bloukrans River.

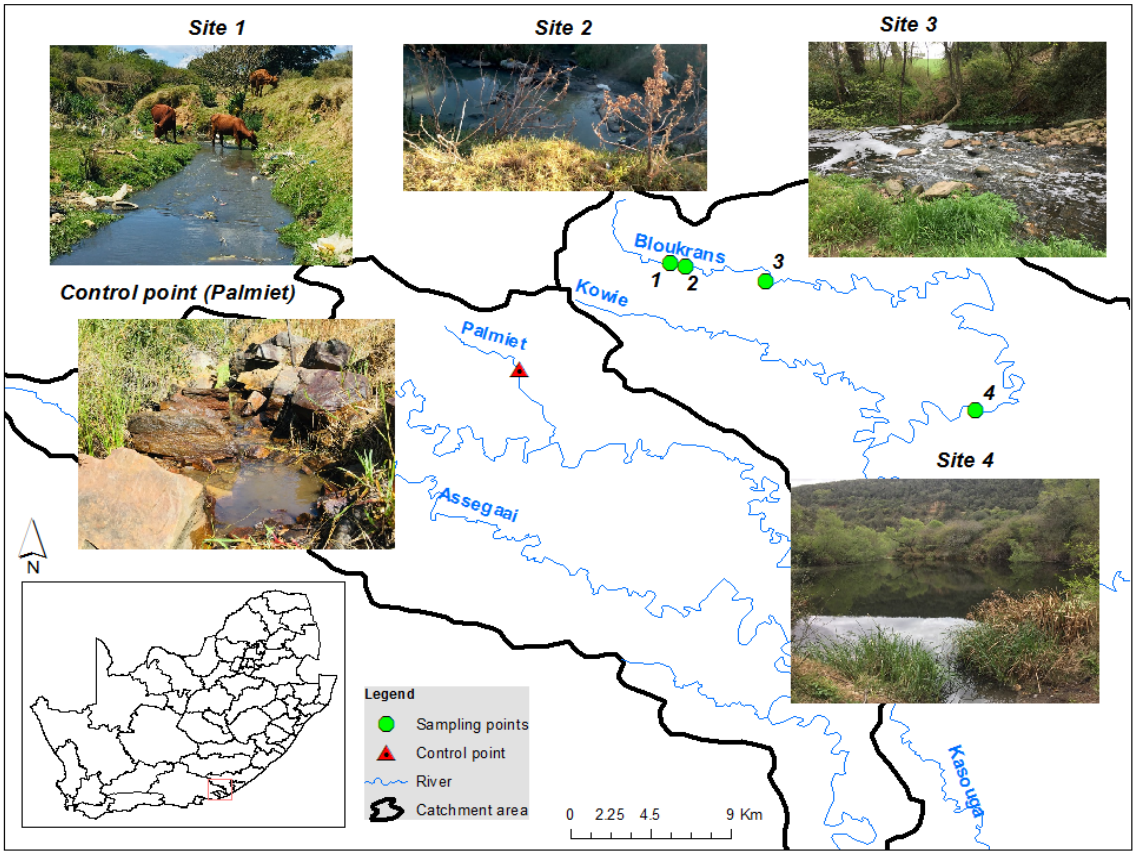

Figure 3-1 Map of Bloukrans/Kowie River

3.1 CONTROL SITE (BC); PALMIET RIVER

Site one (Figure 3-2) lies on Palmiet River, which lies Southwest of Grahamstown. It is used as a control site as it is located away from human settlements and is least likely to be impacted by human activities. Furthermore, The Palmiet River is selected for this study because it is within the same ecoregions as the Kowie River.

Figure 3-2 Palmiet River; the water is clear and odourless, but the site has become more accessible to humans (for leisure and spiritual purposes), resulting in an increase in plastic disposal at the site

3.2 SITE B1:

This site is located in an urban area near a human settlement (Vukani Location). The local people keep animals, and they use this stream as water for their animals. The town is characterised by the unrestricted movement of animals that drop their faecal matter in the streets. Unrestricted movement of animals is a public nuisance, and their faecal matter is carried along with runoff during rains and ends up in streams. The water at this site (Figure 3) is not clear (grey) and unpleasant odours and some plastics characterise it.

Figure 3-3 Site 1, a sampling point at the Bloukrans River, so-called Vukani Bridge. The primary pollution is from nearby human settlements and faecal matter from animals that drink from the river. Water is usually grey as a result of faecal inputs.

3.3 SITE B2

This site lies downstream (1.3 km) of site 2 in an urban area and at the beginning of the Agriculture area. It is downstream of the Belmont Valley Wastewater Treatment Plant (WWTP) discharge point. The Belmont Valley WWTP employ the Conventional WWTP process and applies a biological filtration system for biological treatment. The plant is dysfunctional, and its infrastructure is dilapidated. Water from this site is characterised by unpleasant odours and colour (Figure 3-4)

Figure 3-4 A sampling point of the Bloukrans River, at the effluent discharge point (EDP) of the Belmont Valley Wastewater Treatment Plant (WWTP). The plant infrastructure is dilapidated. Water from this site is grey because of faecal inputs from the WWTP.

3.4 SITE B3:

Site 3 site lies downstream (5 km) of Site 2, in the agriculture area (Figure 3-5). The fast-flowing water is characterised by unpleasant odours and appears grey. The grey colour is suspected to be faecal inputs flowing from Site 2. Farms downstream of this site use water from this river for irrigation. The water in this area is mainly prone to pollution from agriculture runoff.

Figure 3-5 A sampling point of the Bloukrans River surrounded by agricultural lands. Water from this site is mainly used for irrigation. Also, animals access this river to drink and can pollute the river with their faeces.

Site B4

This site is located in a forested area at the end of the agricultural area and downstream 14km of site B4. The water is slow-moving and forms some stagnant pools (Figure 6). The site is accessed by local people for spiritual purposes and also for recreation activities such as swimming.

Figure 3-6 Site B4 Bloukrans Pool, located in a forested area. The water is slow-moving and forms some stagnant pool. This river site is primarily used for baptism and other cultural activities.

CHAPTER 4: INVESTIGATING OPTIMAL METHOD TO RECOVER *CAMPYLOBACTER* CELLS FROM RIVER WATER SAMPLES

4.1 INTRODUCTION

In order to establish true association of source water exposure to increasing *Campylobacteriosis*, an improved understanding of the prevalence of *Campylobacter* in the water is necessary. Current understanding of the diversity and transmission of pathogenic *Campylobacter* is based on the application of highly selective cultivation approaches. Following concentration of *Campylobacter* on a membrane, the bacteria are isolated on agar media directly after concentration or following an enrichment step. (Blaser and Cody, 1986) To inhibit the growth of commensal flora of the water sample, the selective media (blood agar) is supplemented with several antibiotics. There is no universally accepted standard media cultivation for recovery of *Campylobacter.* Campy-Cefex agar, Skirrow agar, Campy-CVA agar (CVA), Karmali agar, modified cefoperazone charcoal deoxycholate agar (mCCDA), Campy-Line agar (CLA), and CAMPY agar/Campy FDA agar are commonly used highly selective media.

To date how to recover *Campylobacter* cells from environmental water samples is still a significant problem for investigating occurrence environmental water samples. Detection of *Campylobacter* in environmental water samples is difficult because they are mostly present in low numbers (Jiang et al., 2015). Concentration either by filtration using membranes of known materials (polycarbonate) and pore sizes (between 0.8-0.1 µm) or by centrifugation are the two standard methods for amass microbial cells from water samples. Previous studies comparing concentration by membrane filtration and centrifugation provide different conclusions (Brindle et al., 1987; Thomson et al., 2008; Wang et al., 2017). Alternatively, *Campylobacter* can be enriched from water samples directly, without prior filtration or centrifugation of water samples (Denis et al., 2011a). This method is recommendable for turbid river water samples. However, the proportion of river water to the broth medium can affect the recoverability of *Campylobacter* using this method. An established protocol to recover *Campylobacter* from water samples is lacking. Besides collecting river water samples, given the low and ununiform distribution of *Campylobacter* in the river a different sampling method – so called Moore Swab method can be applicable (Bisha et al., 2011; Sikorski and Levine, 2020). The method has been applied and is recommended for trapping microbial cells present in environmental water body, including *Campylobacter.*

This aspect of the project aims to establish the optimal method to recover *Campylobacter* cells from river water samples. Recovery of *Campylobacter* cells from river water samples is necessary to enumerate and quantify of *Campylobacter* present in river water samples, for microbial risk assessment. Firstly, the physicochemical quality of the river water and presence of faecal contamination had to be considered, as the physicochemical quality of water determines the occurrence of *Campylobacter.* Besides, *Campylobacter* is highly found in river water that is faecal contaminated, hence the presence of fecal coliforms were confirmed. Different known methods (centrifugation, membrane filtration) for concentrating and recovering microbial cells in environmental water samples were evaluated. Besides collecting river water samples, a different sampling method – so called Moore Swab method was evaluated for its effectiveness in recovering *Campylobacter* cells than sampling river water. Based on average colony count on CCDA plates, an optimal recovery method was selected which can be used to assess the occurrence of *Campylobacter* spp in Bloukrans River water samples by culture based and non-culture based (PCR) methods.

4.2 METHODS

4.2.1 River water sampling

The water samples were collected from different sites along the Bloukrans River system in Grahamstown, Makanda District Municipality, Eastern Cape South Africa (**Table 4-1**). The different sites along the Bloukrans River system represent different level of pollution and turbidity. Water samples were collected in the months of January 2020 to December, 2021. The sampling period covered the four seasons of the year, i.e. winter, spring, summer and autumn Five litre plastic bottles were used to collect the water samples. The bottles were sterilised by first washing with antibacterial soap, rinsed with tap water followed by soaking in 30% HCl for 5 minutes and finally rinsed with sterile distilled water. At the sampling site, the outside of the sterile 5 L bottles were rinsed with 70% Ethanol. The bottle was dipped 10-20 cm below the surface the sampled water body and the sterile 5 L bottles was filled to the brim with the respective sampled water. After sampling, all water samples were stored in a cooler box at 4°C and transported to Rhodes University for analyses within 6 hours of sample collection. Water temperature were measured in the field. Membrane filter was transferred onto MFC culture medium, selective for faecal coliforms in a Petri plate and incubated at 35 ± 0.5°C for 2 h followed by further incubation 44.5 ± 0.2°C for 20 ± 2. Observed blue colonies on MFC media were counted. Where the colonies were too numerous to count, dilutions were made. Turbidity were analysed using a Hach 2100P turbidity meter (Loveland, CO, USA). Faecal coliforms which is an indicator of faecal contamination, (ii) turbidity and river flow, which are indicators for precipitation and surface runoff, (iii) water temperature and (iv) PH (v)Dissolved oxygen (vi) Electroconductivity. The physicochemical quality of the river water and presence of faecal contamination had to be considered, as the physicochemical quality of water determines the occurrence of *Campylobacter.* Besides, *Campylobacter* is highly found in river water that is faecal contaminated.

Table 4-1 The different study sites along the Bloukrans River system in Eastern Cape South Africa and the expected pollution level. BC is a reference site (a Palmiet River site) and was chosen because it is in the same ecoregion as the Bloukrans River.

Site identification	Contamination
B1	Considerable contamination is expected from runoffs from the informal settlement and sewerage from leaking sewer and potable water pipes in the urban area of Grahamstown.
B2	Substantial contamination with insufficiently treated effluents from Belmont Valley Wastewater Treatment Plant (WWTP) in Grahamstown
B3	Considerable contamination mainly impacted by manure runoffs from nearby agricultural lands and excreta from animals that come to drink and graze at the river bank.
B4	The site is called Blaauwkrantz Pool. It is expected to be the least contaminated because it is situated in a forested area at the end of the agricultural area along the Bloukrans River system.
BC (Reference Site)	This is a Palmiet River site. It is pristine compared to the Bloukrans River sites, with less impact from anthropogenic sources.

4.2.2 Moore swab Sampling

Moore swabs were created in the laboratory using cotton gauze strips as described by Sikorski and Levine (2020). Briefly, strips of cotton gauze were cut into 6-inch by 48-inch lengths and folded eight times to form an 8-ply square. Each 6 by 6-inches square-pad was tied by a twine around the centre, and thereafter sterilized in an autoclave. The sterile Moore swabs were deployed at the selected river sites. To secure the swabs, the strings attached to heavy stones found at the river sites. After 72 hours the swabs were collected in a sterile container and transported on ice to the laboratory.

4.2.3 Enumeration of Faecal coliforms concentrations in water samples

mFC plates were prepared by dissolving 52 g of the mFC powder into 1 L of dH_2 O water. The solution was boiled for one minute to ensure that it is completely dissolved. Thereafter, 20-25 mL of this solution was poured each petri-dish and left to solidify. The level of Faecal coliforms was too many to be counted directly, and so each of the water sample was serially diluted. To do this 10 mL of a water sample was diluted by factors of 10. Thereafter, a 10 mL 1/1,000dilution of the water samples was filtered onto a 0.45 µm cellulose-nitrate membrane filter (Sartorius Stedim Biotech GmbH, Goettingen, Germany) using a Millipore manifold filtration system (EZ-Fit™ Manifold, Merck KGaA, Darmstadt, Germany). This was followed by placing the filter at the centre of the plate containing solidified mFC media and incubated at 42-44.5ºC, for 20 ± 2h (Mahmud et al., 2019). Respective water samples were inoculated in triplicates, for each of the sampling sites. Observed blue colonies were counted and recorded as colony forming units per mL (CFU=(no. of colonies X dilution factor)/(Vol of culture plated).

4.2.4 Concentration of microbial cells from water samples

This study assessed three different methods, namely centrifugation, membrane filtration and a combination of centrifugation and membrane filtration for concentrating microbial cells in water samples

4.2.4.1 Concentration of microbial cells from water samples by membrane filtration

150 mL of river water sample was filtered using 0.45 µm pore size cellulose filter paper (Sigma-Aldrich) to trap the bacteria in the water samples. This was performed in duplicate. The filter paper was recovered and chopped into pieces using a sterile pair of scissors wiped with ethanol and then placed into a 1.5 mL tube. Tubes were stored at -20°C, until use for DNA extraction.

4.2.4.2 Concentration of microbial cells from water samples by centrifugation

150 mL of water sample was centrifuged in duplicates at 10 000 x g for 15 minutes (Banting et al., 2016). The pellet was collected and transferred into a 1.5 mL tube and stored at -20°C, until DNA extraction.

4.2.4.3 Concentration of microbial cells from water samples by combining centrifugation and filtration

It was suspected that 150 mL of river water is small for assessing the presence of *Campylobacter* spp., because *Campylobacter* occurs in low number in river water. It is possible to omit *Campylobacter* if a small volume of water is processed. Furthermore, as there is no consensus on the superiority of centrifugation over filtration method and vice versa, for concentrating bacteria from river water centrifugation and filtration method were combined, sequentially, to concentrate *Campylobacter* cells from river water samples. Since water samples are to be centrifuged initially, it is possible to assess a larger volume. 500 mL of each river water sample centrifuged at 14,000 ×g for 20 minutes (Khan et al., 2009a) (Khan et. et al., 2009). 500 mL (a volume less than 1000 mL) was used as recommended by Abulreesh et al. (2017) because the river water samples contain suspended materials. The pellet, which is supposed to contain *Campylobacter* cells, was resuspended in 10 mL of sterile distilled water by vortexing. The supernatant was collected and then filtered through 0.45 µm pore size, 47 mm diameter filter membrane to trap suspended *Campylobacter* that was not pelleted (Vereen et al., 2013). In this way, the problem of membrane filters pores clogging during filtration was not encountered because the centrifugation step reduced the suspended particles in the water samples. The filter paper was recovered and chopped into pieces using a sterile pair of scissors wiped with ethanol and then placed into the centrifuge tube containing the 10 mL resuspended pellet. The resuspended pellet, together with the filter membrane, was collected stored at -20°C, until DNA extraction.

4.2.5 DNA extraction, yield and quality characterisation

Genomic DNA isolation was conducted using the DNeasy Blood Tissue Kit (Qiagen), following manufacturers instruction (Djurhuus et al., 2017). DNA concentration and quality was determined with a NanoDrop ND2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). The NanoDrop measures DNA concentration and the ratio of absorbance at A260/A280 and A260/A230, which assesses the purity of the DNA sample. In general, a 260/280 ratio of ~1.8-1.88 is acknowledged as "pure" for DNA. A reduction of this ratio suggests protein contamination, whereas an increase in this ration suggests RNA contamination of the extracted DNA. (Koetsier, and Cantor, 2013).. Also, 0.7% agarose gel was prepared for gel electrophoresis by mixing 0.7 g of agarose powder in 100 mL of 1 X TAE buffer containing ethidium bromide. Thereafter, 3 µl of the sample and 0.6 µl of 6x loading dye was added to each well. 5 µl of Generuler 1 kb ladder (Thermo Scientific) was loaded in a separate well. The gel was allowed to run at 82 V for 30 minutes. Thereafter, the gel was visualised using a molecular imager ChemiDocTM XRS + BIO-RAD) to capture its image. DNA extraction for the positive controls was also done separately. Modified charcoal cefoperazone deoxycholate agar (mCCDA) plates of dead *Campylobacter jejuni* cells were obtained from the Food chain laboratory in Port Elizabeth, Eastern Cape. DNA from colonies on this plate was isolated by boiling method and used for positive controls.

4.2.6 Culturing *Campylobacter* cells from water samples

This study assessed culturing *Campylobacter* cells from river water samples preprocessed by (i) direct enrichment, without initial concentration, and (ii) Centrifuging followed by membrane filtration. This study assessed the direct enrichment method because it is recommendable for turbid river water samples, containing a lot of suspended particles (Denis et al., 2011a). However, only a small volume of water can be assayed by using the direct enrichment method. This is a problem because of the low number of *Campylobacter* in river water samples because the cells can be missed out. Alternatively, it is possible to assess a larger volume of water samples if initially concentrated by centrifugation. Therefore, this study tested the two methods to recover and grow *Campylobacter* from river water samples. Notably, *Campylobacter* Agar Base Blood Free (CCDA) media was used to isolate *Campylobacter* from the river water samples selectively, because it provides significant recovery rates from environmental water samples (Smith et al., 2015). CCDA contains a combination of oxygen quenching charcoal and cefoperazone. The combination of oxygen quenching Charcoal and Cefoperazone promote the growth of *Campylobacter* spp., (Denis et al., 2011a)(Denis et al., 2011a)(Denis et al., 2011a)(Denis et al., 2011a)(but inhibit the growth of Gram-negative enteric bacilli and some Gram-positive species.

4.2.6.1 Direct enrichment of water sample without prior concentration

River water samples were enriched with Bolton broth supplemented with Bolton antibiotics (Oxoid). in a 1:1 ratio and incubated under microaerophilic atmosphere at 37°C for 48h in order to resuscitate the *Campylobacter* cells (Denis et al., 2011a). Thereafter, 200 µl of the enriched sample was spread plated onto CCDA agar plates (in duplicates), and incubated under microaerophilic atmosphere at 37°C for hours. The Microaerophilic atmosphere was created by placing one Oxoid CampyGen compact sachet in between two plates/flasks, in an anaerobic jar. Two colonies from two different plate were selected randomly and subjected to colony PCR.

4.2.6.2 Culturing Campylobacter cells from water samples concentrated by initial centrifugation followed by membrane filtration

Water samples were concentrated as described in **4.2.4.3**. The resuspended pellet, together with the filter membrane was placed in 90 mL of Bolton broth (BB) (UK Environmental agency, 2018) supplemented with Bolton antibiotics, and incubated at 42ºC in a microaerophilic environment for 48h (Pitkanen and Hanninen,

2017). Thereafter, 200 µL of enrichment culture was collected from below the surface of the enrichment culture, and spread onto the surface of CCDA. This was followed by incubating the CCDA plates at 42ºC for 48 +/-2h under microaerophilic atmospheric conditions (Diergaardt et al., 2004c; Lévesque et al., 2011; Mughini-Gras et al., 2016). Plates were observed for colony growth, as distinct moist, flat, greyish, metallic, spreading colonies grow on the agar surface. Presumptive colonies were subjected to discontinuous streaking on Columbia agar plates (Spellbound Labs, Port Elizabeth, South Africa), containing 5% lysed Horse blood. The procedure was conducted under aseptic conditions in a laminar flow hood. The transfer loop was flamed until it turned orange and allowed to cool down. The Columbia agar plate was opened near the flame and colony picked up using the loop. The loop was reflamed and allowed to cool down, the colony was dragged through one quadrant in four parallel lines. The loop was re-flamed and allowed to cool down. The colony from the streaked parallel lines was dragged through to the next quadrant in four parallel lines. The procedure was continued to the next quadrant and in the last quadrant; a curly streak is made from the third quadrant. The inoculated plates were incubated under microaerophilic conditions at 37ºC for 24 hours. After the process of discontinuous re-streaking was repeated three times, and the isolated colonies of *Campylobacter* spp. were considered pure and subjected to Gram staining, microscopy, and colony PCR,

4.2.7 Gram staining for identification of suspected *Campylobacter* colonies.

Gram staining was performed using the following procedure. The entire procedure was done under aseptic conditions in a laminar flow hood. The transfer loop is immersed in 70% alcohol. The loop is flamed until it turns orange. It is then allowed to cool down before use. The loop was used to collect a small amount of the colony from the agar plate and then smeared on a slide in thin layer and let to dry and fixed (dried over flame, being careful not to cook the organism). Crystal violet solution was poured over the fixed culture for a minute and rinse off with water (being careful not to wash away the smeared organism). Iodine solution was then added onto the smear, which was let to stand for a minute; and then rinsed off with water (being careful not to wash away the smeared organism). Alcohol (decolouriser) was poured over the smear until the runoff is colourless (it takes 5 seconds), and was immediately rinsed off with water (being careful not to wash away the smeared organism). Safranin (Counterstain) was applied next over the smear and let to stand for a minute. It was then rinse off with water (being careful not to wash away the smeared organism). Thereafter the slide was dried with bibulous paper to remove the excess water. The slide was then placed on a compound microscope, and focused to identify whether gram negative/positive (Gram negative appear red/pink in colour, while Grampositive appear blue in colour).

4.2.8 Colony PCR

The experiment was conducted under an aseptic condition in a lamina flow hood. Isolated colony from the discontinuous streaked plate, were collected using an autoclaved toothpick and resuspended in 100 µl of nuclease-free water in a sterile 1.5 mL tube. The tubes were capped and boiled at 100ºC for 10 minutes to lyse the cells. After boiling lysis, the tubes were centrifuged at 20 000 x g for 2 minutes, and the cell debris was discarded (Woodman et al., 2016). The supernatant was used as template for PCR. For the PCR, 50 µl PCR reactions were prepared to consist of 25 µL 2X KAPA Taq Ready Mix (1X), 17 µl PCR-grade water, 1.5 µl of both forward and reverse primers (10 µM) and 5 µL of the boiled bacterial supernatant. Universal 16s rDNA primers 63F (5' CAGGCCTAACACATGCAAGTC 3') and 1387R (5' GGCGGWGTGTACAAGGC 3') (Marchesi et al., 1998; Moura et al., 2009) was used to amplify the bacterial spp. The DNA testing tube along with the contents were placed in the PCR machine and run for 2 hours 9 minutes. The PCR condition is as follows: the initial cycle of denaturation at 95ºC for 5 minutes, thirty cycles of denaturation at 95ºC for 1 minute, annealing at 52ºC for 1 minute and extension at 72ºC for 1.5 min, followed by one cycle of final extension at 72ºC for 5 minutes and held at an infinite cycle at 4ºC. For agarose gel and Electrophoresis, 0.7 g Agarose

powder was be transferred into a conical flask that contained 100 mL of 1X TAE and weighed. The contents were heated up in a microwave for 2-3 minutes until the powder has dissolved and transparent. The volume loss was made up by adding autoclaved MilliQ water; 6 µl Ethidium bromide (10 mg/mL) was added to the solution. Thereafter the solution was poured into a gel dock, with a comb in place. After the solution set, the comb was removed and the gel placed in a gel station. The gel station was filled with 1X TAE solution, 2 cm above the gel. A mixture of the 5 µl PCR product and 1 µl 6x loading dye were added into the wells. Both 6 L control solution and 6 L of the ladder solution (Quick load purple 1 kb DNA Ladder) were also be added in respective wells. The Electrophoresis machine was run for 30 minutes at 90 volts. Thereafter, the gel was placed in a UV machine (molecular imager ChemiDocTM XRS+, supplied by BIO-RAD) to capture its image. For the positive controls, Modified charcoal cefoperazone deoxycholate agar (mCCDA) plates containing dead *Campylobacter jejuni* cells were obtained from the Food chain laboratory in Port Elizabeth, Eastern Cape. DNA was isolated by boiling method using colonies. From the remaining PCR amplicons, 15 µl was sent to Inqaba Technologies (Pretoria, South Africa). The resulting data from Inqaba Technologies was run through FinchTV software to view and arrange the DNA sequence, and the resulting sequence was run through the NCBI BLAST database to identify which sequence belong to which bacteria.

Statistical analysis

Analyses of coefficient of correlation (r) was conducted to understand the relationship between the various water quality parameters and to determine whether significant differences in water quality occur between different seasons, as well indicate the variables responsible for water quality variations. All these statistical analyses were performed in Microsoft Excel, 2010 using the XLSTAT software (Satpathy et al., 2011; Satheeshkumar and Khan, 2012).

4.3 RESULTS AND DISCUSSION

4.3.1 Physicochemical properties and average Faecal coliform count of the water samples collected during the sampling periods

4.3.1.1 Turbidity (NTU)

Turbidity levels of the water samples collected at different sites along the Bloukrans River ranged from 1.94 to 155 NTU. The highest level of turbidity (155 NTU) is recorded in water sampled during Winter at Site B4. The observed increase in the level of turbidity is not expectable, given lesser rains in winter season. However, there could have been a rain event on that day, which could have carried particles into the river. It is also at this Site that the lowest turbidity (1.94 NTU) was recorded. The site is used for recreational. In general most of the sites at Bloukrans River, water samples have turbidity levels above the South African limit (3 NTU)for recreational waters (DWAF, 1996). Water with high turbidity reflects an increased possibility of pathogenic microorganism, and the turbidity recorded at all the Bloukrans River sites (Sites 2 to 6) indicates *Campylobacter* can thrive in the River (Van Dyke et al., 2010) This is because the increasing turbidity levels can prevent disinfection of water disinfected appropriately (DWAF, 1996).

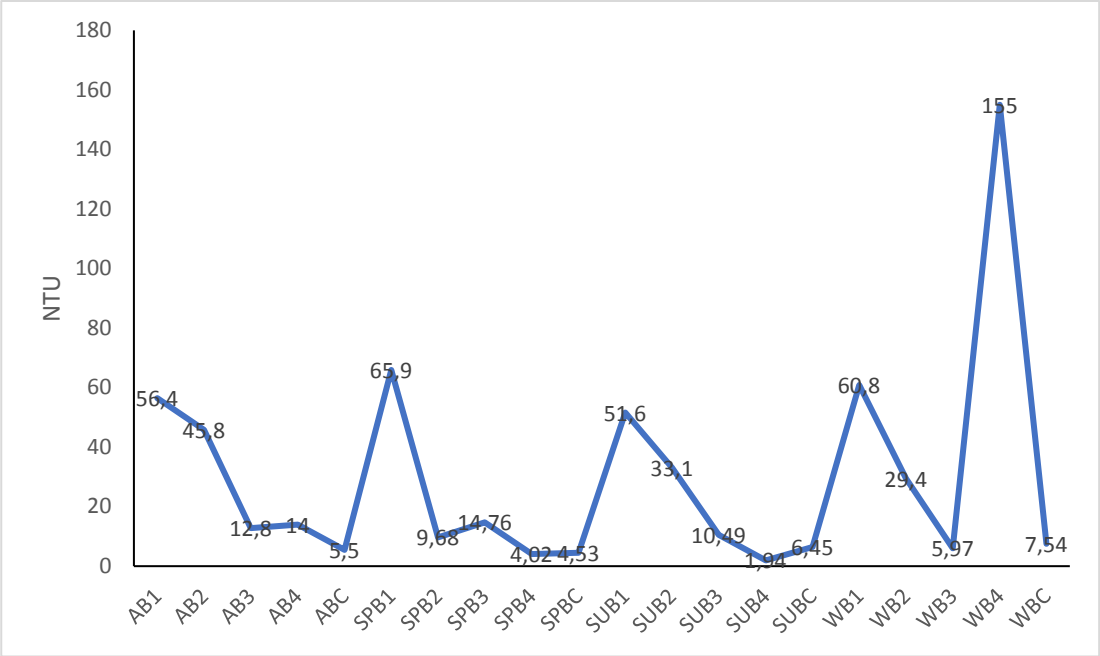

Figure 4-1 Seasonal variation of turbidity along the Bloukrans River system.at four sites in different seasons in South Africa. BC, B1, B2, B3, and B4, are site identification numbers BC is the reference site and most pristine. Autumn season include AB1, AB2, AB3, AB4, and ABC; Spring season include SPB1, SPB2, SPB3, SPB4,and SPBC; Summer season include SUB1, SUB2, SUB3, SUB4, and SUBC; Winter season include WB1,WB2, WB3, WB4, AND WBC.

4.3.1.2 Temperature

Temperatures measurements at the different sites along the Bloukrans River, where water was sampled from ranged from 11.9ºC to 25.5ºC. The highest temperature (25. 5ºC) is recorded at Sites B4 during the summer sampling. Generally, temperature impacts on the levels of pathogenic organisms in surface water. According to microcosm studies, survival of *Campylobacter* in water is said to be improved at low temperatures, especially at around 4ºC rates (Rollins and Colwell, 1986a). However, the maximum temperature at which the survival times become significantly shorter is from 16ºC to 22ºC. Therefore, the water temperatures measurable for Bloukrans River in this study may not suitable for the bacteria to grow. It has been suggested that the warm South African climate causes higher temperatures and longer UV exposure periods, which can to lower potential survival and consequent isolation rates (Diergaardt et al., 2004b). However, one report has shown that *Campylobacter* has the ability to survive at these temperatures (Hazeleger et al., 1998).

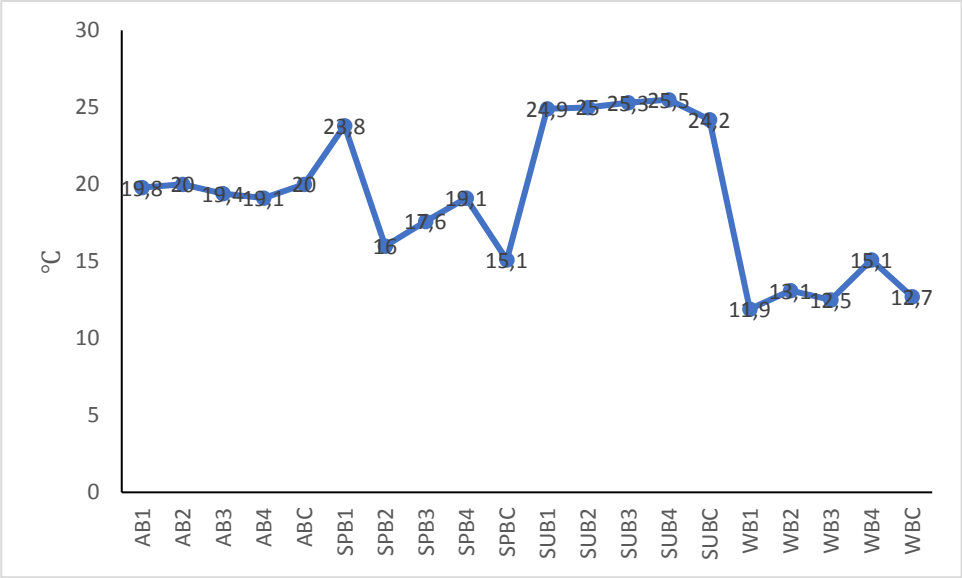

Figure 4-2 Seasonal variation of temperature along the Bloukrans River system.at four sites in different seasons in South Africa. BC, B1, B2, B3, and B4, are site identification numbers BC is the reference site and most pristine. Autumn season include AB1, AB2, AB3, AB4, and ABC; Spring season include SPB1, SPB2, SPB3, SPB4,and SPBC; Summer season include SUB1, SUB2, SUB3, SUB4, and SUBC; Winter season include WB1,WB2, WB3, WB4, AND WBC

4.3.1.3 pH

Water pH measured at collection sites ranged between 3.28 and 8.45. pH remained neutral at all sites during, autumn, summer, and spring. The pH for the sites was within the range for acceptable range (6.5 and 8.2) for freshwaters (Cambers et al., 2008; Sila, 2019). Research revealed that *Campylobacter* could survive in an environment with pH ranging from is 4.9 to 9.5, with their optimal pH is between 6.5 and 7.5 (Forsythe, 2000) Therefore, the range of pH 6.7 and 9.55 presents a viable environment for *Campylobacter* survival and detection. The low pH at the sites B3, B4, B2 during winter is because of chemical pollution from upstream activities as the water was characterised with foam. The source and components of the chemical pollutant is not known.

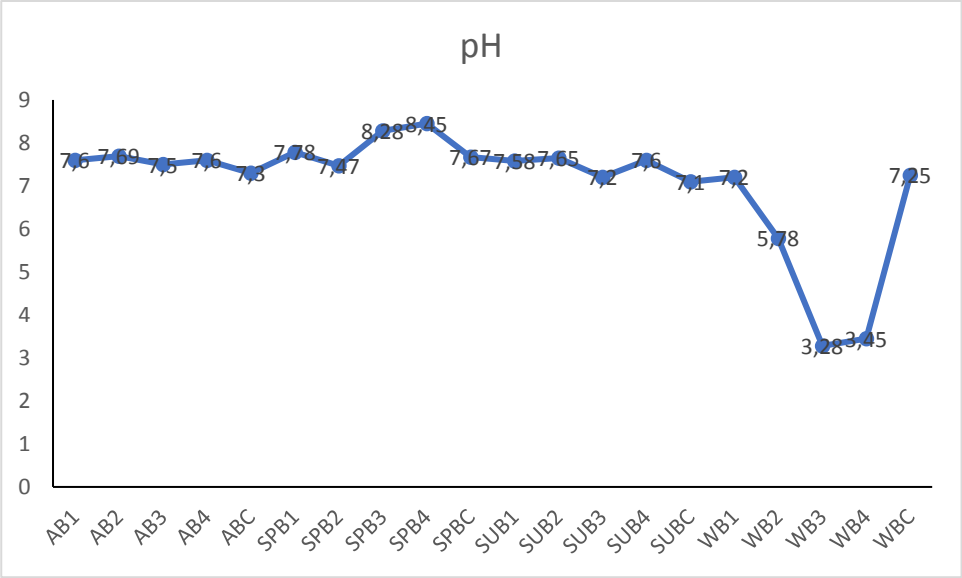

Figure 4-3 Seasonal variation of pH along the Bloukrans River system.at four sites in different seasons in South Africa. BC, B1, B2, B3, and B4, are site identification numbers BC is the reference site and most pristine. Autumn season include AB1, AB2, AB3, AB4, and ABC; Spring season include SPB1, SPB2, SPB3, SPB4,and SPBC; Summer season include SUB1, SUB2, SUB3, SUB4, and SUBC; Winter season include WB1,WB2, WB3, WB4, AND WBC

4.3.1.4 Electrical conductivity (µS/ cm)

Electrical conductivity (EC) of the water samples ranged from 800 to 2430 µS cm^{-1}, and is above the acceptable range of 0-1500 µS/cm established by the Department of Water and Sanitation of South Africa (Gqomfa et al., 2022). The highest EC (2430 µS cm^{-1}) is recorded for water sampled at Site B1 during summer. The Site B1 is impacted substantially by runoffs from the informal settlement and sewerage from leaking sewer and potable water pipes of the urban area of Grahamstown. This explains the high EC value recordable at the site. The site also has a lot of human impacts such as dumping of electronic wastes, etc. It is notable that the average EC value of typical uncontaminated water from a river approximately is 350 µS cm^{-1} (Sila, 2019). Therefore, this suggests alarming pollution at Site B1. Electrical conductivity defines the ability of water to permit electrical current to go through. The increasing EC at this site could also be as a result of the flow of total dissolved salts (TDS) from the insufficiently treated effluents. According to previous studies, water samples with EC values ranging from 24.80-28800.00 µS cm^{-1} permit the survival of *Campylobacter* (Mahagamage et al., 2020). Therefore, with the range of from 175 to 2430 µS cm^{-1} EC values recorded at the different sites, these environments are conducive for *Campylobacter* survival and detection. The reference site has the lowest EC value all season through. This is expected, given that it is the most pristine, relatively.

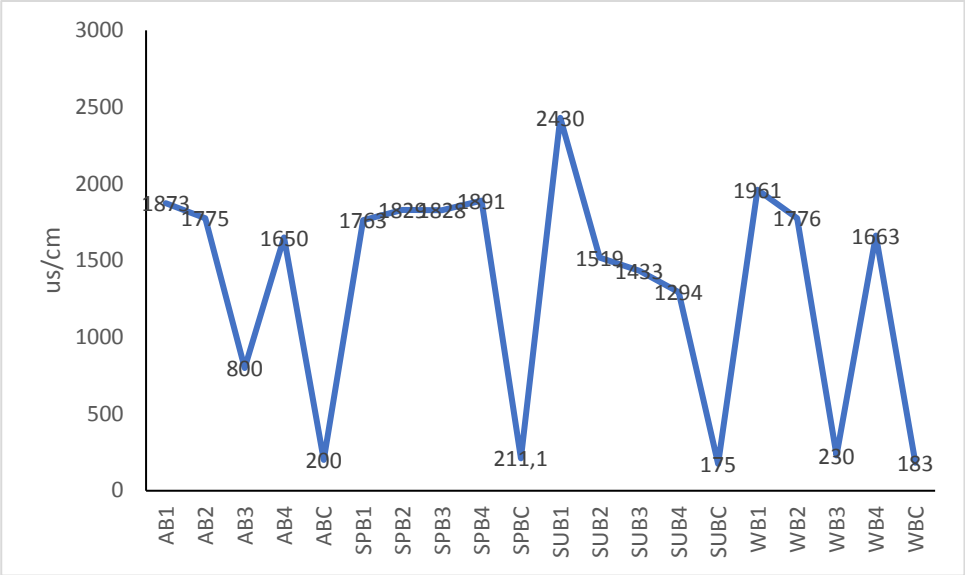

Figure 4-4 3Seasonal variation of electrical conductivity along the Bloukrans River system.at four sites in different seasons in South Africa. BC, B1, B2, B3, and B4, are site identification numbers BC is the reference site and most pristine. Autumn season include AB1, AB2, AB3, AB4, and ABC; Spring season include SPB1, SPB2, SPB3, SPB4,and SPBC; Summer season include SUB1, SUB2, SUB3, SUB4, and SUBC; Winter season include WB1,WB2, WB3, WB4, AND WBC

4.3.1.5 Dissolved oxygen (DO)

Dissolved oxygen concentration, which was recorded for sites along the Bloukrans River, where water samples were collected ranged from 0.26 to 10.98 mg/l. The lowest DO concentration was recorded in spring at Site B1, while the highest DO concentration was recorded at Site B4, in spring also. The range of DO concentration recorded in Site B4 across all seasons is higher than 5.0 mg/l and is within the normal range for excellent quality water for recreational purposes. This observation is understandable because Blaauwkrantz pool is situated far (~ 36 kilometres away from the Belmont Valley WWTP effluent discharge point and is used for swimming purposes. Similarly, the recorded of DO concentration (≥ 8 .0 mg/l), all four seasons is within acceptable water quality limit for recreational purposes, and this is expected as the site is less impacted by anthropogenic pollution. Low DO concentration (≤ 5.0 mg/l) at sites B1, B2 and B3, all through autumn, winter and summer seasons is not surprising. Site B1 is impacted by from runoffs from the nearby informal settlement where there is inadequate sanitation, and the dwellers practice open defecation, as well as sewerage from leaking sewer pipes of the urban area of Grahamstown. Site B2 is impacted by insufficiently treated effluents from Belmont valley wastewater in Grahamstown. Site B3 is impacted by domestic sewage and livestock excreta. DO concentration of at least 3.0 mg/l is the minimum permissible limit by (WHO 2013), for water meant for recreational, e.g. bathing, and fishing). In the present context, the water is not safe for any form of a human. Also, a minimum DO concentration of 3.0 mg/l recommended for protection of aquatic life. With the recordable dissolved oxygen levels below 5.0 mg/l concentrations in Sites B1, B2, and B3, there is the potential for *Campylobacter* to thrive at these sites. *Campylobacter* spp. are microaerophilic, and reduced oxygen tensions increase their survival (Culotti and Packman, 2015; Teh et al., 2017)

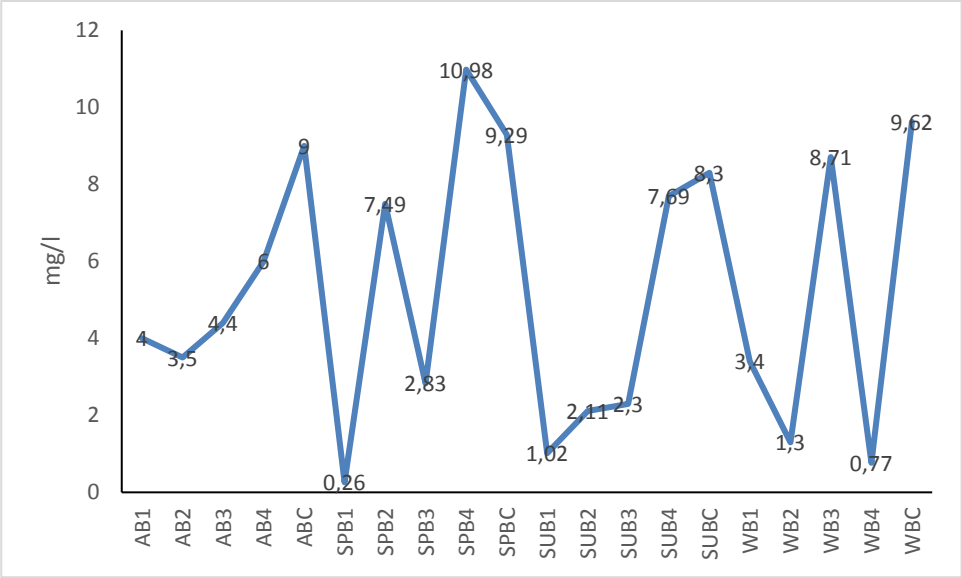

Figure 4-5 43Seasonal variation of electrical conductivity along the Bloukrans River system at four sites in different seasons in South Africa. BC, B1, B2, B3, and B4, are site identification numbers BC is the reference site and most pristine. Autumn season include AB1, AB2, AB3, AB4, and ABC; Spring season include SPB1, SPB2, SPB3, SPB4,and SPBC; Summer season include SUB1, SUB2, SUB3, SUB4, and SUBC; Winter season include WB1,WB2, WB3, WB4, AND WBC

4.3.1.6 Faecal coliform counts

Average total faecal coliform counts for water samples collected at different sites along the Bloukrans River ranged from 0.0-362 CFU 100 mL^{-1}. There were no faecal coliforms detectable in water samples from Sites BC and B4. The allowable limit of total faecal coliform of surface water used for recreational and agricultural water use in the South African Department of Water and Forestry guidelines are (≤130 CFU/100 mL and ≤1 CFU/100 mL, respectively) (WHO 2013, 2018). Therefore, total *faecal coliform* count of water from Sites BC and B4 are within the acceptable water quality for any of these purposes. Water samples collected at Sites B2 and B3 recorded the highest average total faecal coliform counts of (148 CFU 100 mL^{-1} and 362 CFU 100 mL^{-1}, 167 CFU 100 mL^{-1} and 282 CFU 100 mL^{-1}, during spring and summer. Relatively, the average total faecal coliform counts recorded for water samples collected at Site B1 (196 CFU 100 mL^{-1} and 144 CFU 100 mL^{-1}, during summer and spring, respectively) is substantial. Sites B3, B2 and B1 are commonly impacted by substantial contamination with insufficiently treated effluents from Belmont Valley Wastewater Treatment Plant in Grahamstown, domestic sewage and livestock excreta. These results confirm faecal contamination at these sites, which is necessary for high occurrence *Campylobacter* (Pitkanen and Hanninen, 2017).

4.3.2 Relationship between the various water quality parameters and to determine whether significant differences in water quality occur between different seasons

Negative correlation between dissolved oxygen and turbidity is observed and is anticipated. This relationship is observable and measurable readily in aquatic ecosystems (Hall et al., 2015).This phenomenon occurs because dissolved oxygen decline with increasing pollution, which is associated with increasing turbidity. On the other hand, positive correlation is observed with pH and temperature measurements at the river sites. Similarly, positive correlation is observed with conductivity and turbidity measurements at the river sites. The positive correlation between conductivity and turbidity measurements may be because the water is turbid due to suspended particles. As a result the density of water increases, and the denser the water, the more the EC value (similar to saline/sea water) (Prasad, 2018).

As expected, there is enough evidence to show that the physicochemical property of the Bloukrans River differed by season, since the computed p-value is lower than the significance level alpha=0.05.

Table 4-2 Correlation coefficient between the physicochemical characteristics of water characteristics of Bloukrans River

	pH	*Temperature ℃*	*Dissolved Oxygen (mg/l)*	*Conductivity (us/cm)*	*Turbidity (NTU)*
pH	1				
Temperature ℃	0.452817373	1			
Dissolved Oxygen (mg/l)	0.107644224	-0.223585914	1		
Conductivity (us/cm)	0.227898401	0.182406913	-0.64333	1	
Turbidity (NTU)	-0.438705218	-0.102543951	-0.65676	0.449569804	1

Table 4-3 Multivariate Analysis of Variance (MANOVA) table showing the level of significance of the variation of measured physico chemical parameters by season

WILKS' TEST (RAO'S APPROXIMATION):

	Season
LAMBDA	0.023
F OBSERVED VALUES	4.373
DF1	15
DF2	22
F CRITICAL VALUE	2.140
P-VALUE	**0.001**

4.3.3 DNA yield and quality characterisation

Based on 260/280 ratio, the DNA yield from water samples concentrated by membrane filtration (73.43 ng/ul), is highest, followed by DNA yield through sequentially combining centrifugation and membrane filtration (62.85 ng/ul) are close (**Table 4.4**). On the other hand, the DNA yield from water samples concentrated by centrifugation yielded the lowest (47.5 ng/ul). It appears that in the process of centrifugation to pellet cells, insoluble debris is pelleted, which hinders successful extraction of DNA, and causes low DNA yield. Also, the pelleted debris likely contains molecules that cause inhibition or interference with reagents in the DNA extraction kit. Nevertheless, for all the different concentration methods, the extracted DNA is considered to be pure because the recorded 260/280 absorbance ratios of 1.9 value is still within the 1.8 and 2 (Koetsier, and Cantor, 2013). These results suggest that membrane filtration presents the most acceptable method to recover bacteria from the water samples, which allows sufficient amount of DNA to be isolated for downstream analyses to quantify the concentration of pathogens that are present. Cells that were obtained by filtering water samples contain no or minimal interference molecules. *Campylobacter* DNA in the water samples can be present in low amounts. Also, humic substances are common in environmental water samples, which can impact PCR amplification of samples and impact on the reliability of the quantification (Cankar et al., 2006). These explain why the membrane filtration method with high yield and quality of the DNA is selected so that PCR quantification is not hindered. Notably, it is generally difficult to extract a high concentration of DNA from water samples (Roy et al., 2018) . DNA concentration that is as low as 1-4 ng/ul has been recorded. Visible bands were observed on electrophoresis gel DNA extracted from water samples that were concentrated by the three different methods (data not shown).

Table 4-4 Weighted mean yield and quality of DNA amassed from river water samples concentrated by membrane filtration, centrifugation and a combination of membrane filtration and centrifugation

Nucleic acid [ng/µl]			260/280 Absorbance		
Membrane filtration	**Centrifugation**	**Centrifugation and membrane filtration combined**	**Membrane filtration**	**Centrifugation**	**Centrifugation and membrane filtration combined**
73.43	47.5	62.85	1.9	1.91	1.92

Comparative results for culturing *Campylobacter* cells by direct enrichment of water sample without prior concentration, and initial concentration by centrifugation followed by membrane filtration

Table **4-5** presents the results obtained for culturing *Campylobacter* cells by direct enrichment of water sample without prior concentration, and by initially centrifuging the water samples followed by membrane filtration. Growth was observed on mCCDA plates for water samples collected at only two sites (Sites B2 and B3), by using direct enrichment of water sample without prior concentration. Alternatively, growth was observed on mCCDA plates for all the water samples collected from all sites studied, by a prior concentration of the water samples using centrifugation followed by membrane filtration, before culture. This observation could be because the volume of the water samples assayed by using the direct enrichment method is lesser (Denis et al., 2011a). Therefore, because of the low number of *Campylobacter* in river water samples, the cells could have been missed out during water processing stage. These observations suggest that for culture-based enumeration, centrifuging water samples followed by membrane filtration enable a large volume of water to be assayed, and in turn, yield more growth.

Table 4-5 Colony counts for CCDA plates obtained using different methods to recover *Campylobacter* from Bloukrans River water

Site number	Colony counts (CFU/mL)		
	Direct enrichment of water sample without prior concentration	Prior concentration of water samples by centrifugation followed by membrane filtration	Moore swab method
BC	No growth	89	No growth
B1	No growth	324	47
B2	No growth	279	49
B3	263	293	2
B4	321	341	161

4.3.3.1 Colony morphology and identity of presumptive Campylobacter on CCDA agar plates for the water samples processed by direct enrichment without prior concentration

Round, whitish, and moist colonies were observed on the surface of the agar plates, by culturing *Campylobacter* cells from water samples that were processed by direct enrichment without prior concentration (**Figure 4-6**). This is not expected. *Campylobacter* colonies on CCDA are expected to be greyish, with a metallic sheen, moist and flat with the propensity to spread. NCBI BLAST result for sequences of PCR products (**Figure 4-8)** of the two colonies that were picked from two different plates identified both as *Chelatococcus composti*, and not *Campylobacter.* According to research, the temperature required for growth (35-0°C); and pH (5.5-10.0) conditions of *Chelatococcus composti* match those of *Campylobacter* (Zhang et al., 2017). This explains why the bacteria grew on the plates.

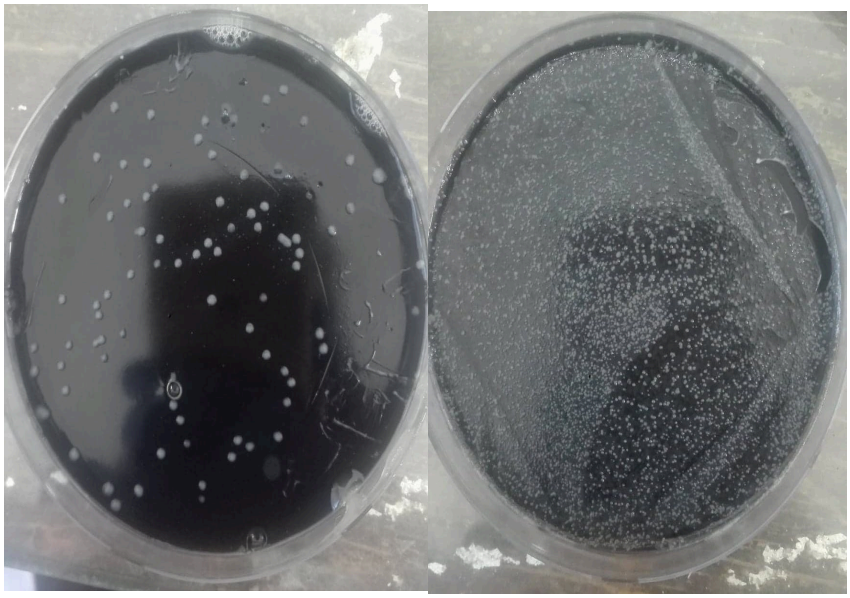

Figure 4-6 Morphology of colonies on CCDA plates, obtained by culturing water samples processed by direct enrichment without prior concentration

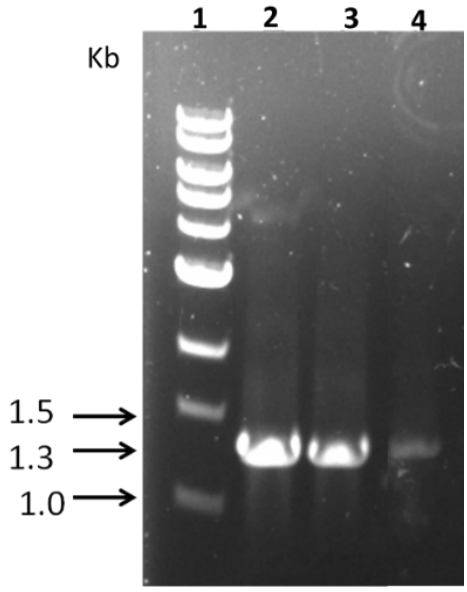

Figure 4-7 Agarose electrophoresis gel showing lane 1: Quick-load Purple 1 kb DNA ladder (BioLabs), lanes 2 and 3: PCR products of colonies from CCDA plates of water samples processed by direct enrichment without prior concentration and lane 4: positive control (PCR product from DNA of dead *Campylobacter jejuni* from the Food chain laboratory in Port Elizabeth, Eastern Cape.

4.3.3.2 Colony morphology and identity of presumptive Campylobacter on CCDA agar plates of water samples concentrated by centrifugation followed by membrane filtration

Culturing *Campylobacter* cells from water samples processed by initial centrifugation followed by membrane filtration produced colonies that are flat, greyish and spreading (**Figure 4 8a**), and grey and moist colonies (**Figure 4-8b**). The observed colony morphology is similar to that expected of *Campylobacter* colonies on CCDA (greyish, with a metallic sheen, moist and flat with the propensity to spread). Also, microscopic examination of Gram staining slides of randomly selected colonies shows curved/spiral pink rods (**Figure 4-4**), which are typically observed for *Campylobacter* cells. NCBI BLAST results for sequences of PCR products of selected colonies from different plates identified them as presumptive colonies, closest hit to *Campylobacter jejuni* strain TERIPS5012 16S ribosomal RNA gene, partial sequence (80% identical). Moore Swab technique yielded fewer number of colonies, compared to the other two methods (**Figure 4-10**). Besides, NCBI BLAST results for sequences of PCR products of selected colonies from the different plates identified their closest hit to an *Escherichia coli* strain, (90% identical).

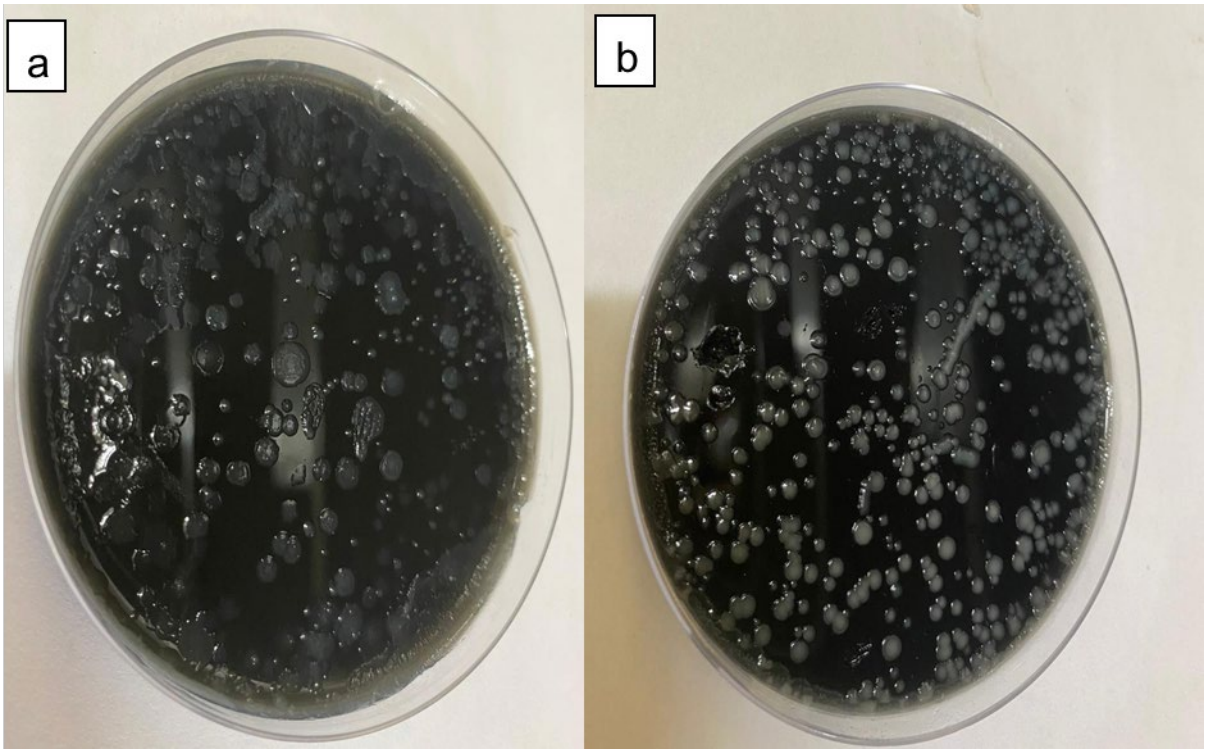

Figure 4-8 Morphology of colonies on CCDA plates, obtained by culturing *Campylobacter* cells from water samples processed by initial centrifugation followed by membrane filtration

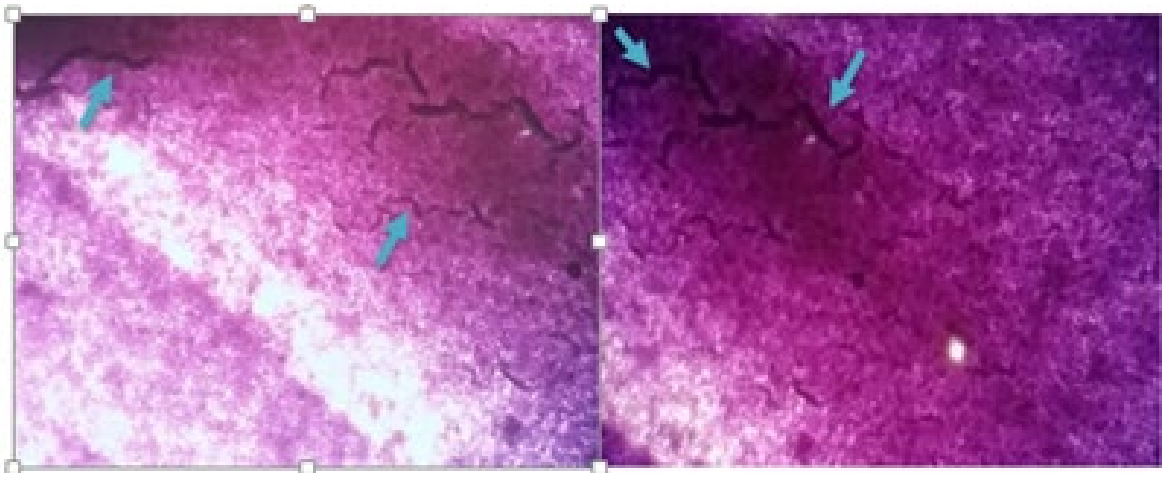

Figure 4-9 Microscopic image of Gram stain for randomly selected colonies from CCDA plates, obtained by culturing *Campylobacter* cells from water samples processed by initial centrifugation followed by membrane filtration. Magnification:1,000.

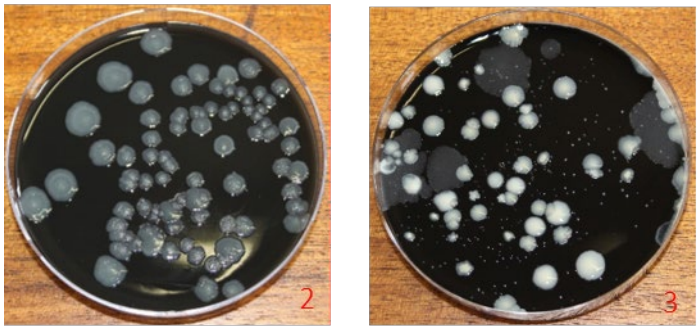

Figure 4-10 Morphology of colonies on CCDA plates, obtained by culturing Campylobacter cells sampled using the Moore swab technique

4.4 SUMMARY AND CONCLUSIONS

Enumeration and quantifying *Campylobacter* is in river water samples is necessary for microbial risk assessment. To enumerate *Campylobacter* in river water samples, the physicochemical quality of the river water, and the presence of faecal contamination had to be considered, as the physicochemical quality of water determines the occurrence of *Campylobacter.* Also, *Campylobacter* is highly found in river water that is faecal contaminated. Physicochemical properties of the water samples indicate *Campylobacter* can thrive in the River.

There are different methods, which are used to enumerate and quantify the concentration of waterborne pathogens present in water, such as culture-based methods, or non-culture based quantitative polymerase chain reaction). The non-culture-based approach is affected by DNA quality and concentration. Two choices of pre-processing water samples, namely (i) initial concentration of water samples by centrifugation followed by membrane filtration, and (ii) direct enrichment without a prior filtration or centrifugation tested yielded different observations. Based on the results described in the previous sections, we can summarize our results by drawing several conclusions. Firstly, for non-culture-based study, water samples processed by centrifugation yields low DNA concentration. It appears that in the process of centrifugation to pellet cells, insoluble debris is pelleted, which hinders successful extraction of DNA, and causes low DNA yield. Secondly,

for culture-based enumeration, direct enrichment of water samples without a prior filtration or centrifugation does not allow a large volume of river water to be assayed. This is a problem because of the low number of *Campylobacter* occurring in environmental water. By assaying a small volume of water, *Campylobacter* can be missed out. Alternatively, centrifuging water samples followed by membrane filtration enables a large volume of water to be assayed, and in turn, yield more growth. Presumptive colonies were detected from culturing the water samples collected by the Moore swab technique. However, the results obtained by 16SrRNA sequence analysis indicated none of these strains were *Campylobacter*, instead they were identified as *E. coli* strain. We can conclude that processing river water samples by membrane filtration approach, or centrifugation followed by membrane filtration to process water samples are promising to recover *Campylobacter* from river water samples for *Campylobacter* enumeration during for microbial risk assessment. The choice of method depends on whether the study is culture or non-culture (DNA) based.

The level of *Campylobacter* reported so far is low, a finding that highlights the importance of testing of *Campylobacter* using different methods instead of using solely culture-based methods. The low *Campylobacter* reported so far is attributed to the limitations and bias of culture-based methods culture methods is unable to give a true picture of *Campylobacter* preponderance in the water sample because the majority failed to grow under laboratory testing conditions. Therefore, in the rest of this study the detection of *Campylobacter* spp. Reported will be based on molecular methods

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CHAPTER 5: OCCURRENCE OF *CAMPYLOBACTER* SPP IN BLOUKRANS RIVER

5.1 INTRODUCTION

Traditional culture methods remain the gold standard for microbiological testing. However, in this study it has been difficult to culture *Campylobacter* spp. from the river water samples. The low detection of *Campylobacter* species reported from the water samples so far was attributed to limitations and bias of culture-based methods. Moreover, not all viable *Campylobacter* species are culturable (Baffone et al., 2006; Silva et al., 2011; Bronowski et al., 2014). Besides, *Campylobacter* that has been exposed to water for a prolonged period of time enter a dormant phase and change their shape from s-shaped to coccal shaped making it hard to culture (Rollins and Colwell, 1986b; Jones et al., 1991; Ikeda and Karlyshev, 2012). Isolation of these bacteria is difficult because they are slow growing, and adaptation to in vitro conditions for growth is difficult (Acheson and Allos, 2001). It is time consuming.

Polymerase chain reaction (PCR) techniques has enabled quicker, with relatively high detection of *Campylobacter* in natural environments. Many PCR methods have been developed to highlight the presence of *Campylobacter* spp. directly in the sample or after enrichment in the culture broths. This is based on 16S rRNA, or the target gene such as flagellin (*flA*), or 16S/23S intergenic space regions. The different PCR methods include conventional PCR (Wegmüller et al., 1993; Moreno et al., 2001, 2003; Nwankwo et al., 2017), reverse transcriptase RT-PCR (Sails et al., 1998; Lin et al., 2009), nested and semi-nested PCR (Waage et al., 1999), real-time / quantitative PCR (Cheng and Griffiths, 2003; Sails et al., 2003; Abu-Halaweh et al., 2005; Ménard et al., 2005; Leblanc-Maridor et al., 2011), multiplex qPCR (Toplak et al., 2012) and multiplex PCR (Denis et al., 1999; Winters and Slavik, 2000). Some of these PCR protocols can be employed directly to detect *Campylobacter* in groundwater, surface water and drinking water without prior cultivation (Kirk and Rowe, 1994; Moore et al., 2001; Yang et al., 2003; Ahmed et al., 2009; Gu et al., 2013). Quantitative polymerase chain reaction (qPCR) can be applied to detect and quantify the amount of target genomic material that is present in a sample without culturing. Furthermore, for risk assessment purposes, viability PCR assay is used to differentiate between live and dead cells (Cangelosi and Meschke, 2014). Viability PCR involves incubating samples with a DNA binding dye, including propidium monoazide (PMA). PMA binds to free DNA (including dead cells that their membranes have been damaged), while only live cells are detected and amplified during PCR (Nocker et al., 2007; Bankier et al., 2018). The advantage of viability PCR is that the DNA of dead *Campylobacter* bacteria is not detected, instead the infectious potential of VBNC state is acknowledged and so the data that is obtained through this method is useful in terms of risk of infection. A significant advantage of PCR technique is that it is a non-culture-based method, allowing fastidious pathogens, such as *Campylobacter* to be studied. This chapter investigates occurrence of *Campylobacter* spp in Bloukrans River based on the data of PCR testing.

5.1.1 PCR based detection of viable *Campylobacter* and virulence genes in the water samples

5.1.1.1 Sample processing and DNA extraction

PCR was conducted to detect the presence of *Campylobacter* spp. and their virulence and antibiotic resistant genes in the water samples. For this part of the study, the method combining centrifugation with membrane filtration (section *3.2.3.2*) was used to recover *Campylobacter* cells from the individual river water samples. In addition, the resuspended pellet was centrifuged to collect about 1.5-2 mL concentrated sample. The concentrated sample was mixed together with chopped filter membrane and treated with PMA. The mix was

overlaid with 500 µl of PMA (50 µm) in a 90 mm Petri dish and incubated in the dark for 10 min. This was followed by exposing the filter to a 500W on ice, keeping it 20 cm away from the light source for 10 min. Following irradiation, the mix was added to the lysis solution and used for DNA extraction. DNA extraction was conducted using DNeasy® PowerSoil® Pro Kit (Qiagen GmbH, Hilden, Germany) according to the kit's protocol, and DNA was eluted in 100 µl of the elution buffer.

5.1.2 PMA-PCR based detection of viable *Campylobacter* bacteria in the respective water samples (TASK 2B)

5.1.3 Water Processing

Each 5L of river water sample was centrifuged at 14,000 ×g for 20 minutes (Khan et al., 2009b). The pellet, which is supposed to contain *Campylobacter* cells, was resuspended in 1-2 mL of sterile distilled water by vertexing. In addition, the supernatant was collected and then filtered through a 0.45 µm pore size 47 mm diameter filter membrane to trap suspended *Campylobacter* that was not pelleted (Vereen et al., 2013). The filter paper was recovered and chopped into pieces using a sterile pair of scissors, wiped with ethanol and placed into the centrifuge tube containing the pellet resuspended in 1 mL of PBS. 400 ul of the concentrated cells is stored for direct DNA extraction. Another 400 ul was incubated with PMAxx (1 µl) and vortexed for 5 mins while inverting and briefly swirling the tubes to enable mixing in the dark in 1.5 ml tubes (Eppendorf) at room temperature (RT). Thereafter, the tube is placed on ice and exposed to UV light at a distance of 10-20 cm while tubes are open. The concentrated cells treated with PMA were stored at -20°C until DNA extraction. DNA extraction was conducted using DNeasy PowerSoil Pro kit (Qiagen GmbH, Hilden, Germany) according to the kit's protocol, and DNA was eluted in 100 µl of the elution buffer. DNA extracts will be stored at -20°C until use.

5.1.4 PCR based detection of viable *Campylobacter* and virulence genes

5.1.4.1 Conventional PCR for presence /absence confirmation

Firstly, conventional PCR was conducted to optimise PCR conditions and detect the presence /absence of *Campylobacter* spp. and associated antibiotics resistant genes and virulence factors (*ciaB, cadF, cdtB, htrB and clpP*) in the respective water samples**.** For all reactions, no template negative controls (sterile water) were added, and the EmeraldAmp GTPCR Master Mix 2X (Takara Biotechnology), was used following the manufacturer's instructions. The reaction mixtures consisted of 25 µL of the Master Mix, 16 µL of nuclease-free water, 2 µL of each primer (20 pM), and 5 µL of DNA, altogether making a final total volume of 50 µL. Amplification of the target genomic fragments was performed using a thermal cycler (MultiGene(r), Labnet International, Edison, USA). Following the reaction, PCR products were analysed using electrophoresis in 1% agarose gel stained with ethidium bromide and then viewed under UV light.

5.1.4.2 Quantification by real-time PCR

Quantitative PCR was conducted using DNA from water samples that indicated presence of the respective targeted genes. Each 20 µL reaction contained 10 µL of Solis Biodyne – HOT FIREPol® EvaGreen®

qPCR Mix Plus (ROX):), 0.4 uL (0.2 µM) of each primer, 5 µL of sample DNA, and nuclease-free water to make it up to 20 µL using the SYBR® Green chemistry. Individual sample PCR was carried out in duplicate, a no template control (NTC) was included in all assays. Amplification was carried out on a Biorad CFX96 Real-Time

PCR system (Biorad, USA) according to the conditions (Henry et al., 2015) (Henry et al., 2015). Melt curve analysis were carried out at the end of 40 cycles to verify primers specificity (Reyneke et al., 2017). Using the amplicons generated from the conventional PCR (section 4.14.1) of positive DNA, standard curve was generated for the qPCR assays. Briefly, amplicons for each target were purified and concentrated using the FavorPrep GEL/ PCR Purification Kit (Promega Corp). The DNA of the purified amplicons were quantified, and used to generate standard curve for the qPCR assays. PCR set of primers and conditions used are listed in **Table 5-1.**

Table 5-1 Primers used in this study

Targets	Primer set		size	References
Campylobacter spp.,16S rRNA	C412F 5'-GGA TGA CAC TTT TCG GAG C-3' C1288R 5'-CAT TGT AGC ACG TGT GTC-3'	Initial denaturation at 94 °C for 1 min, and then 94 ◦C for 1 min, annealing at 58 °C for 1 min and extension at 72 ◦C for 4 min and final extension at 72°C for 5 min.	800 bp	Linton et al. (1997)
cadF	5'- TAT GGT GTA GAA AAA AGT CGC ATC -3' 5'- ATC CGC TCT ACC TTC TTT AGT GTC A -3'	Initial denaturation at 95°C for 2 minutes, followed by 40 cycles at 95°C for 15 sec, 55°C for 35 sec, and 72°C for 1 minute.	90bp	Ghunaim et al. (2015)
cdtB	5'- AAT GCA AGC TGA AGA AGT GAT TGT -3' 5'- AGC ATC ATT TCC ATT GCG AAT -3'		85bp	
ciaB	5'- CAA CTT TAT ATT TGC ACT CCG ATG -3' 5'- GGA ACG ACT TGA GCT GAG AAT AAA C- -3'		74bp	
clpP	5'- TGG GAG CAT TTT TGC TTA GTT G -3' 5'- CTC CAC CTA AAG GTT GAT GAA TCA T -3'		90bp	
htrB	5'- CGC ACC CAA TTT GAC ATA GAA C -3' 5'- TTT TTA GAG CGC TTA GCA TTT GTC T-3'		70bp	
cmeA	5'- TAG CGG CGT AAT AGT AAA TAA AC -3' 5'-ATA AAG AAA TCT GCG TAA ATA GGA -3'	Initial denaturation at 94°C for 7 min, 94°C for 1 min, annealing at 50°C (cmeA, cmeB) and 52°C (cmeC) for 1.5 min, extension at 72°C for 3 min and then, final extension at 72°C for 5 min for 30 cycles.	816 bp	Otigbu et al. (2018a)
cmeB	5'-AGG CGG TTT TGA AAT GTA TGTT -3' 5'- TGT GCC GCT GGG AAA AG -3'		435 bp	
cmeC	5'- CAA GTT GGC GCT GTA GGT GAA -3' 5'- CCCCAATGAAAAATAGGCAGAGTA - -3'		431 bp	
tet(O)	F-5'GGCGTTTTGTTTATGTGCG-3 R-5'ATGGACAACCCGACAGAAGC-3'	Initial denaturalisation at 95°C for 1 min, 95°C for 1 min, 49°C for 1 min and 72°C for 1 min, repeated for 35 cycles.	559 bp	Gibreel et al. (2004b)

5.2 RESULTS AND DISCUSSIONS

A total of 30 water samples were collected. The PCR products with expected size (800 bp) were obtained in 20 out of 30 (66.67%) of the screened samples (Figure 5-1). The sequence obtained for the PCR product showed 96% homology with C. jejuni as evaluated by BLAST. All *Campylobacter* spp. positive water samples were also positive for *Campylobacter* virulence genes (Table 5-2). *Campylobacter* was detected at all the site except for the control site. The occurrence of *Campylobacter* in surface waters often narrates recent fecal contamination by livestock, runoffs from farm animal manure, avian birds and insufficiently treated effluent or leakages from nearby septic tanks (Jones, 2002; Abulreesh et al., 2006). The detection rate of *Campylobacter* spp. in river systems can vary by regions and countries. In this respect, it is noteworthy that in Australia, *Campylobacter* occurrence has been reported to be 25% (Ahmed et al., 2009) 26% in Canada (Jokinen et al., 2011), 35.7% in Ghana (Karikari et al., 2016), 41.5% India (Baserisalehi et al., 2005), , 46.6% to 53.3% in France (Denis et al., 2011b), 53.3% in Norway (Rosef et al., 2001), 70% in Poland (Popowski et al., 1997); and 91% (Siddiqee et al., 2019). The differences observed in the contamination levels from the different studies could also be a result of different methods used for detection (Abulreesh et al., 2006). Never the less, *Campylobacter* contamination (66.67%) reported in this study is almost equal to that (68.7%) reported in Nigeria (Ugboma et al., 2012).

Table 5-2 *Campylobacter* virulence genes in water samples (ND and D signify not detected and detected, respectively)

SITE IDENTIFICATION	Contamination	*Campylobacter* species	Virulence Genes
BC	Control site: Less impact by anthropogenic pollution, except for traditional/cultural rituals that takes place	ND	ND
B1	Considerable contamination is expected from runoffs from the informal settlement and sewerage from leaking sewer and potable water pipes of the urban area of Grahamstown.	D	*CadF, CiaB*
B2	Substantial contamination with insufficiently treated effluents from Belmont Valley Wastewater Treatment Plant (WWTP) in Grahamstown	D	*CadF*
B3	Considerable contamination with domestic sewage, livestock excreta. Also, lies in an area where there is predominant agricultural	D	*CadF, Ctd*
B4	Blaauwkrantz pool, and is expected to be least contaminated because it is situated approximately 36 kilometres downstream of WWTP effluent discharge point, along the Bloukrans River system.	D	*ClpP, Ctd*

The *Campylobacter* from these river water could possess antibiotic resistance genes. All 20 samples were positive for the *Campylobacter* tetO gene. Worse still multi drug resistant *Campylobacter* are potentially present in Bloukrans River water samples. Of the *Campylobacter* spp. positive water samples (n=20), 4 (20%) are cmeA genes positive, 13 (65%) are cmeB genes positive and 2 (10%) were cmeC gene positive (Figure 5-2). The multidrug efflux pump CmeABC genes encode proteins of different structures involved in extruding antimicrobials (Lin et al., 2002). tet(O) is plasmid borne and is acquired by *Campylobacter* through horizontally gene transfer (Connell et al., 2003; Crespo et al., 2016). The binding of the *tetO* genes to an open A site

induces a conformational change leading to the release of the bound tetracycline molecule and so protein elongation is not interrupted (Luangtongkum et al., 2009). *tetO* genes are plasmid-borne and give rise to tremendously high levels of resistance to tetracycline. The presence *tetO* genes in the water samples is expected given the high rate of resistance against tetracycline is commonly occurring in *Campylobacter* isolates from humans (82%), and their use in animal husbandry. There their presence is expected because of the anthropogenic activities around the Bloukrans River, including livestock grazing and Wastewater treatment plant effluents bearing human wastes. Regarding *CmeABC* genes, a local study also reported *Campylobacter* isolates that exhibited multidrug resistance from estuarine water samples (Otigbu et al., 2018b).

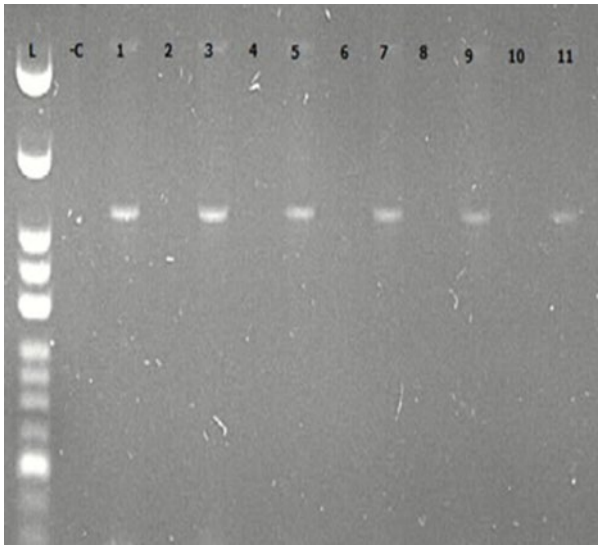

Figure 5-1 PCR products using the 16S rRNA primers specific for *Campylobacter* genus. Lanes:L: Accuris SmartCheck 50bp DNA Ladder; -C: negative control; 1, 3, 5, 7, 9: bands from Bloukrans River water samples; 11 positive control

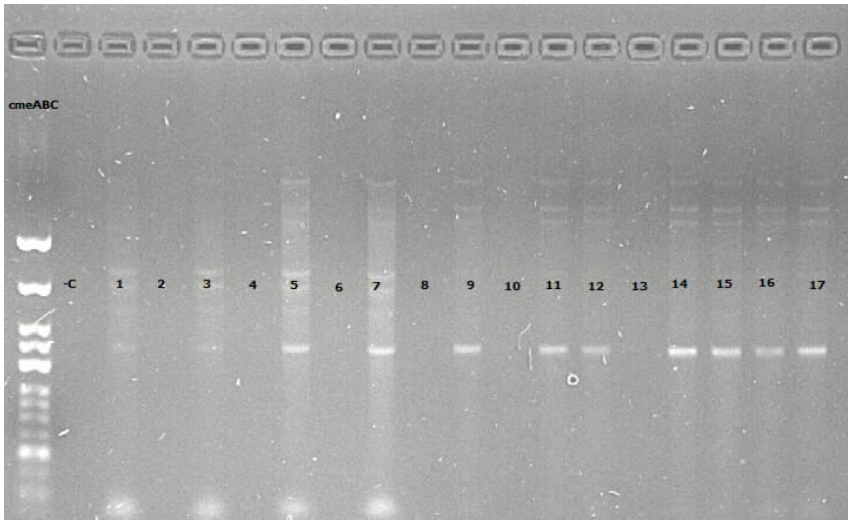

Figure 5-2 PCR products using cmeB and cmeC primers specific for multidrug efflux pump genes. Lanes:L: Accuris SmartCheck 50bp DNA Ladder; -C: negative control; 5, 7, 9: 11 cmeC bands; 14, 15, 16, 17 cmeB bands from Bloukrans River water samples

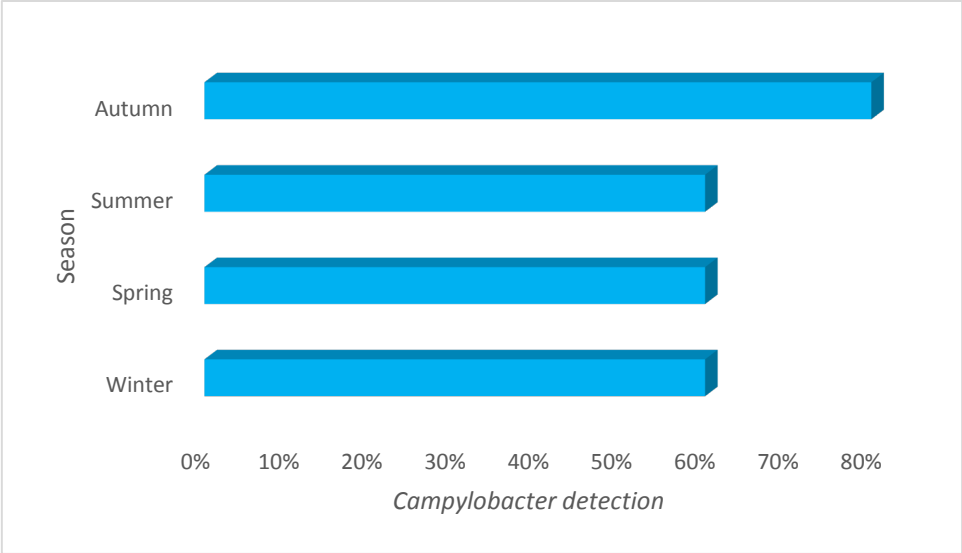

Figure 5-3 The seasonal occurrence of *Campylobacter* spp from the Bloukrans River water samples collected between January 2020 and December 2021

Campylobacter spp. were detected throughout the period of this study. The highest percentage of detection was recorded in autumn (80%), compared to summer (60%), spring (60%) and winter (60%) samples (Figure 5-3). But a weak negative correlation (r= -0.089366) between *Campylobacter* detection and the season was observed. Therefore, the result from this study does not show marked seasonality in *Campylobacter* detection in the river of study. The result of this study is consistent with the result from another local study by Samie et al. (2022), where the detection rate of Campylobacter in young children remained at the same level over 4 years of the study. According to the authors this phenomenon suggests that *Campylobacter* infection might be endemic South Africa. These findings contradict those in most European countries where marked seasonal change in the detection rate of Campylobacter in surface water occurs. There are several studies that have indicated that the detection rate of *Campylobacter* in surface water can vary depending on sampling season. *Campylobacter* isolation rates from surface water were highest in fall/autumn and winter, and lowest in summer and spring (Daczkowska-Kozon and Brzostek-Nowakowska, 2001), in Brittany, France. In a study by Eyles et al. (2003), a larger number of positive samples were obtained during summer and winter, whilst lesser number of positive samples in winter New Zealand, . Variation in *Campylobacter* levels amongst different sites (Figure 5-4) on the Bloukrans River is observed, which reflects the various level and types of anthropogenic activities that occur, respectively. The control site (BC) and this site is least impacted by human activities except for traditional/cultural rituals that takes place. Site (B1) experience considerable contamination from runoffs from the informal settlement and sewerage from leaking sewer, It is also a site where livestock grazes and drinks water. (B2) is influenced by insufficiently treated effluent from wastewater treatment works. The fourth site (B3) lies in an area where the predominant activity is agriculture Also, there is considerable contamination with livestock excreta. The last site, (B4) is a point of human exposure, where recreation and cultural activities usually occurs. It is expected to be least contaminated because it is situated approximately 36 kilometres downstream of WWTP effluent discharge point, along the Bloukrans River system. But it seems to be influenced by upstream activities. Obviously, *Campylobacter* levels are highest at sites B2 and B3, as expected because of influence of wastewater treatment effluents and livestock /agricultural inputs respectively (Eyles et al., 2003b).

The occurrence of *Campylobacter* at the different sites correlates with physico-chemical properties of the sites. Turbidity is a vital environmental variable associated with *Campylobacter* contamination of freshwaters. Sites B2 and B3 with highest *Campylobacter* levels recorded high turbidy and conductivity with the low dissolved oxygen. The conditions in these environments favour *Campylobacter* survival.

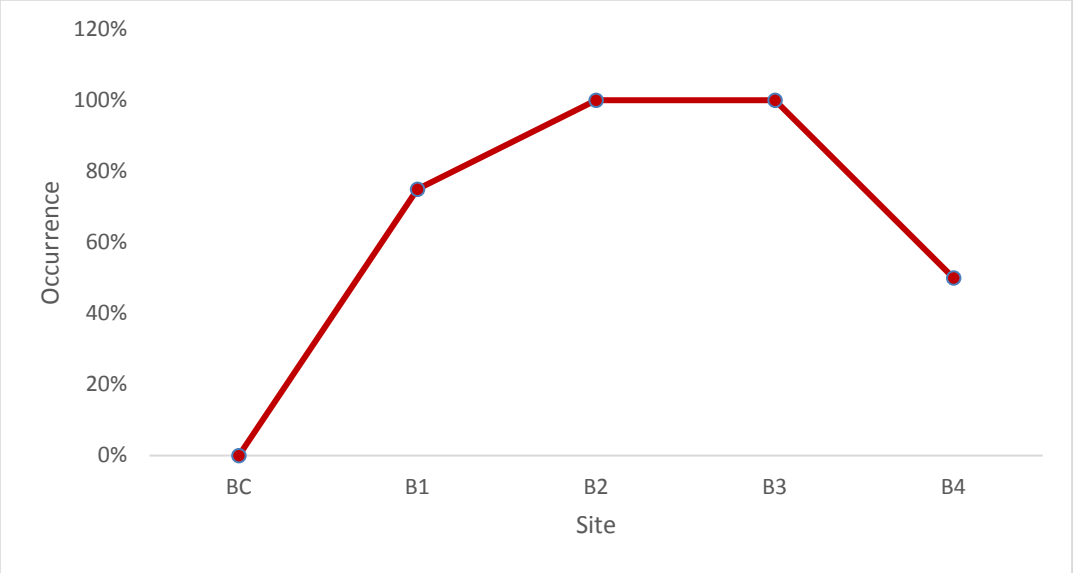

Figure 5-4Spatial occurrence of *Campylobacter* along the Bloukrans River

Table 5-3 Concentrations of *Campylobacter* and their antibiotic resistance genes in Bloukrans River water samples

Site	Season	Target gene	Copy Number/ml
B1	Summer	16S rRNA	4.53E+04
B2	Summer	16S rRNA	1.75E+05
B3	Summer	16S rRNA	9.90E+04
B4	Summer	16S rRNA	2.20E+05
B1	Autumn	16S rRNA	1.16E+04
B2	Autumn	16S rRNA	9.30E+03
B4	Autumn	16S rRNA	3.38E+04
B2	Summer	*tetO*	2.71E+03
B3	Summer	*tetO*	8.09E-03
B4	Summer	*tetO*	4.75E-02
B2	Autumn	*tetO*	1.24E-02
B4	Autumn	*tetO*	9.71E-03
B2	Summer	*cmeB*	2.77E+04
B3	Summer	*cmeB*	3.18E+03
B4	Autumn	*cmeB*	7.80E+01
B2	Summer	*cmeA*	1.33E+04

So far, quantitative PCR detected 9.30E+03 × 101-1.75E+05 gene copies/1 ml of *Campylobacter* 16S rRNA gene in some of those positively identified samples (Table 5-3). Regarding t*etO* and *cmeB* genes, 1.24E-02 – 9.71E-03 gene copies/1 ml and 7.80E+01-2.77E+04 gene copies/1 ml, respectively were detected. Compared to Ahmed et al. (2009) the level of *Campylobacter* in Bloukrans river is high. Given the

low infectious dose (i.e. 500 organisms can cause illness) the concentrations of *Campylobacter* in Bloukrans River could pose significant health risks

5.3 CONCLUSIONS

In conclusion, this study highlights high and continual occurrence of *Campylobacter* spp. in Bloukrans River, which are potentially antibiotic resistant. There was no marked seasonal variation in *Campylobacter* detection rate in the river. Spatial variation along the river was noticeable, with higher *Campylobacter* level at sites that are most impacted by insufficiently treated effluents from wastewater treatment works and livestock excreta. The Bloukrans River serve as a place for spiritual/cultural activities, recreation (mainly for children), source of water for livestock and irrigation. Therefore, it presents a risk of spread of antibiotic resistant *Campylobacter.* It is therefore critical that authorities recognize the possibilities of occurrence of *Campylobacter,* and its transmission through contact with this river, as it may play a significant role in the epidemiology of enteric diseases, including *Campylobacter*iosis in this region.

CHAPTER 6: PREDICTION OF *CAMPYLOBACTERIOSIS* RISK FROM BLOUKRANS RIVER WATER BY APPLYING MACHINE LEARNING ALGORITHMS AND SHOTGUN METAGENOMICS SEQUENCING

6.1 INTRODUCTION

Campylobacter spp. occurrence in Bloukrans River presents a health risk. They can be transmitted to humans through the contact with the contaminated water. Risk assessment is therefore critical in order to quantify human health risks from exposure to the environments Quantitative microbial risk assessment (QMRA) is suggested as the most appropriate method to assess and quantify health risk (Amarasiri et al., 2020). However, a fundamental change in approach have become necessary given the lack of dose-response models for antibiotic resistant bacteria (ARB) and difficulty to culture fastidious pathogens for concentration estimation, which are necessary for QMRA (Schijven et al., 2015; Luther et al., 2017; Ha et al., 2019; Tyagi and Kumar, 2021).

Therefore, MRA can benefit from the spin that Next generation sequencing (NGS) has brought to the investigation of microbial ecology of any environment. NGS metagenomics sequencing enables the study the genetic composition of multiple (if not all) microorganisms, and their repertoire of virulence genes necessary to cause disease in an environmental sample (Riesenfeld et al., 2004; Eidem et al., 2012; Ranjan et al., 2016; Tessler et al., 2017). Machine learning algorithms (MLA) comprise developing and applying computer algorithms in order to identify predictor combinations that will predict the risk outcome (Njage et al., 2018). MLA models depend on extracting “features” from sequence data (LaPierre et al., 2019). These features may signify various aspects of a microbiome, including, taxonomic composition or functional profiles. Using predicted functional profiles and their abundances as features, and applying numerous popular classical machine learning algorithms, it is possible to predict safety of the water samples. For this study, the functional features will be virulence genes of *Campylobacter* origin within the metagenomic data. Various genes have been linked to *Campylobacter* virulence, including but not limited to *ciaB*, *cadF*, cdtB*, htrB* and *clpP* (Abu-Madi et al., 2016). Popular classical machine learning algorithms include Naïve Bayes, logistic regression, decision tree, Support Vector Machines (SVMs), Random Forests (RFs) and K-nearest neighbour that can be used to predict the safety status of water sample (Pasolli et al., 2016b). The behaviour of these features within an environment can be used in hazard identification and characterization studies during risk assessment (Couto et al., 2018). Risk of disease has been associated with occurrence, dominance of or diversity of microbial features in a microbiome (Chen et al., 2022).

There is no established protocol, expertise, capacity and means for NGS microbial risk assessment for environmental water samples, yet. Therefore, a new protocol is developed and applied in this study. Firstly, metagenomic analysis was conducted to reveal the taxonomic diversity, virulence and antibiotic resistance determinants as well as genotype of the river microbial community (hazard identification). It is assumed that the microbiome composition is different between Campylobacter healthy and unhealthy samples. Therefore, subsequently, Machine learning algorithms, Random Forests, and statistical test were applied to identify informative markers “some Campylobacter-specific virulence factors” whose presence significantly differ in healthy and unhealthy metagenomes. Then the potential of the identified relevant features as reliable predictions of Campylobacteriosis safety/risk of Bloukrans River water sample is investigated.

6.2 METHODS

6.2.1 Hazard identification and detection/characterisation of *Campylobacter* AMR determinants and virulence factors in river water samples

Metagenomics methods, based on shotgun high-throughput sequencing, was used to analyse the presence of *Campylobacter ,* and their antibiotic resistance genes (ARGs) and virulence genes in the water samples

6.2.1.1 Shotgun Metagenomic sequencing of water samples

The water samples collected from sites B1 B3 and B4 were selected and subjected to shotgun metagenomics analyses. Each 1L of river water sample was centrifuged at 14,000-×g for 20 minutes (Khan et al., 2009b). The pellet, which is supposed to contain *Campylobacter* cells, was resuspended in 1-2 mL of sterile distilled water by vortexing. In addition, the supernatant was collected and then filtered through a 0.45 µm pore size 47 mm diameter filter membrane to trap suspended *Campylobacter* that was not pelleted (Vereen et al., 2013). The filter paper was recovered and chopped into pieces using a sterile pair of scissors, wiped with ethanol and placed into the centrifuge tube containing the 10 mL resuspended pellet. The resuspended pellet, together with the filter membrane, was collected and stored at -20°C, until DNA extraction. DNA extraction was conducted using Qiagen's DNeasy Blood and Tissue kit (Qiagen GmbH, Hilden, Germany) according to the kit's protocol, and DNA was eluted in 100 µL of the elution buffer. The genomic DNA obtained was pooled together for each sampling event and then sent to Inqaba laboratories South Africa. The DNA was subjected to shotgun sequencing on PacBio SEQUEL IIe system following the procedure & checklist for preparing 10kb Library using SMRTbell Express Template Prep Kit 2.0 for Metagenomics Shotgun Sequencing (PacBio, 2019). Briefly, 10 kb library was prepared using SMRTbell Express Template Prep Kit 2.0. To do this, DNA was sheared to a target size of approximately 10 kb and, after that purified using AMPure PB Beads. Shared DNA was taken through a series of an enzymatic reactions, adapter ligation, nuclease treatment to remove damaged templates, purification using AMPure PB Beads. The two samples were barcoded and thereafter pooled at an equimolar concentration into a single library and sequenced on SMRT Cell 8M. 30 hours sequencing was conducted using the circular consensus method, which generated high quality (>Q20) circular consensus sequencing (Hifi) reads.

6.2.1.2 Assessing the community composition of Bloukrans River metagenome

The community composition of metagenomic reads from the samples was assessed using Kraken2(Wood et al., 2019) combined with the Minikraken_8GB_20200312 database of bacteria, viruses, and archaea complete genomes with default parameters. Kraken2 extracts all *k-mers* (strings for length k) from the metagenomic sequence dataset and compares them with an index of a genome database. Using the result from the Kraken analysis, genera relative abundance was determined using Bracken v2.2 .(Lu et al., 2017)

6.2.1.3 Assembly of metagenomics reads and detection of the antibiotic resistance and virulence genes present in Bloukrans River metagenome

Raw base-called data was transferred into SMRTLink (SMRT (v10.1)) for trimming and assembly. SMRTLink (SMRT (v10.1)) is a suite of bioinformatics software, majorly made for PacBio reads to generate HiFi reads using the CCS algorithm (version 8.0.0.80529). The SMRTLink v10.1 processed the raw data generated from sequencing with the following settings: minimum pass 3, and minimum predicted read quality of 20. To expansively investigate the antibiotic resistome, the NCBI bacterial resistance reference gene, NCBI database ResFinder v.4.1, was used (Feldgarden et al., 2019). ARGs from the assembled contigs were detected using ABRicate version 0.8 (http/;//github.com/tseemann/abricate) (Sanabria et al., 2021) In order to detect virulence

genes, the virulence factor database (VFDB) was used (Chen et al., 2004). The thresholds used for the detection of ARGs and VFs were set at 60% identity and 60% sequence coverage.

6.2.2 Hazard characterisation

The pertinent public health risks of *Campylobacter* spp. are caused by particular virulence genes encompassing its virulome. Established *Campylobacter* pathogens are enriched with repertoire of virulence genes that have high prevalence in human or animal during event of infection (Iraola et al., 2014; Panzenhagen et al., 2021). These virulence genes are informative features and their occurrence individually or in combination can be associated increase in disease incidence (Maury et al., 2016). These genes have been proven to be absent in all *Campylobacter*-negative /healthy samples (Raymond et al., 2016). The presence of these genes associated with each environmental sample can be treated as the dependent variable in risk prediction (Maury et al., 2016). This approach of investigating virulence genes is relevant in this case where there is low *Campylobacter* genome coverage compared to the dominant microbiota, (Yang et al., 2016; Escobar-Zepeda et al., 2016; Couto et al., 2018). It is recognised that dose-response models play a vital role in risk characterization, estimating the probability of illness or infection from pathogen ingestion counts resulting from the exposure assessment. However, applying dose-response relationships is difficult because of the challenge of measuring *Campylobacter* owing to its low concentration in the environment of study, which is relevant to measure response (Abe et al., 2021)

6.2.3 Metagenomic datasets and Bioinformatics analyses

The datasets comprised of metagenomic sequences from water samples from this study and publicly available metagenomic samples (from two main studies) deposited the U.S. National Center for Biotechnology Information's Sequence Read Archive in bioproject number PRJNA786578 (Peterson et al., 2022) and PRJEB8094 (Raymond et al., 2016). The public available metagenomic samples are all human-associated shotgun metagenomic data from patient's faeces during and after diarrheal illness of *Campylobacter* origin. The data PRJNA786578 consists of sequences obtained from sequencing the DNA from *Campylobacter*-positive stool samples that were obtained from patients with lab-confirmed gastroenteritis. On the other hand, the data PRJEB8094 consists of sequences obtained from sequencing the DNA from *Campylobacter*-negative stool sample healthy donors.

6.2.3.1 Retrieval, assembly and detection of virulence genes from publicly available metagenome sequences patients' stool during and after diarrheal illness of Campylobacter origin from NCBI

Retrieval and processing of all metagenomic data was conducted using South Africa's Center for High Performance Computing (CHPC) Lengau Cluster. Raw sequence reads of the publicly available metagenomes were retrieved and stored in-house. The reads were quality trimmed using Trimmomatic (v0.36.5) (Raymond et al., 2016) (SLIDINGWINDOW = 20). Following trimming, metagenome assemblies of each library were generated using metaSPAdes 21 (v3.9.0 and v3.10.1)(Nurk et al., 2017), using . (with the default --meta settings). Parameters employed are as follows: SPAdes (V3.9.0): --meta, SPAdes (V3.10.1): --meta, Trimmomatic (V0.36): -phred33 (Raymond et al., 2016). Virulence genes from the assembled contigs were detected using ABRicate version 0.8 (http/;//github.com/tseemann/abricate) (Sanabria et al., 2021) using the virulence factor database (VFDB) (Chen et al., 2004), with thresholds set at 60% identity and 60% sequence coverage.

6.2.4 Predictive Modelling

The aim is to enable 'biomarker discovery' by detecting relevant discriminative biomarkers that are most useful to discriminate between "healthy" and "unhealthy" metagenomes. To do this the Random Forest (RF) classifier was applied to the *Campylobacter* features /biomarkers (virulence genes) generated from the ABRicate alignment step (http/;//github.com/tseemann/abricate) (Sanabria et al., 2021). Random forest was chosen because it is suitable for datasets that have many features, particularly when each of the features contributes little information (Breiman, 2001). The implemented tool automatically plots the most relevant markers or features with the importance factor along with the average relative abundance (or average presence) associated with the different considered classes. The prediction for healthy and unhealthy samples was performed using a random forest and the results were validated with a logistic regression from the previously selected variables. Random forest analysis was performed with the randomForest package (Liaw & Wiener, 2002). For training and validation of the models, the 50:50 ratio of the dataset was used. The fivefold cross-validation method was used. All analyses were performed using software R.4.1.0. The occurrence of most important risk predictor features (*Campylobacter* spp.) will be compared between the metagenomes. Their occurrence singly or in combination is associated with epidemiological increase in disease incidence (Maury et al., 2016)

6.3 RESULTS AND DISCUSSIONS

6.3.1 Bloukrans River sequencing data

Sequencing generated 1044556, 617825, and 78712, and Hifi reads from the B1, B3, and B4 sites, respectively. For all three samples, reads with 98% per-base accuracy were produced, which guaranteed a low error rate. Therefore, the reads generated are sufficient for the reliable depiction of taxonomy and gene present in samples, and comparable to another study that applied the PacBio Sequel system (Priest et al., 2021; Haro-Moreno et al., 2021). The N50 value for samples indicates that long contigs were produced from this assembly. N50 metrics indicate that the entire assembly is contained in contigs equal to or larger than the values presented in Table 6-1

Table 6-1 Summary statistics of raw and assembled metagenome

SAMPLE NAME	DNA YIELD (NG)	NUMBER OF RAW READS	AVERAGE SEQUENCE LENGTH (bp)	N50	TOTAL ASSEMBLY LENGTH (BP)	NUMBER OF CONTIGS	MAX CONTIG LENGTH (BP)
B1	8860	1044556	6158	15404	80719924	5544	4060645
B3	7590	617825	6158	16682	34445863	2214	276397
B4	3860	78712	6158	36159	4021096	149	181254

6.3.2 Community composition of Bloukrans River metagenome

The taxonomical classification was performed using Kraken on reads and relative abundance (determined by Bracken). *Campylobacter* was identified in water samples from Sites B1 and B3, only. Therefore, we report on those two sites herewith. For Site B1 Bacteroides (6.9%) was the most abundant followed by *Arcobacter* (6.24%), *Acidovorax* (5.38%), *Pseudomonas* (5.07%), *Proteus* (3.28%), *Acinetobacter* (2.94%), *Homo* (2.87%), *Escherichia (*2.04%), and *Aeromonas* (2.03%). In site B3, *Escherichia (*4.13%) was the most

abundant genera, followed by *Arcobacter* (3.69%), *Pseudomonas* (3.59%), *Acidovorax* (3.16%), *Homo* (3.1%) *Bacteroides* (3.02%), *Acinetobacter* (1.03%), *Aeromonas* (0.95%), and *Proteus* (0.09%). For both Sites B1 and B3 *Campylobacter* was relatively few 0.17% and 0.07%, respectively (Figure 6-1).

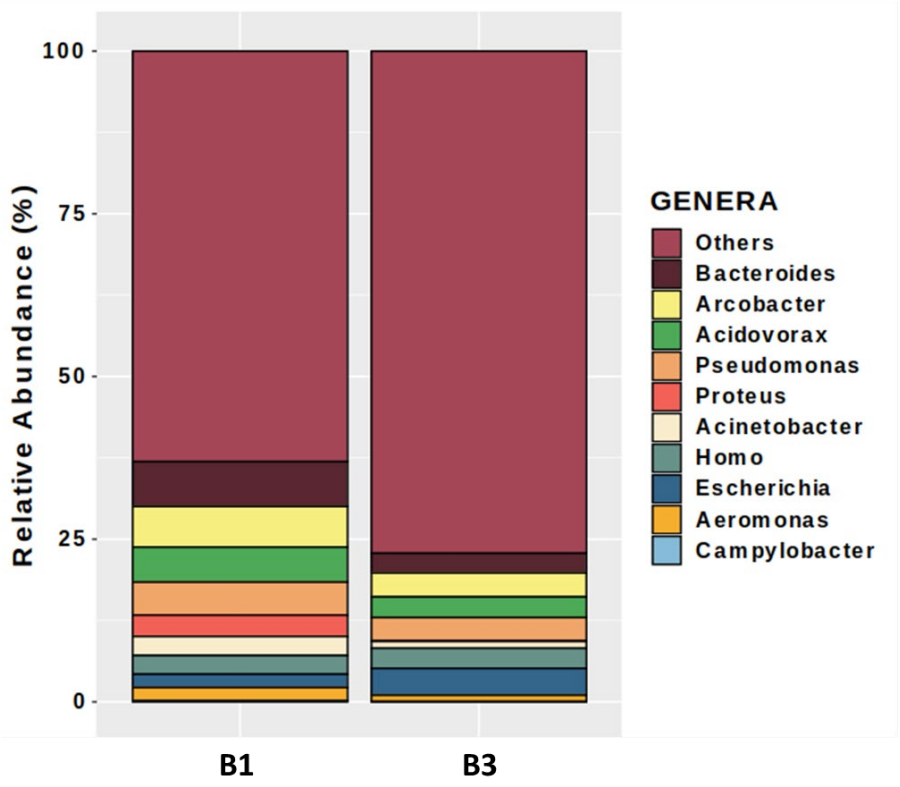

Figure 6-1 Taxonomic composition of Bloukrans River water samples from two different sites along the river The prokaryotic composition in water was determined using Kraken2. Relative abundances (%) of bacterial genera in different sites along the river are presented as stacked bar graphs

6.3.3 *Campylobacter* antibiotic resistance determinants

Screening *Campylobacter* antibiotic resistance determinants in the water samples reveals those conferring resistance to several important antibiotic classes (Ramatla et al., 2022) (Table 6-2). Chloramphenicol resistance is as a result of plasmid-carried- acetyl transferase gene that modifies chloramphenicol (Mikysková, 2018). Therefore, its occurrence in the Bloukrans River is a problem because these genes can be mobilized on plasmids, and therefore can be spread to different pathogens within the same environment through horizontal gene transfer. Therefore, the effective use of this antibiotics for treatment becomes increasingly jeopardized. It is shocking to find these genes in the river because they are usually rare (Schwarz et al., 2004).Chloramphenicol is banned for use in food animals in South Africa (Henton et al., 2011). However, several genes encoding chloramphenicol resistance are found in the water samples. Another study also observed a high percentage of isolates resistant to chloramphenicol in Pigs (Abdalla et al., 2021). The emergence and propagation of antibiotics resistance is multifaceted. There have been instances where an antibiotics selects for the resistance of other substances (Lay et al., 2012; Abdalla et al., 2021). Besides,

frequent conjugation of plasmids carrying ARGs foster plasmid maintenance in a microbial community, even in the absence of that particular antibiotics (He et al., 2020).

Table 6-2Antibiotic resistance genes (ARGs) detected in different sites along the Bloukrans River . The ARGs were detected from contigs using the NCBI bacterial antimicrobial resistance ResFinder database

Sites	Genes	Resistance
B1	*catB2*	chloramphenicol
B1	*catQ*	chloramphenicol
B3	*erm(B)*	Erythromycin; Lincomycin; Clindamycin; Quinupristin; Pristinamycin_IA; Virginiamycin
B1	*floR*	Chloramphenicol; Florfenicol
B3	*floR*	Chloramphenicol; Florfenicol
B4	*mcr-7.1*	Colistin
B1	*tet(W)*	Doxycycline; Tetracycline; Minocyline
B1	*tet(X)*	Doxycycline; Tetracycline; Minocyline; Tigecycline
B1	*tetA (P)*	Doxycycline; Tetracycline

When treatment of human Campylobacteriosis is required, macrolides (erythromycin) is the drug of choice, followed by fluoroquinolones (ciprofloxacin predominantly), and lastly tetracyclines (Sifré et al., 2015; Asakura et al., 2019). However, increasing resistance to these antimicrobials among *Campylobacter* is observed (Zhang et al., 2016; Pergola et al., 2017; Vinueza-Burgos et al., 2017; Tang et al., 2017). Given the indiscriminate use of tetracyclines as food additives in livestock for infection control and prevention as well as growth enhancement, it is not surprising to find several tetracycline genes in the environment (Engberg et al., 2001; Koolman et al., 2015).

6.3.4 *Campylobacter* virulence genes in Bloukrans River

This study reveals the presence of clinically relevant *Campylobacter* virulence genes in Bloukrans River (Figure 6-2). The virulence genes include those responsible for: the chemotaxis protein (*che*), capsule biosynthesis and transport (*fcl, glf, kfiD*), motility and export apparatus protein (*flg*), the flagellar biosynthesis and assembly protein (*flh*), the flagellar motor protein (operon *fli*), virulence factors involved the immune evasion (*, gmhA2*). The virulence genes *cheY, flgG, flhA, flhG, fliE , fliG, fliI, hldE, pseB,* identified are among a 32 essential core genes of the virulome of *Campylobacter* spp, whereas *hldD, kfiD, glf, ghA2 are members of an accessory genes groups.* These genes are amongst those intensely present during development of disease in humans and birds. Most importantly, their presence has been suggested for fast monitoring and confirmation of the presence of *Campylobacter* in samples (Panzenhagen et al., 2021)

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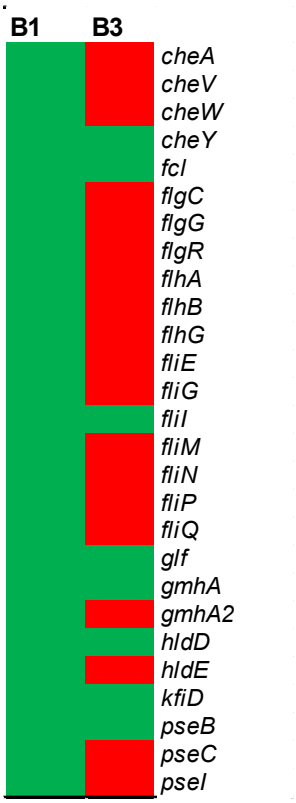

Figure 6-2 Heat map showing presence/absence of *Campylobacter* virulence genes in two sites (B1 and B2) along the Bloukrans River. The ARGs were detected using ABRicate version 0.8 (http/;//github.com/tseemann/abricate) with the virulence factor database (VFDB) (Chen et al., 2004),; green represents presence) and red represents absence. The heat map was generated with Microsoft Excel 2010

6.3.5 Predictive Modelling

6.3.5.1 Random Forest

The classification by Random Forest indicated that 2 variables (biomarkers), *fliP* and *glf* are the most important to classify healthy and unhealthy samples. The Figure 6-3 shows the importance of variables by Random Forest method.

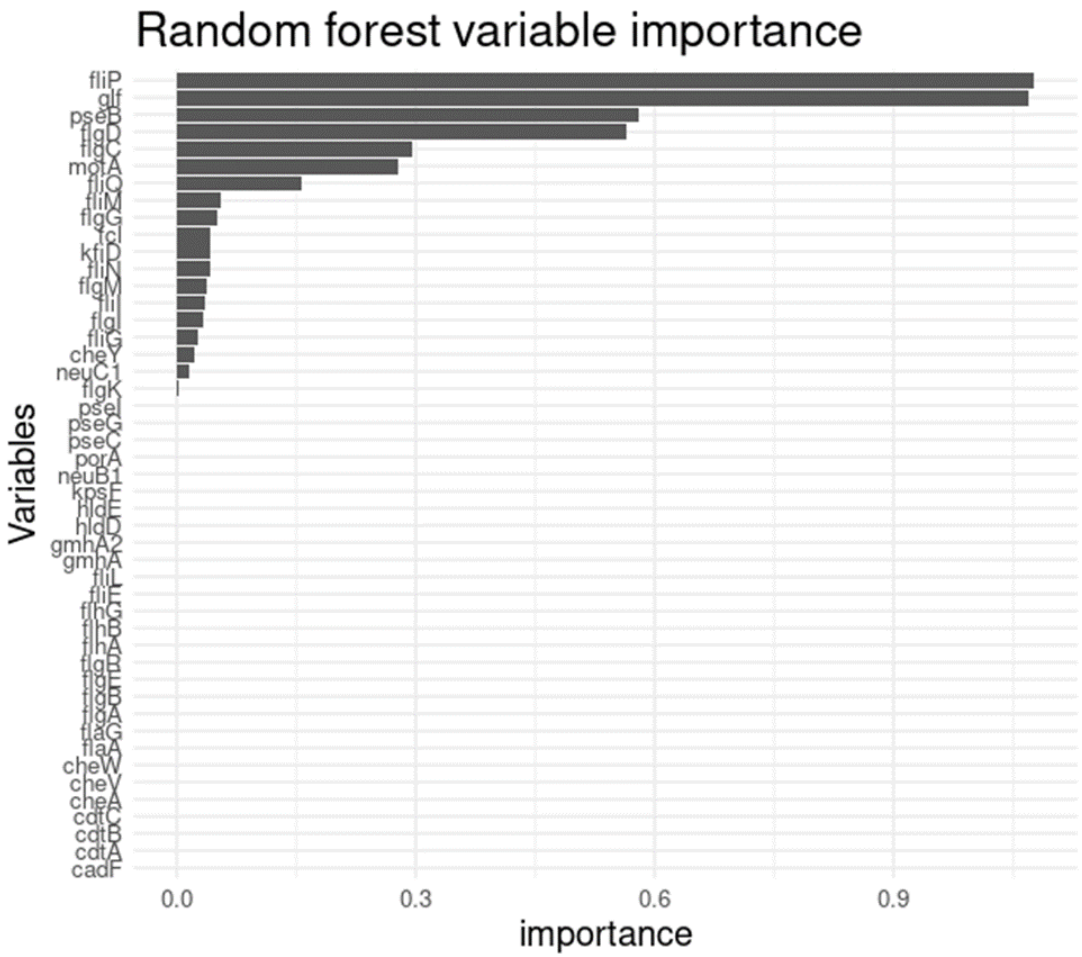

Figure 6-3 Importance of variables (biomarkers) by Random Forest method

The confusion matrix indicated that the model correctly classified 100% of the samples (Table 6-3). Accuracy on testing data:100%>

Table 6-3 Confusion matrix:

	healthy	Un-healthy	class.er-ror
healthy	4	0	0
Un-healthy	1	6	0.142857

6.3.5.2 Logistic model

Cross-validation in the logistic model indicated that 4 biomarkers are important to classify *Campylobacter* healthy and unhealthy samples, being: *flgD*, *fliP*, *glf*, and *PseB*. The logistic model for the selected variables using Random Forest is presented in Figure 6-4.

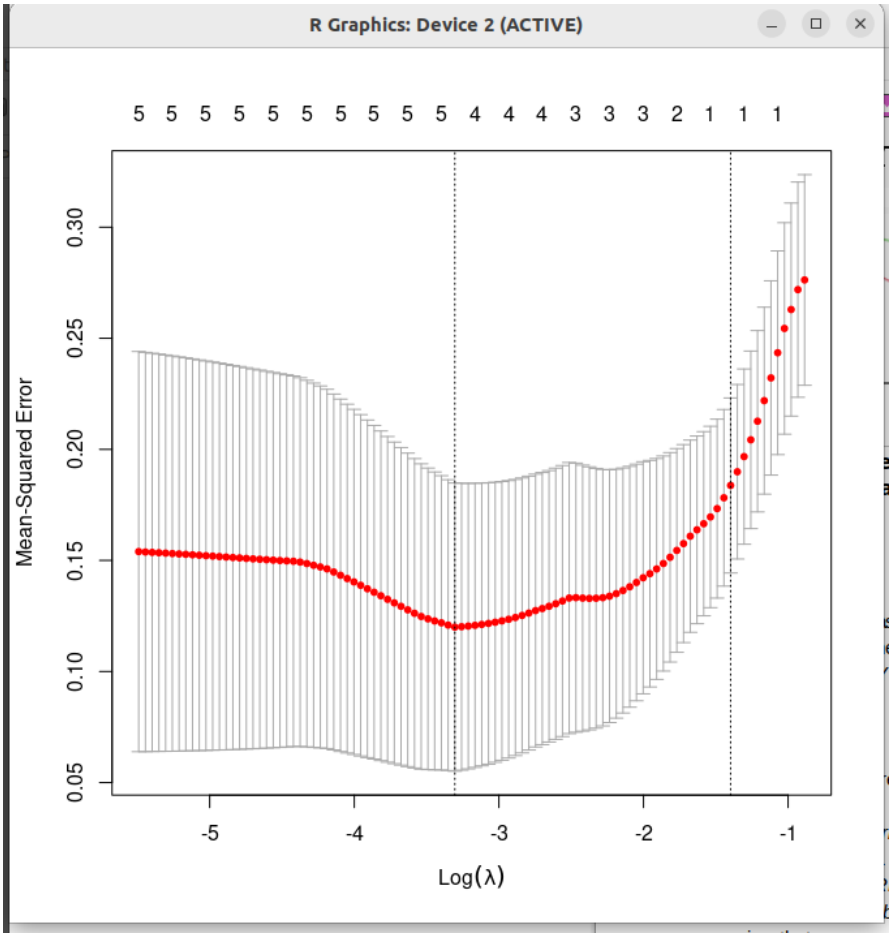

Figure 6-4 Cross-validation (CV) plot of mean squared error (MSE) using the glmnet package in R, and with a range of λ sequences: (The red dotted line is the CV curve and the error bars along the λ sequences are upper and lower standard deviation curves (95% confidence interval). The y-axis represents the MSE for respective values of λ, while the upper x-axis represents the number of predictors (features/biomarkers).

The Table 6-4 show the 4 most important variables and the Mean-squared error for the classification was 0.03671

Table 6-4 Most important variables for the classification of *Campylobacter* healthy and unhealthy metagenomes, as defined by the glm model with LASSO regularization

	Coefficient
Intercept)	0.85993257
cheY	
fcl	
flgC	
flgD	-0.29182980
flgG	
flgI	
flgK	
flgM	
fliG	
fliI	
fliM	
fliN	
flip	-0.52813755
fliQ	
glf	-0.02300176
motA	
neuC1	
pseB	-0.12229317

Considering the above results, the variables defined by the glm model with LASSO regularization are the most important to classify healthy and unhealthy samples. Despite the Random forest model having selected two variables equal to the glm model, it does not clearly define why the other two variables must be dropped as unimportant for classifying the samples. Therefore, we must use the following variables for classifying the samples: *flgD, fliP, glf*, and *pseB*. These 4 genes defined by the model associates with *Campylobacter* unhealthy samples. The use of these genes to distinguish healthy and unhealthy samples cam be justified given the following reasons. Recall that *Campylobacter jejuni* is responsible for ~85% of the human infections. The *flgD* gene encodes the flagellar hook cap protein are amongst human-adapted lineages. Also, varying alleles of this gene have been found to be a defining feature of hyperinvasive *C. jejuni* strains (Baig et al., 2015; Costa and Iraola, 2019). Besides, *flgD* and *pseB* are among the core essential genes of the *C. jejuni* virulome with prevalence above 99.9% amongst clinical isolates (Panzenhagen et al., 2021). The gene, *glf* is involved in capsule biosynthesis and transport in *C. jejuni* and is one of the accessory genes in *C. jejuni* virulome. Lastly, the *FliP* is part of the , *Campylobacter* Type III protein secretion system (T3SS), which is used to inject and secrete putative virulence factors into host cells (Bolton, 2015b). The model was tested on the metagenomic dataset from the river water sample. The variables (biomarkers) occurred in all samples collected from the river water (Table 6-5) indicating a potential risk of disease for population exposed to the river is eminent. The risk of infection upon exposure to the river is of concern given that rivers have been implicated as the leading source of *Campylobacter* (Davies et al., 1995; du Plessis et al., 2015; Siddiqee et al., 2019). Polluted surface water, are suggested waterborne *Campylobacteriosis* outbreak risk factors (Neimann et al., 2003; Kapperud et al., 2003; Evans et al., 2003; Schönberg-Norio et al., 2004; Michaud et al., 2004; Carrique-Mas et al., 2005; Tissier, 2012). In one instance, an outbreak that ensued affected up to 35000 people who consumed contaminated river water. *C. jejuni* and *Cryptosporidium* spp. were responsible for this outbreak (Beaudeau et al., 2008). The risk of Campylobacteroisis is particularly as a result of the ideal growth conditions and their ability to adapt to the harsh conditions in the river environment (Bronowski et al., 2014).

Table 6-5 The occurrence of the variables (biomarkers) as defined by the glm model with LASSO regularization model.

Sites	*flgD*	*fliP*	*glf*	*pseB*	Prediction
B1	0	1	2	2	Positive
B3	1	1	2	1	Positive

In summary, this study revealed the occurrence of *Campylobacter* antibiotic resistance and virulence determinants in Bloukrans River. Potential risk of disease for population exposed to the river is apparent. In theory, restricting the use of antibiotics, such as tetracycline in livestock production systems in South Africa may decrease ARG loads of rivers. However, frequent conjugation of plasmids carrying ARGs foster plasmid maintenance in a microbial community, in the absence of antibiotics, even if the plasmid sustains a fitness cost (He et al., 2020). Therefore, in addition to reducing antibiotics use, strategies to prevent conjugation and promote resistance plasmid loss might be useful. It is acknowledged that the presence of a gene does not necessarily mean phenotypical relevance (i.e. they might be not expressed). However, it noteworthy previous studies have obtained significant level of agreement between predicted resistance by next generation sequencing and phenotypic resistance studied by classical microbiology methods (Koutsoumanis et al., 2019).

The study also develops a predictive model that use critical discriminative biomarkers, *flgD*, *fliP*, *glf*, and *pseB* as an indicator of a *Campylobacter* unhealthy environment. This preliminary research provides an initial context for demonstrating the potential use of shotgun metagenomics data combined with machine learning algorithm to identify and characterise environmental exposure hotspots for Campylobacter spread and risk assessment. Altogether, the findings of this study provide informative as well as practical knowledge of the *Campylobacter* contamination of Bloukrans River and the health-related risk status of individuals upon exposure.

CHAPTER 7: IDENTIFICATION OF THE LEADING SOURCE OF *CAMPYLOBACTER* IN BLOUKRANS RIVER AND LINK TO LOCAL CASES OF HUMAN *CAMPYLOBACTER* INFECTIONS (SOURCE ATTRIBUTION)

7.1 INTRODUCTION

Reducing the burden of Campylobacteroisis requires identification of the leading sources and prioritizing effective and targeted intervention strategies. (Pires et al., 2009, 2014a; Mughini Gras et al., 2012). Tracing the source of the contaminant is key to mitigation and disease prevention. The occurrence of *Campylobacter* in surface waters is as a result of faecal contamination by livestock, runoffs from farm animal manure, avian birds and insufficiently treated wastewater treatment effluent or leakages from nearby septic tanks (Jones, 2002; Abulreesh et al., 2006). Source apportionment is a method of identifying the sources of contamination contributing to a specific environmental system (Chen et al., 2012). Microbial source tracking (MST), is a method of source apportionment and have enabled identification of the origin of contaminating bacteria in contaminated water systems (Newton et al., 2013; Staley et al., 2018; Liu et al., 2018). Microbial source tracking (MST) approaches are means of identifying sources of faecal or bacterial pollution in a target community (Bagi and Skogerbø, 2022). Microbial source tracking (MST) using microbial communities relies on (1) each source having a distinct microbial community composition, or 'fingerprint', and (2) that the source contributions to an environmental sample can be back-calculated by comparing its fingerprint to a range of source fingerprints (Koutsoumanis et al., 2019). Currently, shotgun metagenomic sequencing based MST approaches have been developed, and offer a much wider potential for a collection of microbial diversity (Bacteria, Archaea, Eukaryota, and viruses) to be applied in microbial source tracking, instead of just relying on one or more main bacterial strains or species that may not be present in the sample (McGhee et al., 2020a; Ma et al., 2022)

Furthermore, the proportion of human infections can be attributed to the wellspring of *Campylobacter* in the different reservoirs through the estimation of the extent of subtype sharing between strains that are isolated from human and different reservoirs. For this goal, multi-locus sequencing typing (MLST) is microbiological method that is commonly applied. MLST is based on DNA sequence analyses of seven stable housekeeping genes (Dingle et al., 2002). It is currently the gold standard method. It has improved our knowledge of the *Campylobacter* population and different routes of *Campylobacter* transmission that lead to human diseases (https://pubmlst.org/; Magana et al., 2017). From previous source attribution studies, it is recognised that C*. jejuni* has strong host association (Atterby et al., 2018). Some genotypes are established to be preponderate in wild birds (Waldenström et al., 2002; Broman et al., 2004; Colles et al., 2008b, a), humans (Kinana et al., 2006; Mickan et al., 2007; Dingle et al., 2008), in farm animals (McCarthy et al., 2007; Kinana et al., 2007). The sequences and STs, allele numbers and clonal complexes for these are available in MLST database of *Campylobacter jejuni* and *Campylobacter coli*, with accession numbers 21601-22077, http://pubmlst.org/*Campylobacter* /) (Griekspoor et al., 2013).

It is possible to combine the efficiency of the MLST approach with the ease of culture independent and high throughput metagenomics (Zolfo et al., 2017). In theory Metagenomic datasets can provide typing data for all the species of interest within the sample (Venter et al., 2004). One of the strategies to achieve metagenomic MLST typing is by using metagenomics assemblies. By mapping the assembled metagenomics contigs against the MLST databases, it is possible to extract MLST loci from metagenomes. The problem with this approach is that one can only uncover the strains that are abundant, that is the strains with sufficient depth to assembled

metagenomically. Another approach does not require assembly. Several microbiological methods have been developed for source attribution, which are based on statistical modelling of microorganism subtyping data that are derived from combined surveillance of human cases and pathogen incidences in food, animal and environmental sources (Pires et al., 2009, 2014b; Mughini-Gras et al., 2018).

The objective of this chapter is to identify the leading source of *Campylobacter* contamination of the river and attempt to link to link the *Campylobacter* spp present in water samples to local clinically relevant ones. Large bacterial 16S rRNA marker-gene NGS libraries from different metagenome samples collected from human gut (faeces) and rumen contents from livestock from multiple studies were used to determine the source origins of *Campylobacter* contaminating Bloukrans. Subsequently, subtyping was carried out to link the *Campylobacter* spp present in water samples to local clinically relevant ones.

7.2 METHODS

7.2.1 Source apportionment to identify the sources of microbial contamination of Bloukrans River

The microbial source tracking (MST) method described in McGhee et al. (2020) was used for determining the dominant sources of bacterial pollution in the Bloukrans River. Metagenomic Sequence libraries of publicly available metagenomic studies of the two domesticated ruminant species (cattle and sheep) and two non-domesticated species (reindeer and red deer) (Project: PRJEB34458), and human gut (Project: PRJEB6092) were downloaded from European Nucleotide Archive (ENA). Animal rumen and human gut contents were chosen as likely sources of microorganisms to be found in Bloukrans River water, which tend to have runoff from nearby manure applied crop farms, possible contamination with insufficiently treated effluents from nearby wastewater treatment works, and faecal contamination from livestock grazing and drinking around the rivers. These source samples are likely to contribute organisms found in the sink sample (metagenomic library of the Bloukrans River). All metagenomic samples were pre-processed by removing low quality reads and adaptor sequences using fastp (version 0.2) (Chen et al., 2018a). Taxonomic abundances of the quality filtered metagenomic sequences were obtained using the Kaiju ver. 1.5.0 software. SourceTracker2 was employed to analyse the relative contribution of the microbial taxa from the source samples to the sink sample. The command line SourceTracker2 tool was installed and used with default settings according to the instructions on the software's Github page (https://github.com/biota/sourcetracker2).

7.2.2 Source attribution to link the *Campylobacter* spp. present in water samples to local clinically relevant ones

The source attribution method described in Zolfo et al. (2018) was used in an attempt to link the *Campylobacter* spp. present in water samples to local clinically relevant ones . MetaMLST version 1.1 was applied to the Bloukrans River metagenomic sequence dataset. The raw reads were mapped against the MetaMLST database 2017, which consisting of 113 organisms, 798 loci, 46.2 Mbp and 12,929 total profiles. The mapping was executed using bowtie2 software (Langmead and Salzberg, 2012), version 2.2.6 as previously described (parameters: -D 20 -R 3 -N 0 -L 20 -i S,1,0.50 -a -no-unal) (Asnicar et al., 2017). Samtools v with version 1.3.1 was used to sort alignment files (Li et al., 2009). Only the species that at least one known ST could be detected is reported.

7.3 RESULTS AND DISCUSSIONS

7.3.1 Sources of microbial contamination of Bloukrans River

Animals (livestock) is the predominant contributor of bacterial microbes in Bloukrans River (Figure 7-1). According to the mSourceTracker analysis based on bacterial domain, the value of the relative contribution from animal was estimated to be 28%, 30% and 18% for Sites B1, B3 and B4 along the river, respectively. Alternatively, the value of the relative contribution from human gut was estimated to be 10% for all the sites, respectively. The contribution from unknown sources was estimated to be 62% for sites B1 and B3, and 74% for Site B4. The contribution from Animals is relatively less in Site B4, but contribution from human gut is the same. This is expected given that the site is mainly used for recreational activity, such as swimming and baptism.

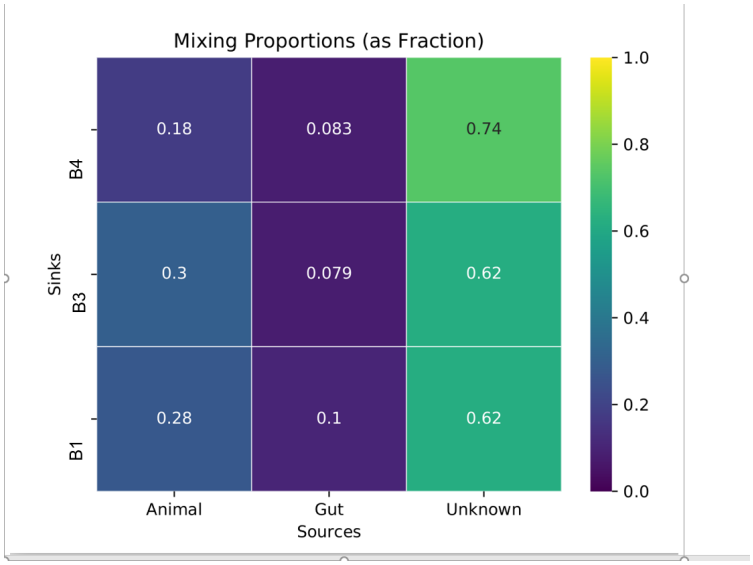

Figure 7-1 Source proportion estimates for water samples collected from three different sites along the Bloukrans River. Heatmaps were produced using mSourceTracker , and shows the proportion that each source contributed to each site along the Bloukrans River for Bacteria. Animal represents rumen contents from livestock, whereas guts represents human gut contents.

These results suggest livestock grazing around the river are the most bacterial pollution contributor to the river compared to effluents from nearby wastewater treatment works bearing human excreta. This phenomenon is not surprising because livestock is commonly viewed as the primary source of freshwater bacterial contamination(Dorner et al., 2004; Espunyes et al., 2021). The high proportion of taxa from unknown sources was likely due to Solid wastes (e.g. garbage, electronic waste, construction waste) generated by individual and residential activities, and toxic wastes (chemicals from improperly disposed wastewater , and surface runoff bearing pesticides used on agricultural areas), all of which selects for organisms from synthetic extreme environments (Selbmann et al., 2013; Maes et al., 2016; Sibanda et al., 2017). Addition of metagenomic samples from extreme environments might decrease the proportion of unknown samples, hypothetically (McGhee et al., 2020b).

7.3.2 Source attribution

Source attribution is conducted here to ascertain transmission of clinically relevant microorganisms from river . Using MetaMLST STs of different species were recovered from the Bloukrans River metagenome (Table 7-1).

Table 7-1 Results of MetaMLST applied to Bloukrans River metageomic dataset. The table reports STs found by profiling the dataset.

Sites	Specie	ST
B1	*E. coli*	ST 624
B1	*Acinetobacter*	ST1346
B1	*Arcobacter*	ST226
B1	*B. cepacia*	ST0
B1	*Achromobacter* --	ST0
B1	*P. fluorescens*	ST0
B1	*S. enterica*	ST3007
B3	*S. enterica*	ST0

One of the STs recovered from Bloukrans River metagenome have been found among clinical isolates previously reported from South Africa. *E. coli* ST624, was defined as a virulent clone, that carry mobile colistin resistance (mcr-1) genes, and have been recovered from urine samples of South African patients (Coetzee et al., 2016; Poirel et al., 2016; Anyanwu et al., 2021). mcr-1 gene is responsible for resistance to colistin (COL), and antibiotics used in livestock production. Therefore, it has been suggested that they are disseminated from the livestock sector into the human population. Their presence in river implicates the river system as a point of dissemination of these livestock originated pathogenic bacteria into the human population (Anyanwu et al., 2021).

The other STs recovered from Bloukrans River metagenome have been reported elsewhere. The ST3007 is animal-derived *Salmonella* ST types, identified in swine in China (ZHAO et al., 2017). Acinetobacter ST1346 harbour carbapenems resistance *NDM* gene and was recovered in human blood, surgical site in Cuba (Pérez et al., 2022). The rest of the species recovered by MetaMLST are equally relevant. *Achromobacter* spp. are ubiquitous in the environment. They are opportunistic pathogens and can cause several types of infections, such as keratoconjunctivitis, pneumonia, endophthalmitis, endocarditis, peritonitis and meningitis (Chalhoub et al., 2022). *Burkholderia cepacia* (*B. cepacia*) can cause severe respiratory infections in cystic (Nunvar et al., 2016). *Pseudomonas fluorescens* occur in the human gut as a low-level commensal, but generally not considered a pathogen in humans. However, recent reports suggests the association of this species with certain human diseases (Biaggini et al., 2015; Nishimura et al., 2017; Quintieri et al., 2020). *Salmonella enterica serovar Typhimurium* is a major enteric pathogen that can infect both animals and humans. Consumption of contaminated water or food or water trigger gastrointestinal disease (Fàbrega and Vila, 2013). *Arcobacter* spp. are emerging pathogens that cause enteritis, serious diarrhoea, bacteraemia and septicaemia, in humans and enteritis, and abortion in animals. Like *Salmonella enterica*, water is a possible route of transmissions of *Arcobacter* spp. in human. The genus Arcobacter is within the *Campylobacter*aceae family with the *Campylobacter* and *Sulfurospirillum* genera (Chieffi et al., 2020). Unfortunately, MetaMLST did not detect ST of *Campylobacter* spp. in the Bloukrans River samples, and this is a problem of missing loci. The sequencing coverage for this metagenomic dataset is probably too low to guarantee the presence of a read containing a given sequence in the targeted *Campylobacter* genome. This is a typical challenge with using shotgun metagenomics dataset in subtyping bacteria(Sanabria et al., 2021). Therefore, the lack of detection

of STs from *Campylobacter* species in the Bloukrans River metagenomic dataset does not necessarily mean thy are absent.

In summary we identified animals as the main contributor of bacterial contamination to Bloukrans River. Rivers are involved in several pathways critical to the possible transmission of pathogens to the general public. The findings from this study suggest that best management practices in animal husbandry, which will control livestock access to rivers may be an effective way to protect rivers from faecal as well as pathogenic microbial contamination. Furthermore, we detected *E. coli* specie with genetic profile that is identical to local human clinical isolates and the river, indicative of possible transmission pathway. This result confirms that exposure to the Bloukrans River is an important route via which people become colonised by pathogens, including the antibiotic resistant ones.

CHAPTER 8: CONCLUSIONS & RECOMMENDATIONS

This study investigated occurrence, and risk to human health, sources of *Campylobacter* species in selected source water in Eastern Cape, South Africa. The understanding is critical for river water quality management in the area. This chapter presents logical inferences drawn from the information contained in the main chapters of this report.

8.1 CONCLUSIONS

Culture-based approach could not give a true picture of *Campylobacter* occurrence in Bloukrans River. It is possible that the organisms could have are in a viable but nonculturable state due to starvation and physical stress in the environment. Therefore, they may not be detectable even if the water is contaminated and infection outbreak is possible.

- A metagenomic approach to evaluating water samples is more efficient, and can be useful for source tracking and the surveillance of pathogens such as *Campylobacter* spp. as well as for monitoring virulence factors and antibiotic resistance genes. Therefore, this approach should be pursued

- The result from this study suggests the level of occurrence of *Campylobacter* and presence of antibiotic resistance genes in the Bloukrans River system presents public health concern if users of river water use collected water without treating the water appropriately before use.

  There was no marked seasonal variation in the prevalence of *Campylobacter* spp. in the Bloukrans River all through period of study, suggesting that *Campylobacter* infection could be endemic in the region.

- Livestock grazing around the river are the most bacterial pollution contributor to the river

- Although findings could not show specifically that *Campylobacter* infection in humans are due to contamination from water, *E. coli* specie from the water were closely related to human isolates suggesting that transmission from the water to humans may occur through direct or indirect contact.

8.2 RECOMMENDATIONS

- Methodological limitations prohibited culture-based study of Campylobacter occurrence in the water samples. It is important that the respective authorities recognize the possibilities of occurrence of these bacteria, even if culture-based methods have reported them absent. Therefore, more efficient methods, such as those based on the genotypic characteristics of these bacteria (PCR, NGS) should be pursued
-
- A key outcome in this project is a set of discriminative biomarkers that may be useful in developing diagnostic tool for water bodies risk assessment, a preventive strategy against *Campylobacter* outbreak.

- Research is needed to enhance source control, including identifying best livestock management strategies to reduce livestock roaming and presence around the river and improving the knowledge base to support policies to reduce diffuse pollution from agricultural systems. By doing so, policy and

operational adjustments can be implemented to mitigate pathogenic bacteria pollution of rivers by livestock and protect human health

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