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The application of wastewater-based epidemiology to Hepatitis E virus in South Africa

by
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Declaration

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Abstract

Background

Hepatitis E virus (HEV) has a global presence, but the highest burden of disease is found in Africa and Asia. Unfortunately, in Sub-Saharan Africa, there is a gap in knowledge on the molecular epidemiology of the disease, as often serological methods only are applied. In recent years a high seroprevalence of the virus has been detected in populations in the Western Cape province. Evidence of viraemia has been published on a few South African patients from the Western Cape. Viral ribonucleic acid (RNA) was also detected in swine samples from an abattoir and in porcine meat products in Cape Town. Based on the existing literature, HEV genotype 3 has been detected in patients and swine samples. In this study, the aim was to determine whether HEV was circulating in communities in the Western Cape by testing wastewater extracts for the presence of HEV RNA. The study also sought to determine the HEV genotype in wastewater sample extracts.

Methods

One hundred and forty-three wastewater extracts were tested for the presence of HEV RNA using real-time polymerase chain reaction (PCR). Samples were sourced from four locations: two wastewater treatment works and sewer manholes from two Stellenbosch University residences. Nested reverse-transcriptase PCR (RT-PCR) targeting three regions of the HEV genome was applied to samples which tested positive for HEV RNA. The sequenced regions were a portion of open reading frame (ORF) 2 covering 347 base pairs (bp), a 286 bp region of ORF1 and a 126 bp region of the overlapping ORF2/3. The generated PCR products were then sequenced with Sanger sequencing. The generated consensus sequences were placed in a phylogenetic tree with reference sequences for HEV genotypes 1 to 8, to determine which genotype is likely circulating in the selected communities.

Results

Out of the 143 wastewater samples, 130 valid results were generated. Out of 130, 21 (16.2%%) were positive and 109 (83.9%) were negative. Of the 21 positive samples, 5 (23.8%) were collected from Athlone wastewater treatment works (WWTW), 9 (42.9%) from Zandvliet WWTW, 1 (4.8%) from Meerhoff residence in Tygerberg and 6 (28.6%) from Metanoia residence in Stellenbosch. The positive samples had a median cycle threshold value of 37.9 (interquartile range: 36.7-39.7). Out of the 21 positive samples, 4 (19.1%) sequences were obtained from the ORF2/3 region. The sequences clustered most closely with a sequence from a South African patient and HEV genotype 3 reference sequences.

Conclusions

Based on the real-time PCR results, it appears that HEV is circulating among communities in the Western Cape province. Based on the detection of HEV genotype 3 in the wastewater samples, it suggests that zoonotic transmission may be the mostly likely route of infections. Further investigation to identify porcine and other food products which may be contaminated with HEV are necessary to break chains of transmission.

[454 Words]

Opsomming

Agtergrond

Hepatitis E virus (HEV) het ‘n wêreldwye teenwoordigheid, maar die hoogste siektelas is in Afrika en Asië ondervind. In Sub-Sahara Afrika, is daar ongelukkig ‘n gaping in die kennis van die molekulêre epidemiologie van die siekte, omdat serologiese metodes alleen dikwels gebruik word. ‘n Hoë seroprevalensie van die virus is onlangs in bevolkings in die Wes-Kaap provinsie opgespoor. Bewyse van viremie was in ‘n paar Suid-Afrikaanse patiënte gedokumenteer. Virale ribonukleïensuur (RNS) was ook in vark monsters van ‘n slagpale en in varkvleisprodukte in Kaapstad bespeur. Gebaseer op die bestaande literatuur is HEV genotipe 3 in pasiënte en vark monsters bespeur. In hierdie studie was die doel om vas te stel of HEV in gemeenskappe in die Wes-Kaap sirkuleer deur afvalwaterekstrakte vir HEV RNS te toets. Hierdie studie het ook probeer om die HEV genotipe in afvalwatermonster ekstrakte te bepaal.

Metodes

Een honderd-drie-veertig afvalwaterektrakte was vir die teenwoordighied van HEV RNA getoets met intydse polimerase kettingsreaksie (PKR). Monsters was van vier liggins verkry: twee afvalwaterbehandelingswerke en riool mangate van twee Universiteit Stellenbos koshuise. Geneste omgekeerede transkripsie-PKR was gedoen op monsters wat positief getoets het vir HEV RNS. Die bepaalde streke was ‘n gedeelte van oop leesraam (OLR) 2 wat 347 base pair dek, ‘n 286 bp gedeelte van OLR1 en ‘n 126 bp OLR van OLR2/3. Die PKR produkte het toe volgorderbepaaling ondergaan met Sanger-volgorderbepaling. Die gegenereerde konsensus volgordes was dan met verwysing volgordes vir HEV genotipes een tot agt in ‘n filogenetiese boom geplaas. Dit was gebruik om die waarskynlike sirkulerende genotipe in die geselekteerde gemeenskappe te bepaal.

Resultate

Uit die die 143 afvalwatermonsters, het 130 geldige resultate gegenereer. Uit die 130 resultate, is 21 (16.2%) positief en 109 (83.9%) negatief. Uit die 21 positiewe monsters, is 5 (23.8%) van Athlone afvalwaterbehandelingswerke (AWBW) ingesamel, 9 (42.9%) van Zandvliet AWBW, 1(4.8%) van Meerhoff koshuis in Tygerberg en 6 (28.6%) van Metanoia koshuis in Stellenbos. Die positiewe monsters het ‘n mediaan waarde van 37.9 (interkwartielvariasiewydte: 36.7-39.7). Uit die 21 positiewe monsters, is 4 (19.1%) volgordes van die ORF2/3 streek verkry. Die volgordes het die nouste saam met ‘n positiewe volgorde van ‘n Suid-Afrikaanse pasiënt en HEV genotipe 3 verwysingvolgordes gegroepeer.

Gevolgtrekkings:

Gebaseer op die intydse PKR resultate, blyk dit dat HEV onder gemeenskappe in the Wes-Kaap sirkuleer. Gebaseer op die opsoring van HEV genotipe 3 in die afvalwatermonsters, blyk dit dat soönotiese oordrag heel waarskynlik die roete van infeksies is. Verdere ondersoek is nodig om vark en ander kosprodukte wat dalk met HEV besmet is te identifiseer, om die kettings van oordrag te breek.

[419 Woorde]

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List of Abbreviations

BLAST: Basic Local Alignment Search Tool
bp: base pair
CAR: Central African Republic
CDC: Centers for Disease Control
cDNA: complementary deoxyribonucleic acid
COVID-19: coronavirus disease 2019
DNA: deoxyribonucleic acid
dNTPs: deoxynucleoside triphosphates
EDTA: ethylenediaminetetraacetic acid
EIA: enzyme immunoassay
ELISA: enzyme-linked immunosorbent assay
ER: endoplasmic reticulum
FAM: fluorescein amidites
GBS: Guillain-Barré Syndrome
GC: guanine-cytosine
HCC: hepatocellular carcinoma
HEV: Hepatitis E virus
HIV: human immunodeficiency virus
HREC: Health Research Ethics Committee
IC: internal control
ICTV: International Committee on Taxonomy of Viruses
IgG: immunoglobulin G
IgM: immunoglobulin M
kb: kilobase
MGB: minor groove binder
NA: neuralgic amyotrophy
NAT: nucleic acid amplification techniques
NGS: next generation sequencing
NHLS: National Health Laboratory Service
NIH: National Institute of Health
NRF: National Research Foundation
nt: nucleotide
NTC: non-template control
ONT: Oxford Nanopore Technology
ORF: open reading frame
PBS: phosphate-buffered saline
PC: positive control

PCR: polymerase chain reaction

PEI: Paul Ehrlich Institute

PRF: Poliomyelitis Research Foundation

qPCR: quantitative polymerase chain reaction

REC:BES: Research Ethics Committee: Biological and Environmental Safety

RNA: ribonucleic acid

RNase: ribonuclease

RT: reverse transcriptase

RT-PCR: reverse transcriptase polymerase chain reaction

RT-qPCR: reverse transcriptase quantitative polymerase chain reaction

SAMRC: South African Medical Research Council

SARS-CoV-2: severe acute respiratory syndrome coronavirus 2

SNF: supernatant fluid

SU: Stellenbosch University

UAE: United Arab Emirates

USA: United States of America

TAE: Tris-acetate-ethylenediaminetetraacetic acid

Tm: melting temperature

WBE: wastewater-based epidemiology

WHO: World Health Organisation

WRAIR: Walter Reed Army Institute of Research

WWTWs: wastewater treatment works

Chapter 1: Background

Infection of the liver with Hepatitis E is caused by the hepatitis E virus (HEV). The virus can cause acute, chronic, or fulminant hepatitis. HEV has global presence, but its molecular epidemiology varies. HEV-1 and HEV-2 are transmitted faecal-orally and are associated with water-borne outbreaks, particularly in underdeveloped countries (Kamar et al., 2014; Sayed et al., 2015). These genotypes are found in Africa, Asia, and Central America (Webb and Dalton, 2019). Genotypes 3 (HEV-3) and 4 (HEV-4) are transmitted zoonotically and can infect swine, wild boar, rabbits, and deer. HEV-3 is thought to be more prevalent in developed countries and HEV-4 has mainly been found in Japan and China (Webb and Dalton, 2019). Developing nations have the highest burden of disease, and despite major hepatitis E outbreaks occurring in Africa, limited studies have been conducted on the continent. Molecular methods are not often applied due to the lack of technical and financial capacity, with serology being used to confirm infection but being unable to inform the reservoir of infection (Bagulo et al., 2020). Regions where outbreaks occur include refugee camps, emergency shelters in conflict-affected areas, places affected by floods or monsoons and settings where plumbing and sanitation infrastructure are not well developed (Pallerla et al., 2020). The use of serology to diagnose HEV infection in these regions requires funding and trained personnel. Molecular tests require more specialised equipment, laboratory access and trained technicians. Samples from these regions may need to be transported to referral laboratories for testing, but this is expensive especially ensuring maintenance of the cold chain to ensure sample integrity. As a result, few molecular epidemiology studies have been conducted in Sub-Saharan Africa, resulting in a knowledge gap.

In South Africa, several serological studies testing for HEV in both human and swine samples have been conducted. Case studies have been reported from Cape Town of infection with HEV-3 (Andersson et al., 2013; Madden et al., 2014; Andersson et al., 2015). Swine serum samples from Cape Town have tested positive for total HEV antibody and HEV ribonucleic acid (RNA) was detected in pork liver products in Cape Town in 2014 (Van Helden et al., 2017; Korsman et al., 2019). A 2022 study performed on pregnant women in Pretoria found a low prevalence of HEV based on serological testing (Simani et al., 2022). One master's study was conducted in Pretoria in 2004 which tested swine and wastewater samples for the presence of HEV. They detected HEV RNA and confirmed it with Sanger sequencing in 2/199 (1%) wastewater samples (Williams, 2004). Based on these findings, it would suggest that HEV may be more endemic to the Western Cape compared to Gauteng, although the true burden of infection is unknown. Minimal studies have been conducted on HEV seroprevalence in Gauteng, and most reports of HEV infection in South African patients published after 2000 have been conducted in the Western Cape.

In developed nations, such as Germany, Switzerland and Italy, wastewater-based epidemiology (WBE) has been applied to gain insight into the circulation of HEV on a large scale (Masclaux et al., 2013; Iaconelli et al., 2017; Beyer et al., 2020; Iaconelli et al., 2020). These studies tested wastewater samples for the presence of HEV RNA and inferred whether the disease was prevalent in selected communities. In some cases, they were able to sequence HEV genome regions from wastewater samples and determine the genotype of the

detected strains (Beyer et al., 2020; Iaconelli et al., 2020). They could place their findings of the clinical data available for their region and motivate future studies and awareness of HEV from a public health perspective. Comparatively few similar studies have been conducted in Africa. Testing of wastewater samples for HEV RNA has been performed in Egypt (North Africa), and Cameroon (Central Africa) (Kamel et al., 2011; Fatawou et al., 2023), but to the author's knowledge, no published studies have been conducted in South Africa.

HEV infections are often asymptomatic, and clinical records do not reflect the true burden of infection. Wastewater samples provide a non-invasive insight into whether the virus is being shed (and therefore whether individuals are infected) in a community. WBE has been used to monitor the circulation of chemicals and microbes for years, and it became a highly valuable tool during the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic (Johnson et al., 2021). The South African Medical Research Council (SAMRC) launched the SARS-CoV-2 wastewater surveillance dashboard in 2020 to be used as an early warning system for coronavirus disease 2019 (COVID-19) infections. The total RNA which they extracted in 2021 for SARS-CoV-2 surveillance was stored at -80°C.

In this study, stored nucleic acid extracts from SARS-CoV-2 surveillance were tested for the presence of HEV RNA. This allowed insight into whether HEV was circulating at the time of sampling in communities in the Western Cape. Sequencing of HEV genome regions from positive samples was attempted to increase insight into the molecular epidemiology of HEV in South Africa.

Chapter 2: Literature Review

2.1. Introduction

Hepatitis E virus infection causes disease in the liver known as hepatitis E. Hepatitis E presents in the forms of acute hepatitis, fulminant hepatitis and, in certain cases, chronic hepatitis. The World Health Organisation (WHO) estimates that the disease caused 44 000 deaths in 2015 alone and that 20 million cases of HEV infections occur globally each year (WHO., 2023). Most cases of hepatitis E infection are acute and self-limiting, and symptoms include fever, nausea, vomiting, malaise, and hepatomegaly. The virus has an incubation period ranging from 2 to 10 weeks. Asymptomatically infected individuals in outbreak settings have been recorded as having elevated liver enzyme levels, HEV viraemia and normal serum bilirubin levels (Aggarwal and Jameel, 2011). Elevated serum bilirubin levels have also been described and elevated liver enzyme levels appear to be a more commonly detected marker of infection (Gerolami et al., 2011; Andersson et al,. 2013; Kamar et al, 2014).

HEV superinfection in patients with pre-existing asymptomatic liver disease can result in acute-on-chronic liver disease and decompensated cirrhosis (Kumar and Saraswat, 2013). Chronic hepatitis E has been reported in immunocompromised patients such as individuals with human immunodeficiency virus (HIV) disease, solid organ transplant recipients, patients undergoing chemotherapy, and patients on immunosuppressive therapy due to autoimmune diseases (Kamar et al., 2013; Andersson et al., 2015; Pischke et al., 2019). Liver cirrhosis can result from chronic hepatitis E, and it has been hypothesised that HEV may play a role in the development of hepatocellular carcinoma (HCC) (Colson et al., 2019). A study from Cameroon found a higher seroprevalence of HEV in patients with HCC compared to those with chronic liver disease without HCC. It was theorised that the presence of pre-existing liver tumours might make patients more susceptible to HEV infection or that the infection may promote the development of liver disease towards cancer in patients with chronic hepatitis B virus or hepatitis C virus infections (Amougou Atsama et al., 2017).

Although hepatitis E occurs globally, the greatest burden of disease is in Africa and Asia (Raji et al., 2022). In Africa, multiple outbreaks and sporadic cases have been recorded, including one of the single largest recorded HEV outbreaks in the world which occurred in Kitgum, Uganda between October 2007 and June 2009 resulting in more than 10 000 people being infected and 160 deaths (Teshale et al., 2010; Raji et al., 2022). The most severe burden of disease is found in pregnant women with recorded case fatality rates ranging from 12.5% to 42.1% (Kim et al., 2014). Despite the heavy burden of disease there are still knowledge gaps regarding the epidemiology and particularly the molecular characteristics of HEV in Africa. Lack of resources in places where these outbreaks occur and lack of access to molecular methods contribute to this gap.

2.2. Virology and transmission of HEV

HEV has a positive-sense, single-stranded RNA genome which ranges in length from 6.4 kilobases to 7.2 kilobases (Purdy et al., 2017). The virus is non-enveloped when detected in faeces and bile but quasi-enveloped in blood (Primadharsini et al., 2021). HEV belongs to the Hepeviridae family under the subfamily *Orthohepevirinae* and the genus *Paslahepevirus* (Smith et al., 2020). The genus contains 8 genotypes of the species *Paslahepevirus balayani*, of which HEV-1, HEV-2, HEV-3, and HEV-4 commonly infect humans. The subfamily *Orthohepevirinae* also contains the genera *Avihepevirus* (which infects birds), *Rocahepevirus* (which infects rodents, shrews, and other carnivores) and *Chirohepevirus* (which infects bats) (Purdy et al., 2022).

These four genotypes which infect humans all have the same serotype (Kmush et al., 2013; Lhomme et al., 2016). HEV-1 and HEV-2 are transmitted faecal-orally, especially through the consumption of contaminated water (Kamar et al., 2014). These genotypes are most associated with HEV outbreaks in Africa, especially in areas where access to proper sanitation and clean water is limited such as refugee camps or informal settlements (Sayed et al., 2015; WHO, 2023). On the other hand, HEV-3 and HEV-4 infect other mammals besides humans and are transmitted zoonotically, often via the consumption of undercooked, infected meat products. Fruit, vegetables, and shellfish have been known to become contaminated with HEV where pig slurry was used as manure (Treagus et al., 2021). Meat products from pigs, wild boar and rabbits can all be infected (Primadharsini et al., 2021). HEV-3 and HEV-4 are associated with sporadic cases of HEV infection in developed countries. HEV is now recognised as an emerging public health concern in Europe with several indigenous infections being reported (Aspinall et al., 2017; Davis et al., 2021). Transmission of HEV via transfusion of tainted blood products has occurred, and countries such as the United Kingdom, Ireland and the Netherlands now screen donated blood for HEV (Webb and Dalton, 2019; Primadharsini et al., 2021).

2.3. The pathogenesis of HEV in pregnancy

Pregnant women in developing countries have been recorded as experiencing severe disease due to HEV, especially in their third trimester (Lhomme, 2016; Webb and Dalton, 2019). HEV-infected pregnant women have an increased risk of fulminant hepatic failure and acute liver failure, with mortality rates ranging from 20 to 25% in developing countries (Kamar et al., 2014). In a systematic review of HEV focused on Africa, case fatality rates in the general population ranged between 0% and 17.8%, compared to 10.4% to 42.1% in pregnant women (Kim et al., 2014). Infants acquiring HEV via vertical transmission have low birthweight and stillbirths have also been recorded (Wu et al., 2020).

Hormonal, immune, and viral factors are all considered to play a role in the effect of HEV infection during pregnancy (Gouilly et al., 2018; Wu et al., 2020). Altered maternal immune responses due to increased levels of progesterone, oestrogen, and human chronic gonadotropin have been linked to increased viral replication and a suppressive effect on cell-mediated immunity (Wu et al., 2020). Cellular immunity is also decreased during pregnancy due to a decrease in CD4 count, an increase in CD8 count resulting in a downregulated

CD4/CD8 ratio (Gouilly et al., 2018). Another host factor is the nutritional status of the mother because malnutrition and folate deficiency can increase the risk of viral infections (Pérez-Gracia et al., 2017).

Pregnant women experience higher rates of morbidity and mortality due to HEV infection than the general population. The HEV genotype is related to disease severity in pregnancy, with HEV-1 and HEV-2 being associated with higher morbidity and mortality than HEV-3 or HEV-4 (Gouilly et al., 2018). HEV-1 is associated with outbreaks which have occurred in Africa (Kmush et al., 2013; Kim et al., 2014). The pathogenesis of HEV is not completely understood, partially due to the lack of an appropriate in vitro cell culture model, and this may be part of the reason that the severe outcomes in pregnancy are not well understood (Kenney and Meng, 2019; Wu et al., 2020).

A study comparing the replication of HEV-1 and HEV-3 in an ex vivo maternal-foetal interface found that HEV-1was associated with greater viral replication, tissue necrosis, and tissue apoptosis than HEV-3 (Gouilly et al., 2018). The study used organ culture from maternal decidua and foetal placenta, infecting one culture set with HEV-1 and the other with HEV-3, to observe differences in replication and tissue injury. HEV-1 infection resulted in alterations of the placental barrier architecture. A feature unique to the genome of HEV-1 is ORF4 which produces the ORF4 protein under conditions of endoplasmic reticulum (ER) stress (Kenney and Meng, 2019). The ORF4 protein activates the host RNA-dependent RNA polymerase and increases viral replication. Pregnancy may increase ER stress triggering ORF4 protein production resulting in increased viral replication and contributing to more severe disease outcomes (Primadharsini et al., 2021).

2.4. HEV in immunocompromised individuals

An immunocompromised individual is defined by the reduced capability of their immune system to defend the body from infection. There are several reasons why a patient may be immunocompromised, including disease and immunosuppressive treatments. In the case of cancer patients, the immunosuppressive effects of both haematological malignancy and chemotherapy make them more vulnerable to chronic HEV infection (Tamura et al., 2007; Alric et al., 2010). Solid-organ transplant recipients undergo immunosuppressive therapy to prevent and treat allograft rejection (Dropulic and Lederman, 2016). Infections that would usually be self-limiting or subclinical can persist in these individuals. Over time it has appeared that such patients are more susceptible to chronic infection with HEV-3 and HEV-4 (Webb and Dalton, 2019). In cases of chronic hepatitis E in immunocompromised patients, viraemia is detected but they can be anti-HEV IgG and anti-HEV IgM negative (Kamar et al., 2011). Fatigue is the most common symptom of chronic hepatitis E, but infection can be asymptomatic with only elevated liver enzymes as an indicator (Kamar et al., 2011; Aslan and Balaban., 2020). Despite the lack of symptoms, chronic infection affects the structure of the liver and can cause rapidly progressing cirrhosis, to the point where a liver transplant may be necessary (Gérolami and Moal, 2008). The use of tacrolimus rather than cyclosporin A as immunosuppressive treatment in solid-organ recipients has been identified as a risk factor for chronic hepatitis E progression (Kamar et al., 2011). This is thought to be due to tacrolimus being more immunosuppressive than cyclosporin A.

Progression of chronic hepatitis E has also been associated with low CD4+ T lymphocyte cell counts in people living with HIV, especially cell counts below 200 cells/mm^3 (Colson et al., 2009; Dalton et al., 2009; Kenfak-Foguena et al., 2011). While studies on the effects of antiretroviral therapy on HEV infection are limited, there are two documented cases of chronic HEV infection in HIV patients where the HEV infection cleared after antiretroviral therapy and HIV viral load suppression (Kenfak-Foguena et al., 2011; Andersson et al., 2013). Since immunosuppression is a risk factor for chronic HEV infection, immune reconstitution may increase the chance of clearing HEV infection.

2.5. Extrahepatic manifestations of HEV

HEV infections have been recorded to affect systems and organs outside the liver. These include the nervous system, the circulatory system, the kidneys, and the pancreas. Neurological extra-hepatic manifestations are most common (Lhomme et al., 2016). Most of these cases have been documented in Europe and are associated with HEV-3 but neurological manifestations associated with HEV-1 has been reported in Asia (Webb and Dalton, 2019). These include Guillain-Barré Syndrome (GBS), Neuralgic amyotrophy (NA), Bell's Palsy, and encephalitis/ myelitis (Lhomme et al., 2016; Webb and Dalton; 2019; Aslan et al., 2020). The pathophysiology causing these outcomes is not yet fully determined, but there is speculation. HEV infection triggers the body's immune response, and auto-immune host response may be responsible for the observed neurological manifestations. It is also possible that HEV's tropism may not be limited to hepatocytes, as it has been documented to replicate in human-derived neuronal cell lines (Drave et al., 2016).

GBS usually occurs after an infection, whether viral or bacterial. The host immune system generates antibodies in response to the infection, but they cross-react with complex lipids present in grey matter (gangliosides) at nerve membranes (van den Berg et al., 2014). This cross-reactivity can cause acute flaccid paralysis, including rapidly progressing symmetrical motor paralysis (van den Berg et al., 2014; Fousekis et al., 2020). NA is also known as brachial neuritis and similarly to GBS, is thought to be a result of an autoimmune response after infection (Cheung et al., 2012; Webb and Dalton, 2019). It is characterised by severe pain followed by weakness in the dominant shoulder and arm (Webb and Dalton, 2019; Aslan and Balaban, 2020). HEV-associated NA appears to have more severe outcomes, including injury beyond the brachial plexus (the nerve network present in the shoulder which sends signals to the arms and hands) and involvement of the phrenic nerve (which provides signals to the diaphragm for breathing) (Webb and Dalton, 2019; Fousekis et al., 2020).

Decrease in kidney function during HEV infection has been documented in both immunocompetent and immunocompromised patients (Webb and Dalton, 2019). A retrospective study assessed kidney function in 51 solid-organ transplant recipients who were infected with HEV (Kamar et al., 2012). They observed membranoproliferative glomerulonephritis based on kidney biopsies and found that more than half of the patients had cryoglobulinemia which cleared once the HEV infection resolved. HEV-1 infection resulting in pancreatitis has been documented, this manifestation has also been associated with other hepatitis viruses (hepatitis A, B, and C) (Kamar et al., 2017; Aslan and Balaban, 2020).

2.6. Treatment and Prevention

While there is no gold standard for hepatitis E treatment, ribavirin and interferon-alpha have been used as treatment in certain contexts (Haagsma et al., 2010; Kamar et al., 2010; Gerolami et al., 2011; Goyal et al., 2012; De Winter et al., 2018). In cases of acute hepatitis E in immunocompetent individuals, the virus usually clears spontaneously (Aslan and Balaban, 2020). The benefits of therapy with ribavirin in treating acute hepatitis in immunocompetent patients have not been determined (Gerolami et al., 2011). Treatment of chronic hepatitis E with ribavirin has been documented, but further studies are needed to elucidate the mechanism of action, determine optimal dosage, and determine treatment duration (De Winter et al., 2018). Individuals who are on immunosuppressive therapy have cleared HEV after reduction of therapy dosage (Andersson et al., 2015; Pischke et al., 2019; Lhomme et al., 2020). Pegylated interferon alpha has been used to treat chronic hepatitis E in liver transplant recipients, although it is not used to treat other solid organ transplant recipients as it increases the risk of acute rejection (Haagsma et al., 2010; Kamat et al., 2010; Aslan and Balaban, 2020). Ribavirin and interferon-alpha are contra-indicated in pregnant women, making treatment options for acute infection in pregnant women more limited (Wu et al., 2020).

2.7. Vaccination against hepatitis E

The Hecolin® (HEV 239) vaccine has been licensed in China and was launched in 2012, but is only available in China (Zhang et al., 2016; WHO, 2023). The vaccine is in the form of virus-like particles based on amino acids 386-606 of the ORF2 region of the HEV genome (Velavan et al., 2021). It was originally designed to target HEV-1 but has demonstrated efficacy against HEV-4 (Lynch et al., 2023). It was recommended for outbreak response by the WHO in 2015 and was used in an outbreak response campaign in South Sudan in 2022. The campaign was launched by Médecins Sans Frontières and South Sudan's Ministry of Health at Bentiu internally displaced persons camp (WHO, 2023). The vaccine has not been pre-qualified by the WHO, which has recommended additional areas of study including the use of the vaccine in pregnant women, immunocompromised individuals and people living with chronic liver disease (Lynch et al., 2023).

2.8. Current known epidemiology of HEV in Africa

A systemic review published in 2014 compiled the existing data on outbreaks and sporadic cases of HEV in Africa (Kim et al., 2014). At the time of the review being published, only twenty-seven African countries had data on the prevalence of HEV infection, while outbreaks had been recorded in 12 African countries and sporadic cases in nine. They found that studies on the seroprevalence of HEV antibodies in Africa have been conducted since 1986 and have focused on various groups, including blood donors, pregnant women, pig handlers and patients with chronic liver disease. A common limitation with many of the studies on the prevalence of hepatitis E antibodies conducted in the 1990s and earlier is the diagnostic performance and validity of the old assays resulting in widely differing seroprevalences (Kmush et al., 2013; Bagulo et al., 2020). For example, serological studies conducted in South Africa in the 1990s found seroprevalences ranging from 2.1% to 10.7% (Grabow et al., 1994; Tucker et al., 1996; Kim et al., 2014) but a study conducted and

published in the Western Cape in 2016 found an HEV seroprevalence of 27.9% among 1 161 individuals and another study published in 2020 found an HEV seroprevalence of 42.8% among 250 blood donors in the Western Cape (Madden et al., 2016; Maponga et al., 2020). A recent study conducted in a region close to Pretoria found a low seroprevalence of anti-HEV immunoglobulin (Ig) G among pregnant women, with 12/384 (3.1%) of women testing positive for anti-HEV IgG (Simani et al., 2022). None of the women that were tested had a positive anti-HEV-IgM or detectable HEV RNA. The difference in seroprevalence between the Pretoria study and the Western Cape studies may be due to hepatitis E seroprevalence differing across regions, sample populations or time of sampling.

The studies conducted have used a variety of assays with varying performance characteristics (Kim et al., 2014; Bagulo et al., 2020). Detection of anti-HEV IgG indicates previous exposure to infection, while detection of anti-HEV IgM indicates an active or recent infection. In studies in Sub-Saharan Africa, anti-HEV IgG assays or total antibody assays were used, making it difficult to determine what proportion of a population was actively infected or previously exposed (Bagulo et al., 2020). An enzyme-linked immunosorbent assay (ELISA) to detect anti-HEV IgG was developed by Beijing Wantai Pharmacy Enterprise Co., Ltd. and is currently the most used test for HEV IgM (Kmush et al., 2015; Talapko et al., 2021). It uses a 211 amino acid section of recombinant ORF2 protein derived from HEV-1 in a solid-phase indirect ELISA to detect anti-HEV IgG (Kmush et al., 2015; Shrestha et al., 2016). The Wantai test is considered as having a high sensitivity and specificity compared to other Enzyme Immunoassays (EIAs) for HEV IgG but has not been established as a "gold standard" for HEV antibody testing (Kmush et al., 2015; Bagulo et al., 2020; Talapko et al., 2021). It was found to have a higher sensitivity than the Walter Reed Army Institute of Research (WRAIR) in-house assay which was developed and used by many studies in the early 2000s (Kmush et al., 2015). The WRAIR assay makes use of a recombinant protein from ORF2 (the capsid protein region), but it was truncated and expressed in a baculovirus system (Kmush et al., 2015). An Australian study found poor agreement between the Wantai kit and the MP Diagnostics: HEV IgG ELISA (Shrestha et al., 2016). The MP Diagnostics Kit makes use of 3 recombinant proteins: a 42 amino acid sequence from the ORF2 region of HEV-2, a 33 amino acid sequence from the ORF3 region from HEV-3 and an ORF3 sequence from HEV-1 (Shrestha et al., 2016).

Variability in anti-HEV antibody test sensitivity and specificity are known problems (Shrestha et al., 2016; Talapko et al., 2021). EIAs detecting HEV antigen (viral capsid protein produced from ORF2 or phosphoprotein produced from ORF3) detect HEV infection more directly, but many of these assays have low sensitivity and they have not been used as frequently as anti-HEV antibody tests (Talapko et al., 2021). Viral replication of HEV in the body may see a sharp decrease as the immune system counteracts the infection, this causes a drop in HEV RNA viral load and thus the concentration of antigens in the body may fade quickly. Antibodies such as IgM and IgG are reliably detected for a longer period after infection compared to antigens. Apart from antigens, HEV RNA can also be detected to confirm infection. In acute infection, HEV RNA is detectable in the blood for 2 weeks after symptoms manifest and in stool for 5 weeks after the onset of symptoms (Kamar et al., 2014). Nucleic acid testing methods include nested RT-PCR, real-time RT-PCR and loop-mediated isothermal amplification assay (Talapko et al., 2021).

HEV antibody seroprevalence studies have focused on unique sub-groups making it challenging to compare results across studies. It is also difficult to determine the impact of HEV and extrapolate results to a population level when sample groups may not be representative of a country's population. All these factors result in a small pool of studies with findings that are difficult to compare to each other. Even so, it is clear that HEV has a heavy impact across the continent and is likely endemic in more regions than is currently known.

A review published in 2013 covered the epidemiology of HEV in both African and Asian low- and middle-income countries (Kmush et al., 2013). The prevalence of anti-HEV in African nations ranged from 4.3% in Tunisia in 2008 to 84.3% among pregnant women in Egypt. The Egyptian study population consisted of 2 428 pregnant women and the serological assay used was a National Institute of Health (NIH) in-house EIA (Stoszek et al., 2006). The EIA used a capture antigen targeting a truncated HEV protein capsid translated from the ORF2 region of the HEV genome. The Tunisian study tested for the presence of HEV in a group of 1 505 individuals with a mean age of 21 years using an ELISA by GeneLabs Diagnostic (Rezig et al., 2008). These outcomes demonstrate that it is difficult to compare study results when different demographics are targeted. Egypt is also known to contain regions where HEV is endemic and displays an uncommon pattern of affecting young children to a greater extent (Kmush et al., 2013).

2.9. Different age-specific patterns of HEV observed in Africa

Two age-specific patterns of HEV infection are present in the global epidemiology of HEV and both patterns are present in Africa (Labrique et al,. 2010; Kmush et al., 2013). In one pattern, the disease attack rate is higher in teenagers and young adults (ages 15 to 40 years) compared to children (ages 0 to 15 years). Disease attack rate being the frequency at which new infections of a disease occur in an at-risk population during a defined period, such as an epidemic or outbreak (Centers for Disease Control [CDC], 2019). This age-specific pattern is observed in endemic African countries but also globally in non-endemic regions. One would expect a disease which is transmitted faecal-orally to be more common in children than adults as observed for hepatitis A, but many studies have reported increase in anti-HEV prevalence with age (Kmush et al., 2013; Iaconelli et al., 2017; Raji et al., 2022). The actual recorded seroprevalences are unexpected and the reason for the pattern has not been clearly explained.

There are various theories around the cause of young adults and adults being more frequently affected by HEV than children. One theory is that a higher incidence of HEV is observed in adults since they ingest more quantities of possibly contaminated food and water than children and have increased mobility with access to high-risk environments (Labrique et al., 2010). It was hypothesised that in some regions this may reflect the improvement of sanitation and infrastructure over time, resulting in less exposure to HEV-1 and HEV-2 in children (Labrique, et al., 2010). It is also suspected that age-specific immune responses play a role, for example HEV IgG may form in children and rapidly wane or immunity gained in early childhood could be lost before adolescence (Labrique, et al., 2010; Kmush et al., 2013). However, it is generally accepted that the presence of anti-HEV IgG can be detected for months to years after infection (Kamar et al., 2014; Kupke and Werner, 2021).

In the second pattern children have higher seroprevalence rates than adults. This pattern is uncommon but has been observed in Egypt and Sudan (Hyams et al., 1992; Fix et al., 2000; Mudawi, 2008; Kmush et al., 2013). It is suspected that hepatotropic co-infections with hepatitis B and C, which are common in Egypt, may increase HEV susceptibility at a young age, or that local factors such as viral subtype, infectious dose, and host genetic factors could influence the observed pattern (Zaki et al., 2008; Labrique et al., 2010; Kmush et al., 2013). Although it should be noted that the use of in-house diagnostic tests, and the known variability in anti-HEV antibody test sensitivity and specificity may have skewed results, causing this pattern to be observed.

2.10. Zoonotic reservoirs of HEV in Africa

Most studies on HEV in Africa have focused on human cases while making use of already limited resources. Zoonotic reservoirs of the disease have not been well-studied in Africa even though they are important due to the genetic variability and cross-species transmission potential of HEV (Primadharsini et al., 2021). A systematic review of HEV infection of animals in Africa found that viral prevalence or seroprevalence has been detected in pigs, dromedaries, cows, goats, sheep, donkeys, and rats (Modiyinji et al., 2021). Of all the animals covered in the review, pigs had the highest anti-HEV IgG seroprevalence of 35.1% while dromedaries had a seroprevalence of 14.8%. It is known that the consumption of undercooked pork meat products from HEV-infected animals can lead to infection with HEV-3 and HEV-4, although these genotypes are linked to sporadic cases of the disease which occur in developed countries. In Africa, HEV-3 has been detected in pigs in Cameroon, Burkina Faso, Democratic Republic of Congo, Madagascar, São Tomé, and Principe (Kaba et al., 2010; Temmam et al., 2013; de Paula et al., 2013; Traoré et al., 2015; Mesquita et al., 2019; Modiyinji et al., 2021).

In South Africa, HEV-3 RNA was detected in pork liver spread from supermarkets in Cape Town and in swine faecal samples obtained from the Eastern Cape province (Adelabu et al., 2017; Korsman et al., 2019). A large proportion of swine samples from a Cape Town abattoir was found to be either HEV IgM or IgG positive (Van Helden et al., 2017). They found 61% (575/947) swine serum samples to be seropositive for total antibodies to HEV while viral RNA was detected in 4 out of 45 serum samples. The high seroprevalence of HEV in animals recorded so far indicates that further study is needed into zoonotic transmission of HEV in Africa.

There has been a case of zoonotic infection of a patient in the United Arab Emirates (UAE) with HEV-7 which infects dromedaries (and is another genotype of HEV belonging to the species *Paslahepevirus balayani*) due to the regular consumption of camel milk and meat (Lee et al., 2016). This case demonstrated that HEV-7 can cross species barriers and infect humans. One study focused on detecting HEV in camels in North and East Africa, UAE, and Pakistan detected HEV-7 in Somalia, Sudan, Egypt, and Kenya (Rasche et al., 2016). Another study conducted in Nigeria detected an HEV antibody seroprevalence in 30.7% of 88 camels (Adamu et al., 2022).

Individuals who are in close contact with swine and camels such as butchers, farmers, pastoralists, forest workers and veterinarians should be made aware of the risk of contracting this disease and proper infection control mechanisms should be in place. HEV is genetically diverse and can evolve enabling it to transmit across different species. Understanding the mutations in strains and subtypes circulating across the continent is crucial in HEV surveillance efforts (Primadharsini et al., 2021).

2.11. The limits of serology and the known molecular epidemiology of HEV in Africa

The four HEV genotypes which commonly infect humans share one serotype which allows for straightforward serology-based testing to confirm outbreaks and sporadic cases, but this type of testing does have limitations. Serological tests do not determine the genotype of the virus which is causing infection, and these genotypes differ in routes of transmission, potential for causing chronic disease and in the severity of disease they cause (Primadharsini et al., 2019). Chronic HEV infections have been observed in patients infected with HEV-3, and genotypes HEV-1 and HEV-2 cause excess mortality in pregnancy compared to HEV-3 and HEV-4 (Kamar et al., 2014).

Molecular methods such as reverse transcriptase polymerase chain reaction (RT-PCR) and sequencing can provide more information on the molecular epidemiology of HEV in Africa. A review of HEV studies in sub-Saharan Africa found that molecular methods were not applied routinely and when implemented, mostly targeted the ORF1 and ORF2 regions of the HEV genome (Bagulo et al., 2020). Data on prevalent genotypes is only available for a few African countries and based on a review conducted in 2014, it appeared that HEV-1, HEV-2, and HEV-3 are present in Africa, with HEV-1 detected most frequently while HEV-2 and HEV-3 were detected at similar rates (Kim et al., 2014). The most prevalent genotype, HEV-1, has been detected in human faecal samples from outbreaks in Central African Republic (CAR), Namibia, Sudan and from Egypt (Divizia et al., 1999; He et al., 2000; van Cuyck et al., 2003; Nicand et al., 2005; Escribà et al., 2008). HEV-2 has been detected in human faecal samples from HEV outbreaks in CAR and Namibia and in a patient faecal sample in Chad (van Cuyck et al., 2003; Maila et al., 2004; Escribà et al., 2008). HEV-3 has been detected in patient serum and faecal samples in Nigeria, a faecal sample from a 9-year-old patient in Egypt, and in pig liver samples in Madagascar (Buisson et al., 2000; Kamel et al., 2011; Temmam et al., 2013).

2.12. Wastewater-based epidemiology and its application to HEV

Molecular testing has a clear benefit in addressing the knowledge gap regarding HEV in Africa, but the application of molecular methods to clinical samples is not the only means of gaining more information on the epidemiology of HEV in communities. Due to the emerging number of indigenous cases of HEV in developed nations, several countries have implemented wastewater surveillance systems and studies have applied WBE to HEV to observe the circulation, frequency of detection, and molecular epidemiology of the virus. Wastewater surveillance is the systematic and continuous testing of wastewater to gain insight into a chosen microbe or analyte to the benefit of public health while WBE describes the scientific field which links the levels of pathogens, microbes, chemicals, or analytes found in wastewater to public health on a population

level (Larsen et al., 2021). Most studies test influent wastewater samples which are raw wastewater samples that have not been processed while effluent wastewater samples have undergone processing at a wastewater treatment plant.

The detection of pathogens in wastewater has been performed for centuries. An early example of pathogens present in water was the application of germ theory by John Snow in 1854. He determined that water pumps in Broad Street, London were contaminated with cholera and that this was causing the observed epidemic (Cameron and Jones, 1983; Kilaru et al., 2021). In 1939 poliovirus was detected in wastewater and the WHO later published official guidelines for the detection of poliovirus in wastewater to supplement acute flaccid paralysis surveillance (Paul, Trask and Gard, 1940; WHO, 2003). Viruses such as rotavirus, coxsackie viruses, and hepatitis A have been detected using environmental surveillance. Bacteria such as *Salmonella enterica* serotype typhi, *Escherichia coli*, and *Campylobacter jejuni*, have also been detected (Chahal et al., 2016; Kilaru et al., 2021). Researchers are increasingly employing WBE to detect antimicrobial resistance genes (Dai et al., 2022; Singer et al., 2023).

In 2008, the term sewage epidemiology was used to describe surveillance for residues of illicit drugs in wastewater (Zuccato et al., 2008). In 2014, the term WBE took the place of sewage-based epidemiology and its usage to describe the detection of markers in wastewater became more frequent (Larsen et al., 2021). Several phrases have been used to describe the detection of markers in sewage or the environment (Larsen et al., 2021; Singer et al., 2023). The term "surveillance" indicates an ongoing analysis of wastewater samples at a set frequency, with the purpose of public health action (Larsen et al., 2021). "Environmental surveillance" is considered a broader term which includes wastewater surveillance but also includes studies on the air, soil, and high-touch surfaces (Kilaru et al., 2021; Larsen et al., 2021). Most recently, WBE and wastewater surveillance became topics of interest as they were applied during the COVID-19 pandemic. Where information was needed on a large population scale and healthcare systems were already overloaded, wastewater surveillance was a relatively cost-effective alternative method that could give insight into the spread of the virus, function as an early warning system, track the circulation of variants of concern, detect mutations, and provide information beyond hospital admissions during lockdown (Johnson et al., 2022; Singer et al., 2023).

A German study found 84% of the 111 influent samples and 31% of the 83 effluent samples from an urban wastewater treatment plant to be HEV RNA positive (Beyer et al., 2020). The HEV positive influent samples had a median concentration of $3x10^3$ HEV copies/100 mL while the HEV positive effluent samples had a lower median concentration of $1x10^3$ HEV copies/ 100 mL. Seasonality based on rainfall events was detected with an increased viral load being present in river surface water during heavy rainfall events, although viral load levels did not differ between summer and winter seasons. Subtypes HEV-3c and HEV-3f were found in sequenced samples, with HEV-3c being most prevalent, which correlated with the subtypes detected in HEV patients in the area.

An Italian study which surveyed urban wastewater samples from 2011 to 2019 detected HEV in 5.4% out of 1374 raw sewage samples from 20 regions across the country (Iaconelli et al., 2020). HEV was detected at a significantly higher rate in Central Italy (7.1% of samples were HEV RNA positive) compared to detection across all regions (5.4%). The predominant genotype in the country was determined to be HEV-3.

In a study conducted in Scotland, 15 influent wastewater samples were tested for HEV RNA and 14 were found to be positive (Smith et al., 2016). Only HEV-3 was detected in the samples despite HEV-1 being reported in 11% of Scottish and 30 % of English patients. It was noted that despite the number of HEV-1 cases reported, a great proportion of the Scottish population may be asymptomatically infected with HEV-3 leading to mainly HEV-3 being represented in wastewater samples.

Although these studies used different methods a generally similar workflow has been employed. This workflow consists of sample collection, RNA extraction, RNA concentration, HEV detection, HEV quantification, sequencing, phylogenetic analysis, and statistical analysis. HEV RNA-containing samples were identified using real-time PCR and sequencing was applied to determine the genotypes present in samples. Where quantitative RT-PCR is used, the viral concentration of HEV present in samples can be determined (Masclaux et al., 2013; Iaconelli et al., 2017; Beyer et al., 2020). In terms of sequencing, fragments of either the ORF1 or ORF2 region are commonly amplified (Smith et al., 2016; Iaconelli et al., 2017; Beyer et al., 2020). Use of the ORF2 region allows for the viral genotype and subtype to be determined (Iaconelli et al., 2017). WBE allows for HEV circulation patterns and genotype prevalence to be determined in various communities in a non-intrusive way. Wastewater surveillance has been used in South Africa, although in recent years HEV has not been a focus.

2.13. The application of WBE to HEV in Africa

Very few African nations have applied WBE to HEV, they include Cameroon, Tunisia, and Egypt. Two studies were conducted in Egypt in 1998 and 2000 where wastewater samples and workers in wastewater treatment plants were tested for HEV. In the study published in 2000, HEV RNA was not detected in 24 influent and effluent wastewater samples while in the 1998 study HEV RNA was detected in influent wastewater samples (El-Esnawy et al., 1998; El-Esnawy, 2000). Further details on African studies which applied WBE to HEV can be seen in Table 2.1. In the Cameroonian study listed in Table 2.1, they were able to obtain one sequence which clustered with HEV-3 subtype 3a. HEV-3 was considered uncommon in Africa, however these recent studies published have detected HEV-3 in Northern, and Sub-Saharan Africa. In humans, HEV-3 has been associated with both acute and chronic cases of hepatitis E in South Africa (Andersson et al., 2013; Andersson et al., 2015; Madden et al., 2016).

Table 2.1: Details of African studies which aimed to detect HEV RNA in wastewater

Country	Influent Samples	Effluent/Treated Samples	Detection Method	Positive Rate	Genotype	Reference
Egypt	18	18	RT-PCR	27.78%(10)	n/a	El-Esnawy et al., 1998
Egypt	12	12	Nested RT-PCR	0%	n/a	El-Esnawy et al., 2000
Egypt	38	38	RT-PCR and real-time RT-PCR	0%	n/a	Kamel et al., 2011
Tunisia	79	71	Nested RT-PCR	2% (3)	HEV-1, HEV-3	Béji-Hamza et al., 2015
Cameroon	157	0	Real-time RT-PCR	1.9% (3)	HEV-3	Fatawou et al., 2022

In 2004, a study conducted in the Gauteng province of South Africa applied WBE techniques to HEV (Williams, 2004). The study involved testing domestic sewage samples and swine faeces for HEV RNA and determining the HEV genotypes present. The sewage samples were collected from May to October 2003 from the East Rand Water Board. They made use of a two-step RT- PCR for HEV detection. Amplified HEV RNA was purified and sequenced, allowing for genotyping. The strains obtained from domestic sewage samples clustered with HEV-1 sequences from India, Nepal and Burma while swine strains clustered with HEV-3 sequences from the United States of America (USA) and Japan. They found HEV RNA in 9/199 (4.5%) wastewater samples of which 2 (1%) were confirmed using Sanger sequencing.

WBE techniques have developed since 2004 and the field has been especially relevant recently due to its utility in SARS-CoV-2 surveillance. It would be prudent to apply the improved techniques to HEV and build upon the wastewater surveillance infrastructure already in use in South Africa, and the Western Cape specifically, to address the gap in knowledge surrounding the molecular epidemiology of HEV in the country. The clinical suspicion for hepatitis E infection in South Africa is generally low and diagnostic testing is infrequent despite the high seroprevalence. WBE may provide a good indication of the burden/threat of HEV on public health.

A One Health approach is necessary in understanding the threat of HEV because of the potential for zoonotic transmission. Within other countries, different animals, apart from pigs, have been shown to harbour HEV. In addition to zoonotic transmission following consumption of contaminated pork products (animal health), there is the additional risk that faecal material from piggeries can end up in rivers and dams (environmental health) from which rural communities obtain untreated water for household consumption thereby increasing the risk of acquiring infection (human health). WBE can potentially be used to understand and mitigate against this risk.

2.14. The application of wastewater surveillance to SARS-CoV-2 in South Africa

The SAMRC put the SARS-CoV-2 wastewater surveillance dashboard in place to monitor the concentration of SARS-CoV-2 RNA in wastewater in different regions and provinces in the country (Street et al., 2020, 2021). The first report of a COVID-19 case in South Africa was in March 2020, although the WHO had already declared Coronavirus as a Public Health Emergency of Global Concern (Department of Health, 2020a; Department of Health, 2020b). The SAMRC had sampling pilot studies testing sewage for SARS-CoV-2 RNA using real-time PCR underway in October 2020 and the wastewater surveillance dashboard was officially launched in the first week of June in 2021 (Schütz, 2020; Bhengu, 2021). At the time it was launched, wastewater from the Eastern Cape, Gauteng, Limpopo, and the Western Cape were being tested for SARS-CoV-2 RNA. The dashboard is an interactive tool which is available and online and can be accessed by the public. The dashboard is available at: https://www.samrc.ac.za/wbe/index.html .

This was done after considering the factors limiting SARS-CoV-2 surveillance in South Africa which included the state of health systems infrastructure, operational issues, lack of access to personal protective equipment and tests as well as asymptomatic cases which were underreported. Increasing concentrations of SARS-CoV-2 RNA in wastewater correlated and preceded increased confirmed cases of SARS-CoV-2 infections in the Western Cape (Johnson et al., 2021). WBE methods have been applied to other enteric viruses such as polio, norovirus, calicivirus and hepatitis A (Street et al., 2021). The methodology is a non-invasive way to obtain samples which represent a large population, at a relatively low cost compared to clinical studies (Singer et al., 2023). It can be applied to a broad range of pathogens whether they are transmitted faecal-orally or not, provided that the appropriate marker is determined.

2.15. Problem Statement

Despite the limited volume and quality of data available, the burden of hepatitis E infection in Africa is a relevant public health threat. It is likely that the number of HEV-infected individuals and deaths caused by HEV is currently underestimated due to the asymptomatic course of the disease, limited access to reliable serological tests and overall lack of resources to study the virus. The molecular epidemiology of the disease in Africa is also unclear in regions where few or no studies have taken place. Based on the existing knowledge gaps and resource limitations, the potential value of wastewater surveillance and its application to HEV is evident. In the African context, molecular data on HEV is necessary and wastewater surveillance can include real-time PCR and sequencing to gain molecular information without the collection of clinical samples. In the South African context, the SARS-CoV-2 wastewater surveillance is already in place which shows the potential to apply WBE to HEV and other enteric viruses. Overall, HEV is a global emerging public health issue which causes significant morbidity and mortality in Africa and more effort and resources should be invested on its prevention, treatment, diagnosis and understanding its molecular epidemiology across the continent.

2.16. Aims and objectives

This study aimed to determine whether HEV RNA was present in untreated wastewater in the Western Cape province of South Africa. The secondary aims of this study were to determine the genotypes of detected HEV RNA and to detect any trends in HEV circulation from January to November 2021 in four selected collection locations in the Western Cape.

The specific objectives of the study were as follows:

- To test processed wastewater samples for the presence of HEV using real-time RT-PCR;
- To sequence regions of the HEV genome from HEV RNA positive samples using Sanger sequencing;
- To phylogenetically analyse and determine the genotype of sequenced samples;
- To statistically analyse the proportion of HEV RNA positive samples from each collection location to detect temporal or geographical trends.

Chapter 3: Materials and Methods

3.1. Ethics

This study was approved by the Research Ethics Committee: Biological and Environmental Safety (REC:BES) of Stellenbosch University (SU), reference number: BEE-2022-24569. The ethics approval form is attached as Addendum A.

3.2. Study Design and Sampling

3.2.1. Study Design

The research project was a retrospective analytical study.

In November of 2020, the SAMRC launched the SARS-CoV-2 Wastewater Surveillance Dashboard (Mlambo, 2022). Since the launch of the dashboard, RNA has been extracted from various wastewater treatment works (WWTWs) in South Africa and tested for SARS-CoV-2. The increasing or decreasing SARS-CoV-2 RNA levels in wastewater were used as an early indicator of COVID-19 case trends. This allowed the dashboard to function as an early warning system for SARS-CoV-2. Monitoring of the wastewater gave a non-invasive snapshot into the presence of SARS-CoV-2 within communities across the country. These RNA extracts have been stored at -80°C and a selected number of extracts from 2021 sourced from Athlone WWTW, Zandvliet WWTW, Meerhoff student residence and Metanoia student residence sewage were tested for the presence of HEV RNA in this project.

3.2.2. Wastewater sample collection and RNA extraction

Influent wastewater extracts chosen for analysis were collected from two WWTWs in the Cape Town Metropole and manholes from sewer manholes from two SU residences (one in Stellenbosch and one in Tygerberg) from various time periods over 11 months as shown in Table 3.1, below. From the Athlone and Zandvliet WWTWs, 39 and 37 samples respectively were collected from January to September 2021. Zandvliet WWTW serves around 755 000 people while Athlone WWTW serves around 385 000 people (Johnson et al., 2021). From the Meerhoff residence on the Tygerberg campus, 31 samples were collected from May to September 2021. The residence has a capacity of 225 students (Meerhoff Residence, 2023). From the Metanoia residence on the Stellenbosch campus, 36 samples were collected from May to November 2021. The Metanoia residence has a capacity of 501 students. In total, 143 samples were tested in this study (Metanoia Residence, 2023). The locations and catchment areas of the WWTWs are shown in Figure 3.1. The geographical location of all 4 collection sites is shown in Figure 3.2.

Due to the various alert level restrictions in place in South Africa during the COVID-19 pandemic, the occupation of the residences varied throughout the year. The country was on adjusted alert level 1 in May 2021 (South African Government, 2021). The country was on adjusted alert levels 3 and 4 from June 2021 to 12 September 2021. For the remainder of September, the country was on adjusted alert level 2 and from October 2021 onwards it was on adjusted alert level 1. Residence students may have elected to live and work from home due to the ongoing pandemic and access to online learning facilities. The recess in June would also have

seen a lower percentage of students present, the same can be said after November at the end of the academic year. Overall, it should be noted that it is unlikely that the residences were occupied to capacity, and it is expected that fewer students occupied the residence in June and November compared to the rest of the year.

Table 3.1: Sample details for the wastewater extracts collected in 2021

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov
Athlone WWTW	39 samples										
Zandvliet WWTW	37 samples										
Meerhoff					31 samples						
Metanoia					36 samples						

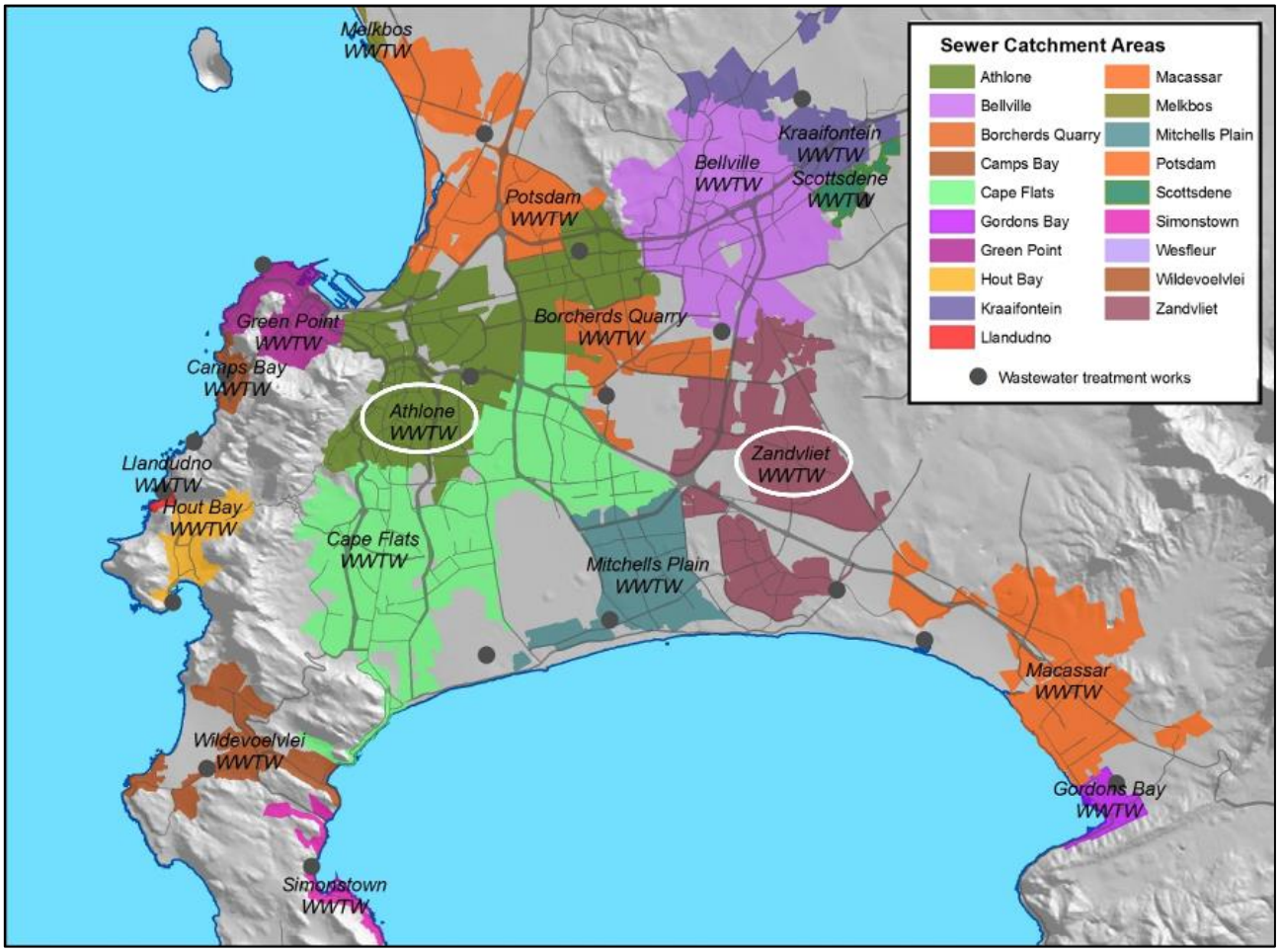

Figure 3.1: A map showing the locations and catchment areas of WWTWs within the Cape Peninsula. Athlone WWTW and Zandvliet WWTW are circled in white.
(City of Cape Town, Media Office, 2018)

Samples from the WWTWs were collected by grab sampling of raw, untreated wastewater on Monday mornings by trained wastewater samplers (SAMRC, 2020). The sampling teams included staff and students from the Division of Medical Virology working in collaboration with the SAMRC. The samples were transported under refrigerated conditions to the SAMRC laboratory shortly after collection. Student residence samples were taken using passive samplers lowered into manholes using nylon string. The samplers were 3D printed passive sample devices based on a freely available design (Schang et al., 2021; Mangwana et al., 2022). They were filled with sterile medical gauze to capture wastewater and solid matter (Mangwana et al., 2022). The sample devices were placed in manholes for 21 hours each, samples were taken twice a week (Mangwana et al., 2022).

Figure 3.2: A map showing the locations and catchment areas of wastewater treatments works and two university residences which were the collection sites in this study

3.2.3. Extraction of nucleic acid from wastewater samples using the RNeasy PowerSoil Kit

RNA was extracted from influent wastewater samples using the RNeasy PowerSoil Kit (QIAGEN) at the SAMRC by staff and students as part of the wastewater surveillance activities. The extraction method was described by Johnson et al. (2021) and is a modification of the protocol published by Peccia et al. (2020). The RNeasy PowerSoil Kit (QIAGEN) is used to extract nucleic acid from soil or environmental samples, and sufficiently remove the contaminants present in soil that would inhibit downstream PCR applications.

3.2.3.1. Principle of the RNeasy PowerSoil Kit

The first stage of the protocol uses organic phenol-chloroform extraction to lyse cells, remove contaminants and acquire an aqueous phase containing both deoxyribonucleic acid (DNA) and RNA. The second stage makes use of anion-exchange gravity flow columns.

In the organic extraction phase, the pellet is added to PowerBead tubes containing PowerBead solution and silica carbide beads. The PowerBead solution includes including sodium dodecyl sulphate and Solution SR1 which disrupt cell membranes while the beads mechanically shear cells. The tubes are vortexed which releases nucleic acid and cell debris from the pellet. Solution IRS precipitates deoxyribonucleases & ribonucleases in the tube, neutralizing them to preserve nucleic acid integrity. Phenol-chloroform is then added to the PowerBead tube. The phenol-chloroform mixture is immiscible in water. Vortexing the tube homogenizes the lysed cell contents with the phenol-chloroform, and centrifugation separates the nucleic acid and contaminants (protein, lipids, cell debris) into an upper aqueous phase and lower solvent phase (McKiernan and Danielson, 2017). The aqueous phase containing total nucleic acid is less dense than the phenol-chloroform mixture which has trapped proteins, lipids, and other cell debris. The aqueous phase is separated into a clean tube and incubated with isopropanol. This causes the nucleic acid to precipitate. The tube is then centrifuged, this draws the nucleic acid pellet to the bottom of the tube, the supernatant fluid (SNF) does not contain nucleic acid and is discarded.

The resultant resuspended pellet from the organic extraction phase will then undergo further purification and concentration via anion-exchange gravity flow columns. This is where the final wash and elution steps occur. The precipitated total nucleic acid is bound to the column matrix due to their opposite polarity. While bound, it is washed with a salt solution to remove any remaining unbound contaminants. The column is then washed with a different salt solution, causing the polarity of the column to change and preferentially release RNA rather than DNA from the column. This RNA is then precipitated using 100% isopropanol and finally suspended in nuclease-free water.

3.2.3.2. Extraction of RNA from wastewater samples using the RNeasy PowerSoil Kit

Briefly, between 50 to 100 mL of influent wastewater sample was centrifuged at 2500 x *g* for 20 minutes, yielding SNF and a pellet. Between 45 and 48 mL of SNF was discarded, and 2-5 mL of the remaining pellet were added to a 15 mL PowerBead tube. PowerBead solution, Solution SR1, and Solution IRS were then added to the tube. An equal volume of phenol/chloroform was used to separate the mixture into layers.

The upper aqueous layer was then transferred to a clean 15 mL collection tube. Solution SR3 was added to the tube and the solution was vortexed and centrifuged. The mixture was transferred to a new 15 mL collection tube, where solution SR4 was added and incubated for 30 minutes. The tube was centrifuged at 2500 x *g* for 30 minutes. The SNF was discarded, and the collection tube was inverted and placed on a paper towel for 5 minutes. Solution SR5 was added to the tube and the pellet in the tube was resuspended.

A JetStar Mini Column was prepared and the RNA isolation sample in the collection tube was added to it. The Jet Star Mini Column was transferred to a new tube, solution SR6 was added and allowed to gravity flow into the collection tube. The RNA was eluted from the JetStar Mini Column and combined with solution SR4. The RNA was incubated with solution SR4 at -20°C for 10 minutes before centrifugation at 15 000 x *g* for 15 minutes. The pelleted RNA was resuspended in 50 to 100 µL of Solution SR7. Following SARS-CoV-2 RNA testing at the SAMRC, the nucleic acid extracts were stored at -80°C.

Volumes of RNA extracts for this study were limited due to use in multiple studies, hence the nucleic acid was diluted 4X in nuclease-free water for HEV RNA testing. For the wastewater samples tested in this project, 5 µL of the resuspended RNA was combined with 15 µL of nuclease-free water in clean, labelled 1.5 mL Eppendorf tubes. Where extracts tested positive for HEV using a real-time qPCR assay, further samples of nucleic acid were requested for sequencing purposes. Where additional sample volume was available, one more aliquot of 20 µL was taken in the same dilution described above.

3.3. Real-time PCR (detection of HEV in wastewater extracts)

3.3.1. Determining the limit of detection of the real-time PCR assay

The limit of detection of an assay is defined as "the lowest analyte concentration likely to be reasonably distinguished from the limit of blank and at which detection is feasible" (Armbruster and Pry, 2008). In the context of this project, the limit of detection of the real-time PCR assay would be the lowest concentration of HEV RNA expressed in IU/mL that is detected by the assay, producing a valid positive result. The 1st WHO International Standard for HEV RNA nucleic acid amplification techniques (NAT)-based assays (PEI code 6329/10) obtained from the Paul Erlich Institute (Germany) was used to determine the limit of detection from wastewater samples. The vial containing lyophilised standard was reconstituted with 500 µL of nuclease free water for an effective concentration of 125 000 IU/mL of HEV RNA. A 10-fold dilution series ranging from a concentration of 125 000 IU/mL to 12.5 IU/mL was prepared from the reconstituted standard before extraction. Another set of the dilution series was prepared and not extracted, for use in the spiking experiment.

To determine the limit of detection of the real-time PCR assay used in this project, the HEV standard dilution series was used to spike wastewater samples which were then extracted as routinely done by the SAMRC laboratory and underwent real-time PCR HEV RNA detection as detailed in Section 3.2. Klipheuwel WWTW is located in Cape Town, north of the 4 collection sites. It is roughly 33.1_km from Athlone WWTW, 39.9 km from Zandvliet WWTW, 31.3 km from Metanoia Residence in Stellenbosch and 25.2 km from Meerhoff Residence in Tygerberg.

Briefly, a wastewater sample collected from Klipheuwel WWTW on 27 March 2023 was split into 4 aliquots of 50 mL each. One of the aliquots was left unspiked while three aliquots were spiked with 200 µL of a dilution series of HEV Standard. This resulted in a dilution series consisting of 10 IU/mL, 1 IU/mL, and 0.1 IU/mL of HEV RNA standard. The aliquots then underwent extraction (as described in section 3.2.3) and real-time PCR analysis using the QuantiFast Pathogen RT-PCR and IC Kit (QIAGEN) described in section 3.3.2. The assay was able to detect HEV RNA in the 10 IU/mL sample and the 1 IU/mL sample. HEV RNA was not detected in the 0.1 IU/mL sample. The amplification curve is shown in Figure 3.3 and the Ct value results are shown in Table 3.2. No HEV RNA was detected in the wastewater extract which was not spiked. The limit of detection was set to 10 IU/mL since this was the concentration that could be consistently detected.

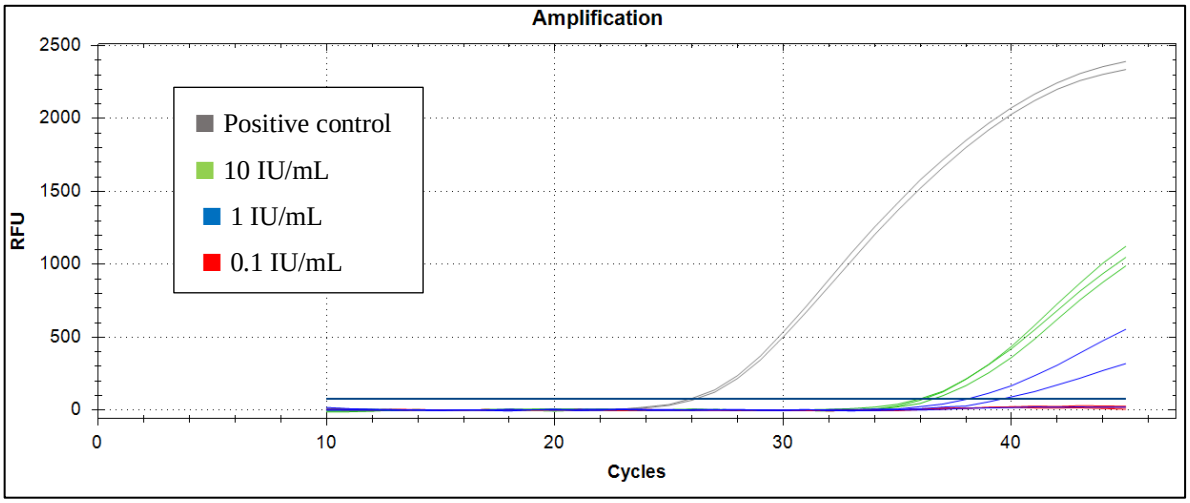

Figure 3.3: Amplification curve showing real-time RT-PCR assay detection in spiked wastewater extracts

Table 3.2: Ct values results of the spiking experiment used to determine the limit of detection, corresponding to Figure 3.3

Specimen	HEV RNA (Ct)	IC (Ct)
10 IU/mL	36.54	33.13
10 IU/mL	36.77	33.32
1 IU/mL	n/a	33.89
1 IU/mL	38.93	33.60
0.1 IU/mL	n/a	33.87
0.1 IU/mL	n/a	33.54
Unspiked	n/a	33.28
Unspiked	n/a	33.34

3.3.2. Detection of HEV RNA in wastewater extracts

One hundred and forty-three wastewater extracts were tested using a modified protocol of a previously published method (Jothikumar et al., 2006) and an HEV probe modified by (Garson et al., 2012). The modified probe had the addition of a minor groove binding modification to increase sensitivity. In this study, the QuantiFast Pathogen RT-PCR and IC Kit (QIAGEN) was used. The region targeted by the primers and probe was 5261-5330 of the HEV genome, using the reference sequence with GenBank accession number: M73218 (Tam et al., 1991; Jothikumar et al., 2006).

Real-time PCR combines the amplification reaction of conventional PCR with fluorescence detection, allowing the relative quantity of amplified product to be detected in real-time (Engstrom-Melnyk et al., 2015). The reaction makes use of probes and primers which target a specific DNA sequence. As the reaction progresses, replication of the target sequence occurs, and the number of fluorescent probes increases. Once this fluorescent signal exceeds a threshold of background noise, it is detected by the fluorescence detection module of a specialised real-time thermocycler. The cycle number at which this detection occurs is called the Ct (Stephenson, 2010). Samples containing a higher concentration of the target sequence will cross this threshold sooner, having a lower Ct, while samples with a low concentration of the target sequence cross later, having a higher Ct. HEV is an RNA virus and thus a reverse transcription step is required to generate complementary DNA (cDNA) before amplification. In this kit, a reverse transcriptase (RT) mix was included.

Different fluorescent molecules, or fluorophores, used in real-time reactions have differing emission spectra, meaning they emit light at different wavelengths. A multiplex real-time PCR makes use of two or more fluorophores with emission spectra that do not overlap to amplify multiple target sequences. Each target sequence will have a specific probe and primers. In this project, TaqMan probes were used. The TaqMan probe consists of an oligonucleotide that is complementary to the target sequence, a report dye, and a quencher (Stephenson, 2010). The reporter dye used in this study was fluorescein amidites (FAM). The quencher prevents the fluorescent signal of the reporter dye from being detected when the two are close together. During replication, the polymerase enzyme will partially cleave the TaqMan probe from the target sequence it has hybridised with, releasing the reporter dye. The reporter dye will then fluoresce, which is detected by the thermal cycler.

The real-time HEV assay primers used had been tested on an environmental sample of surface water previously and proven effective (Jothikumar et al., 2006). However, the extraction method differed to the kit used in this study and we used the HEV probe with a minor groove binder (MGB) (Garson et al., 2012).

The QuantiFast Pathogen RT-PCR and IC Kit (QIAGEN) included internal control (IC) RNA and an IC assay which made use of the VIC fluorophore. The amplification of the IC served as confirmation that the PCR was successful, and that negative results of HEV RNA in a sample were due to the absence of HEV RNA and not failure of the PCR. In cases where the ICs did not amplify, the reaction result was considered invalid. In the initial protocol, the IC assay and RNA were combined with the kit assay and template RNA as a multiplex reaction, the reaction composition is shown in Table 3.3, below. When both the IC and HEV amplified, the

result was considered positive. When the IC amplified but HEV did not, the result was considered negative. When the IC did not amplify, the result was considered invalid whether HEV amplified or not. Due to a high number of invalid results using the initial protocol, the IC and HEV reactions were separated into two wells as depicted in Table 3.4, below. One well would contain the IC assay (IC probe and primer mix), IC RNA and extracted RNA; the second well would contain the HEV assay (HEV probe and primer mix) and extracted RNA. Separating the reactions removed the competition for master mix components (RT, DNA polymerase and deoxynucleoside triphosphates (dNTPs)), between the two assays thus removing interference between them which had led to reduced amplification. This adjustment led to a reduced number of invalid results and was applied to the remainder of the samples.

Table 3.3: QuantiFast Pathogen PCR and IC Kit reaction composition with combined assays

Reagent	Concentration Stock	Concentration Final	µL per 1 reaction
QuantiFast Master mix	5x	1x	2.50
RT mix	100x	1x	0.13
HEV forward primer	20 uM	300 nM	0.19
HEV2 reverse primer	20 uM	300 nM	0.19
HEV probe	10 uM	250 nM	0.315
IC Assay	10x	1x	1.25
IC RNA	10x	1x	1.25
Template	Diverse	Diverse	2.50
Nuclease-Free Water			4.19
		Total volume:	12.50

Table 3.4: QuantiFast Pathogen PCR and IC Kit reaction composition with separated HEV and IC assays

Reagent	Concentration Stock	Concentration Final	µL per 1 reaction (HEV assay)	µL per 1 reaction (IC assay)
QuantiFast Master mix	5x	1x	2.50	2.50
RT mix	100x	1x	0.125	0.125
HEV forward primer	20 uM	300 nM	0.19	-
HEV2 reverse primer	20 uM	300 nM	0.19	-
HEV probe	10 uM	250 nM	0.315	-
IC Assay	10x	1x	-	1.25
IC RNA	10x	1x	-	1.25
Template	Diverse	Diverse	2.50	2.50
Nuclease-Free Water			6.805	7.375
		Total volume:	12.50	12.50

A non-template control where the template was replaced by nuclease-free water was included in every run. The region targeted by the primers and probe is indicated in Tables 3.5 and 3.6.

Table 3.5: Primer details for the HEV-specific primers used in real-time PCR

Primer	Sequence	Binding Sites
HEV Primer 1A (forward)	5'-GGT GGT TTC TGG GGT GAC-3'	5261-5278
HEV Primer 2A (reverse)	5'-AGG GGT TGG TTG GAT GAA-3'	5284-5301

Table 3.6: Probe details for the HEV-specific probe used in real-time PCR

Probe	Sequence	Binding Sites
HEVPRobeJHEVPh	6-FAM-TGA TTC TCA GCC CTT CGC- MGB	5313-5330

The reaction conditions are detailed in Table 3.7. These conditions are based on the manufacturer's instructions and the method is established in the National Health Laboratory Service (NHLS) Tygerberg Medical Virology laboratory where it is used in routine clinical testing for HEV.

Table 3.7: Real-time PCR Cycling Conditions

Stage	Temperature	Time	Number of Cycles
Reverse-transcription	50°C	20 minutes	1
Initial PCR activation	95°C	5 minutes	1
Denaturation	95°C	15 seconds	44
Annealing/extension	60°C	1 minute	

3.4. Statistical Analysis

The proportion of positive samples from each location was calculated and represented in graph form. The positive results as a proportion of total samples tested from each month were calculated and represented as a stacked bar graph. Calculations and generation of graphics were done in Excel Version 2304 (Build 16.0.16327.20200).

3.5. Nested RT- PCR

Sanger sequencing was attempted on all samples that tested positive for HEV RNA on real-time RT-PCR, regardless of their Ct value. Nested PCR reactions were performed following previously described methods with some modifications (Zhai et al., 2006; Blackard et al., 2009).

3.5.1. Pre-nested and nested reaction details

A 286 bp region of ORF1, a 126 bp region of ORF2/3 and a 347 bp region of ORF2 were targeted. The genome regions targeted by all inner primers are illustrated in Figure 3.4 below. The nested primers used to target the ORF1 and ORF2/3 regions were developed by Blackard et al. (2009). Primer details, minimum and maximum melting temperature (Tm) and guanine-cytosine (GC) content are shown in Table 3.8. Nested PCR reactions targeting a 347 bp region of the ORF2 region were done using primers developed by Zhai et al. (2006), primer details are shown in Table 3.9.

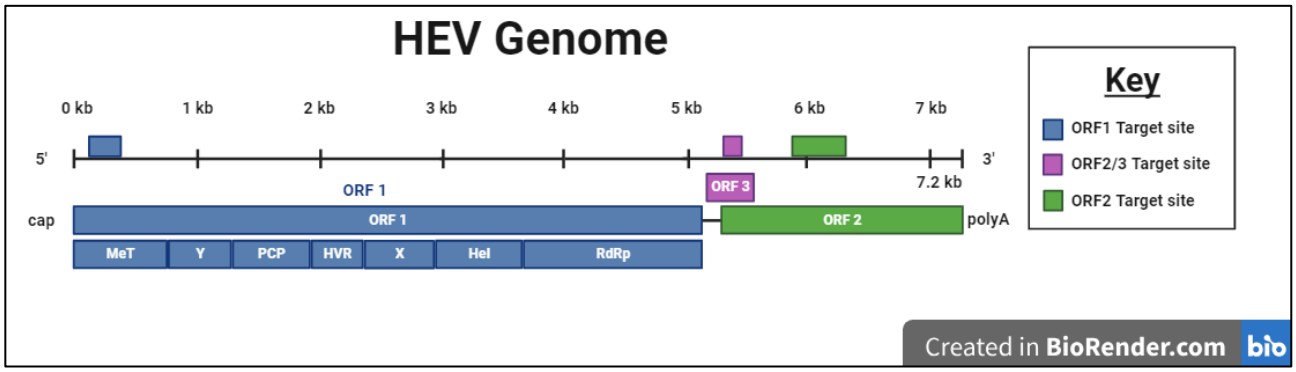

Figure 3.4: A schematic of the HEV genome with the regions targeted in nested RT- PCR (Created in BioRender)

Table 3.8: Nested RT-PCR primer details for ORF1 and ORF2/3

Name	Sequence	Binding sites	Tm min (°C)	Tm max (°C)	GC content (%)
Outer Primer Sets:					
ORF1 OS	CTGGCATYACTACTGCYATTGAGC	64-87	55.68	59.09	45
ORF1 OA	CCATCRARRCAGTAAGTGCGGTC	459-581	55.27	60.62	47
ORF2/3 OS	GCRGTGGTTTCTGGGGTGAC	5279-5298	55.88	57.93	60
ORF2/3OA	CTGGGMTYGGTCDCGCCAAG	5321-5485	55.88	62.03	60
Inner Primer Sets:					
ORF1 IS	CTGCCYTKGCGAATGCTGTGG	112-132	56.31	60.21	57
ORF1 IA	GGCAGWRTACCARCGCTGAACATC	375-398	57.38	60.8	50
ORF2/3 IS	GYTGATTCTCAGCCCTTCGC	5302-5321	53.83	55.88	55
ORF2/3IA	GMYTGGTCDCGCCAAGHGGA	5419-5428	53.83	62.02	55

Table 3.9: Nested RT-PCR primer details for ORF2

Name	Sequence	Binding sites	GC content (%)
Outer primer set:			
Meng F_0	AAYTATGCMCAGTACCGGGTTG	5687-5708	50
Meng R_0	CCCTTATCCTGCTGAGCATTCTC	6395-6417	52
Inner primer set:			
Meng F_1	GTYATGYTYTGCATACATGGCT	5972-5993	57
Meng R_1	AGCCGACGAAATYAATTGTGTC	6298-6319	50

The Superscript II One-Step RT-PCR Kit (ThermoFisher Scientific) was used to generate cDNA in the pre-nested reactions targeting the ORF1 and ORF2/3 regions. In this reaction, cDNA is generated using the extracted viral RNA as a template via the SuperScript™ II RT enzyme. The Platinum Taq DNA Polymerase present in the kit allows for dNTPs complementary to the target sequence to be aligned in such a way that strands of DNA are formed and sealed into place by DNA ligase. This amplifies the cDNA generated with the RT enzyme, resulting in a double-stranded cDNA PCR product. The outer primer sets are used in this reaction. The final cDNA product is held at 4°C until the nested reaction can occur. The pre-nested reaction compositions and cycling conditions are shown in Tables 3.10 and 3.11.

Table 3.10: Pre-nested reaction composition targeting HEV regions ORF1 and ORF2/3

Reagent	µL per 1 reaction
2X Reaction Mix	12.500 µL
Forward Primer (outer sense)	0.625 µL
Reverse Primer (outer anti-sense)	0.625 µL
Superscript™ II RT/Platinum Taq DNA Polymerase mix	0.250 µL
Nuclease Free Water	6.000 µL
Template	***5.000 µL***
Total Volume:	**25.000 µL**

Table 3.11: Pre-nested cycling conditions targeting HEV genomic regions ORF1 and ORF2/3

Stage	Temperature	Time	Number of Cycles
Reverse-transcription	50°C	60 minutes	1
Reverse-transcriptase deactivation/ initial denaturation	94°C	10 minutes	1
Denaturation	94°C	1 minute	40
Annealing	53°C	45 seconds	
Extension	72°C	1 minute	
Final extension	72°C	10 minutes	1
Hold	4°C	∞	

The nested reactions for the ORF1 and ORF2/3 regions were performed using 2x MyFi Mix (Bioline) with the reaction composition and cycling conditions detailed in Table 3.12 and Table 3.13, below. This reaction uses the pre-nested PCR product as a template, but uses the inner primer sets to target generate more copies of a small, specific product.

Table 3.12: Nested reaction composition targeting HEV regions ORF1 and ORF2/3

Reagent	µL per 1 reaction
2x MyFi Mix	25.0 µL
Forward primer (inner sense)	1.0 µL
Reverse primer (inner ant-sense)	1.0 µL
Nuclease-free water	8.0 µL
Template	***10.0 µL***
Total Volume:	**45 µL**

Table 3.13: Nested reaction cycling conditions when targeting HEV genomic regions ORF1 and ORF2/3

Stage	Temperature	Time	Number of Cycles
Initial denaturation	95°C	1 minute	1
Denaturation	95°C	15 seconds	35
Annealing	53°C	45 seconds	
Extension	72°C	15 seconds	
Final extension	72°C	1 minute	1
Hold	4°C	∞	

The pre-nested reaction targeting the ORF2 region made use of the SuperScript IV RT enzyme (ThermoFisher), the reaction composition and cycling conditions are shown in Tables 3.14 and 3.15. This reaction follows the same principle as the pre-nested reactions which targeted the ORF1 and ORF2/3 regions. The SuperScript™ IV RT enzyme generate cDNA using the extracted RNA as a template, using the outer primer set. The 2X Platinum SuperFi enzyme then amplifies the generated cDNA, resulting in a double-stranded DNA PCR product. The nested reaction also used MyFi Mix (Bioline) with the inner primer set, the reaction composition and cycling conditions are seen in Tables 3.16 and 3.17.

Table 3.14: Pre-nested reaction composition targeting HEV region ORF2

Reagent	µL per 1 reaction
2X Platinum SuperFi RT-PCR Master Mix	12.5 µL
Forward Primer (F_0)	0.625 µL
Reverse Primer (R_0)	0.625 µL
Superscript™ IV RT	0.25 µL
Nuclease Free Water	6.0 µL
Template	***5 µL***
Total Volume:	**25 µL**

Table 3.15: Pre-nested reaction cycling conditions when targeting HEV genomic region ORF2

Stage	Temperature	Time	Number of Cycles
Reverse-transcription	42°C	10 minutes	1
Reverse-transcriptase deactivation/ initial denaturation	98°C	2 minutes	1
Denaturation	98°C	10 seconds	40
Annealing	42°C	10 seconds	
Extension	72°C	30 seconds	
Final extension	72°C	5 minutes	1
Hold	4°C	∞	

Table 3.16: Nested reaction composition targeting HEV region ORF2

Reagent	µL per 1 reaction
2x MyFi Mix	25.0 µL
Forward primer (F_1)	1.0 µL
Reverse primer (R_1)	1.0 µL
Nuclease-free water	18.0 µL
Template	***5.0 µL***
Total Volume:	**50 µL**

Table 3.17: Nested reaction cycling conditions when targeting HEV genomic region ORF2

Stage	Temperature	Time	Number of Cycles
Initial denaturation	94°C	5 minutes	1
Denaturation	94°C	1 minute	40
Annealing	42°C	1 minute	
Extension	72°C	2 minutes	
Final extension	72°C	5 minutes	1
Hold	4°C	∞	

The pre-nested and nested PCR products were visualised using gel electrophoresis as detailed in the next section. Nested RT-PCR, and subsequently sequencing, was attempted on all samples that tested positive for HEV RNA on real-time RT-PCR, regardless of their Ct value.

3.5.2. Gel electrophoresis visualisation

A 2% agarose gel was prepared using Tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer. For the preparation of 2% gel, 2 g of agarose powder was weighed and added to 100 mL of 1x TAE buffer. The mixture was heated in a microwave until the powder fully dissolved in the buffer, with care taken to not boil over. The mixture was then left to cool on a magnetic stirrer for around 15 minutes. Once the mixture was cool to the touch, it was poured into a casting tray and well combs were added. The gel was left to set in the tray for around 45 minutes. Once the gel was set, it was removed from the tray and the well combs were carefully removed. The gel was then placed in an electrophoresis gel tray containing 1x TAE buffer, the volume of buffer was sufficient to cover the surface of the gel.

Once the gel had set, the samples were prepared for loading. For every 5 µL of sample, 1 µL of Novel Juice (GeneDirex) was added for visualisation of PCR products. The sample and Novel Juice were combined by pipetting on a parafilm sheet. The 1kilobase (kb) plus DNA Ladder RTU (GeneDirex) was also run alongside the samples; it was combined with Novel Juice in the same way as the samples.

The solution composed of Novel Juice and sample was loaded into the gel using a pipette. The voltage of the electrophoresis power unit was set to 90 volts and the gel was run for 50 minutes. The migration of tracking dyes in the Novel Juice were observed to ensure that the samples did not run off the gel. Upon completion of the run, the gel was carefully removed from the gel box and placed in the BIO-RAD Chemi-Doc Imaging System (Bio-Rad Laboratories) for visualisation. The ladder allowed for the approximate fragment sizes amplified in samples to be observed. The expected fragment size for the ORF1 target was 286 bp, for the ORF2/3 the target was 126 bp, and for the ORF2 target it was 347 bp. When band sizes of the expected size were present, it was an indicator that amplification of the desired region occurred. An example of this is given in Figure 3.5.

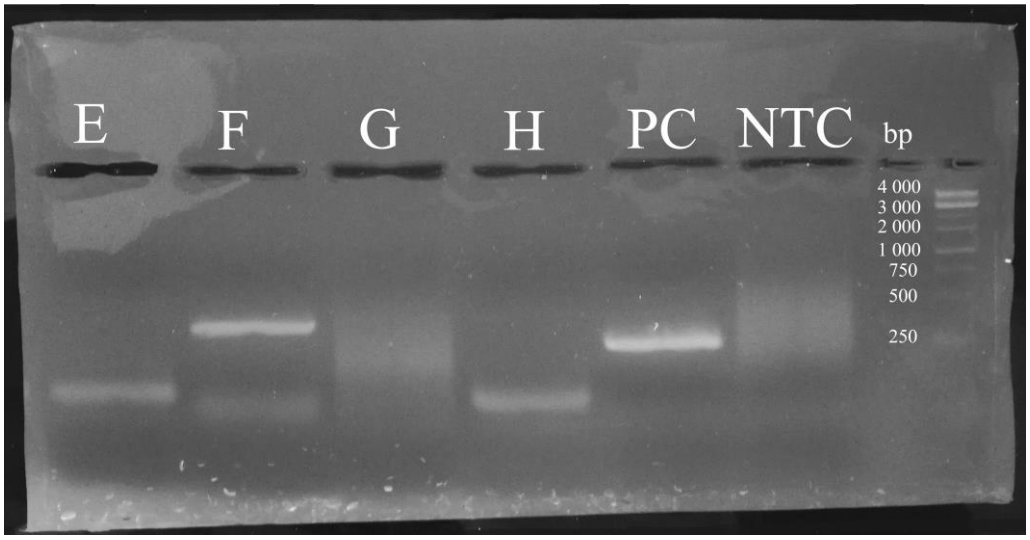

Figure 3.5: A gel electrophoresis visualisation of the nested products amplifying the ORF1 region (at a size of 286 bp) of the HEV genome. Samples are labelled: “E”, “F”, “G” and “H”. The positive control is labelled “PC” and the non-template control is labelled “NTC”. In this figure, Sample F is aligned with the positive, while samples E, G and H do not show a band in line with the positive control.

3.6. PCR purification and quantification

Following gel electrophoresis visualisation, the nested PCR products were purified. This step removes the dNTPs, primers, and other reagents used in the nested reactions, and leaves behind the targeted fragments. A column-based purification method and a gel purification method were both used in this project. The column-based method was initially used, and downstream sequences appeared to contain excessive background noise. The gel purification method was used to try and reduce background noise and improve downstream sequencing quality. The concentration and purity of the purified products using either method was determined using a spectrophotometer. If samples were of adequate concentration and purity, they were subjected to Sanger sequencing.

3.6.1. Purification of nested RT-PCR products

3.6.1.1. Gel purification

To reduce background noise and contaminants present in samples, a gel excision purification method was applied to nested products. The Wizard ® SV Gel and PCR Clean-Up System (Promega) was used according to the manufacturer's instructions. The entire nested PCR product (a volume of 45 µL) was combined with 9 µL of Novel Juice (Bio-Helix) by pipetting. The mixture was then loaded onto a gel. Once the gel was run, it was briefly placed on a Chromate-Vue Model TM-20 Transilluminator (Analytik Jena UVP) ultra-violet transilluminator, and the DNA band of the correct size was excised and placed in a clean, empty 1.5 mL Eppendorf tube. The excisions were made as close to the visible band as possible, to reduce gel volume, but with care not to nick DNA.

After the gel slice was weighed, 10 µL of Membrane Binding Solution was added per 10 mg of gel. The tube was vortexed to mix and incubated at 65°C until the gel slice was dissolved. An SV Minicolumn was placed in a collection tube and the gel mixture was added to the column. The tube was incubated for 1 minute at room temperature and then centrifuged at 16 100 x *g*. The flowthrough was discarded before washing. For gel slices that weighed more than 350 mg, the binding step was repeated using half of the dissolved gel slice at a time. The flowthrough was discarded after loading the first half and before spinning the second half.

To wash the column, 700 µL of Membrane Wash Solution was added. The tube was centrifuged at 16 100 x *g* for 1 minute, after which the flowthrough was discarded. The step was repeated using 500 µL of Membrane Wash Solution and centrifuged at the same speed for 5 minutes. The flowthrough was discarded. The tube was then spun at 16 100 x *g* for one minute to evaporate remaining ethanol.

To elute, the Mini-column was placed in a clean, labelled, 1.5 mL Eppendorf tube and 50 µL of nuclease-free water was added directly to the column membrane. The tube was incubated for one minute, and then centrifuged at 16 100 x *g* to elute the DNA. The DNA was then stored at 4°C or -20°C.

3.6.1.2. Column-based purification

The Wizard SV Gel and PCR Clean-Up System (Promega) was also used for the column-based purification method according to the manufacturer's instructions. For PCR product preparation, 45 µL of Membrane Binding Solution were combined with 45 µL of nested PCR product. An SV Minicolumn was placed into a clean collection tube and the product was added to the column. The product was incubated in the assembly for 1 minute at room temperature and was then centrifuged at 16,100 x *g* for 1 minute. The flowthrough was discarded and the Minicolumn was placed back in the same collection tube.

The DNA washing and elution steps which follow are the same as those used for gel purification detailed in section 3.6.1.1. The only difference being that the final elution occurred in 20 µL rather than 50 µL of nuclease-free water.

The resultant purified sample was quantified immediately using the NanoDrop One spectrophotometer (Thermo Scientific) or stored at 4°C until quantification and sequencing reactions.

3.6.2. Quantification of purified nested RT-PCR products with a spectrophotometer

The NanoDrop One (ThermoFisher scientific) spectrophotometer was used to measure the concentration and determine purity of nested PCR products which had undergone purification with the QIAquick column kit (QIAGEN) or the Wizard SV Gel and PCR Clean-Up System (Promega). The instrument was blanked using 1.5 µL of Elution Buffer (the same buffer used to elute DNA in purification). To read each sample, 1.5 µL of sample was loaded onto the spectrophotometer pedestal. Concentration readings were expressed as ng/µL and absorbance ratios (260/280 and 260/230) noted for purity. A concentration above 30 ng/ µL is ideal for sequencing. A 260/280 ratio of between 1.8 and 2 indicated acceptable DNA purity for sequencing. Due to the RNA being sourced from environmental samples and in most cases diluted, samples with low DNA concentrations (of around 20 ng/µL) were still sequenced.

3.7. Sanger Sequencing

The forward and reverse primers used in the nested reactions were used for Sanger sequencing. For each sample a well with forward primer and a separate well with reverse primer was prepared. The BigDye Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific) was used for the sequencing reaction with reaction composition detailed in Table 3.18, below. The sequencing reaction cycling conditions are show in Table 3.19.

Table 3.18: Sequencing reaction composition

Reagent	Volume (µL) per reaction
Terminator Ready	1
Sequencing Buffer	3
Primer	1
Nuclease-Free Water	4
Template	1
Total Volume:	**10**

Table 3.19: Sanger sequencing reaction conditions

Stage	Temperature	Time	Number of Cycles
Denaturation	96°C	10 seconds	25
Annealing	50°C	5 seconds	
Extension	60°C	4 minutes	
Hold	4°C	∞	

Following the sequencing reaction, clean-up was performed using the BigDye XTerminator Purification Kit (ThermoFisher Scientific). A master mix was prepared using 49.5 µL of SAM solution and 11 µL of XTerminator solution per well. The master mix was vortexed thoroughly and 55 µL was pipetted into each well. Taking care to vortex the master mix between pipetting. The plate was then sealed and placed onto a shaker for 45 minutes at 1805 rpm. The plate was stored at 4°C or immediately loaded onto the ABI 3500XL genetic analyser (ThermoFisher Scientific).

3.8. Phylogenetic Analysis

Forward and reverse chromatograms obtained from Sanger sequencing were used to construct consensus sequences *de novo* in Geneious Prime. The consensus sequences were then put through Basic Local Alignment Search Tool (BLAST) against the GenBank NIH database to confirm that the sequences corresponded to HEV. The sequences were then aligned to reference sequences of member species as listed by the International Committee on Taxonomy of Viruses (ICTV) as part of an online chapter on the family Hepeviridae (Purdy et al., 2022). Additional references for HEV genotypes 6, 7, and 8 were included from a proposed list of reference sequences published by Smith et al., (2020). The member species sequences included are listed in Table 3.20, below. Only full genome reference sequences were used, that is, sequences with a length of around 7.2 kb and there was at least one sequence from each HEV genotype included in the analysis. More sequences of genotypes 1 to 4 were included, as these commonly infect people. There was a higher number of full-length genome sequences available for HEV-3 across a variety of countries. This is likely due to HEV-3 being more prevalent in developed countries which are more likely to have the resources required for sequencing.

Multiple sequence alignment and construction of phylogenetic trees were performed in MEGA 11 by ClustalW (Tamura et al., 2021). The region of interest was then extracted from the multiple sequence alignment and used to construct a phylogenetic tree using the Maximum-Likelihood method and Tamura-Nei model in MEGA 11 (Felsenstein, 1985; Tamura and Nei, 1993; Tamura et al., 2021). Initial trees for the heuristic search were obtained automatically by applying neighbour-join and BioNJ algorithms to a matric of pairwise distance estimated using the Tamura-Nei model and selecting the topology with superior log likelihood value (Tamura and Nei, 1993).

Table 3.20: Member species as detailed by the ICTV and Smith et al. (2020)

Genotype	Virus name	Country of origin	GenBank Accession number	Available sequence
HEV-1a	Human HEV-1a	Myanmar (formerly Burma)	M73218	Complete coding genome
HEV-1a	Human HEV-1a	China	L08816	Complete coding genome
HEV-2a	Human HEV-2a	Mexico	KX578717	Partial genome
HEV-3a	Swine HEV-3a	USA	AF082843	Complete coding genome
HEV-4a	Swine HEV-4a	China	EF077630	Complete genome
HEV-5a	Wild boar HEV-5a	Japan	AB573435	Complete coding genome
HEV-6a	Wild boar HEV-6a	Japan	AB602441	Complete coding genome
HEV-6	Wild boar HEV-6	Japan	AB856243	Complete genome
HEV-7a	Camel HEV-7a	UAE	KJ496143	Complete genome
HEV-7	Camel HEV-7	UAE	KJ496144	Complete genome
HEV-8a	Camel HEV-8a	China	KX387865	Complete genome
HEV-8	Camel HEV-8	China	MH410174	Complete genome

Additional full-length reference sequences of genotypes HEV-1, HEV-2, HEV-3 and HEV-4 were included based on the reference sequences used by Blackard et al. (2009). They are listed in Table 3.21 below.

Table 3.21: Full-length HEV genomes included as reference sequences for genotyping

Genotype	Country of origin	GenBank Accession number
HEV-1	Chad	AY204877.1
HEV-2	Nigeria	MH809516.1
HEV-3	Sweden	EU360977.1
HEV-3	Japan	AB369687.1
HEV-3	France	KF922359.1
HEV-3	Japan	AB780453.1
HEV-3	Japan	AB246676.1
HEV-3	USA	AF082843.1
HEV-4	China	AJ272108.1
HEV-4	Japan	AB220979.1
HEV-4	Japan	AB220975.1

Chapter 4: Results

4.1. Real-time PCR (detection of HEV in wastewater extracts)

In total, 143 wastewater nucleic acid extract samples were tested for the presence of HEV RNA using the QuantiFast RT-PCR + IC Kit (QIAGEN). Of these, 130 (90.9%) samples yielded valid results. A total of 43 results were considered invalid because the IC failed to amplify. Invalid samples were re-tested at least once. Overall, 21/130 (16.2%) samples tested positive for HEV RNA, and 109/130 (83.9%) samples tested negative for HEV RNA. The median Ct value on the positive samples was 37.9 (interquartile range: 36.7-39.7). The number of positive and negative samples from each month across all four locations are summarised in Table 4.1. The positive and negative samples from each location organised by month are shown in Figures 4.1, 4.2, 4.3, 4.4 and 4.5.

Table 4.1: The number of positive and negative samples detected from each collection site

Collection Site	Positive Samples	Negative Samples	Total Samples	% Positive per site
Athlone WWTW	5	29	34	15%
Zandvliet WWTW	9	22	31	29%
Meerhoff Residence	1	28	29	3%
Metanoia Residence	6	30	36	17%
Column Totals:	21	109	130	

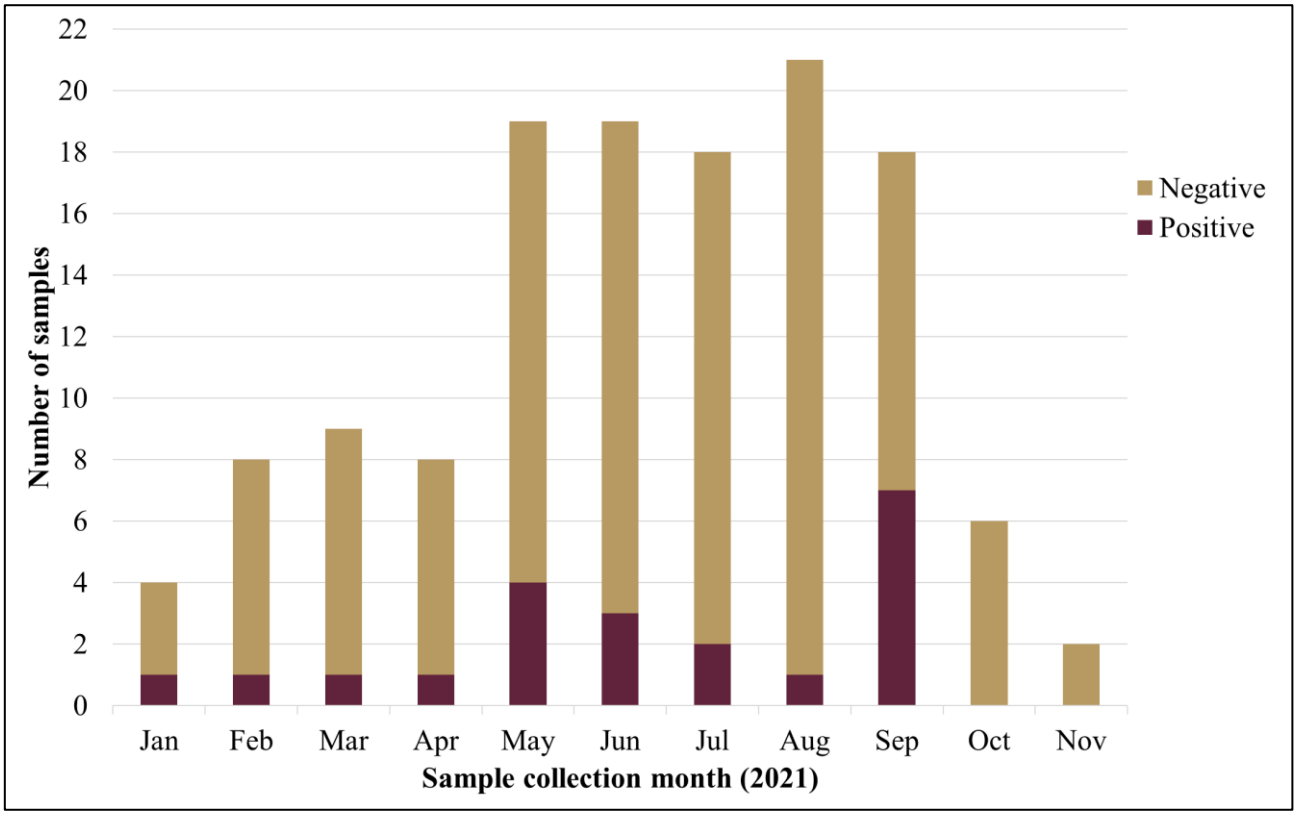

Figure 4.1: The number of positive and negative samples detected across all collection sites

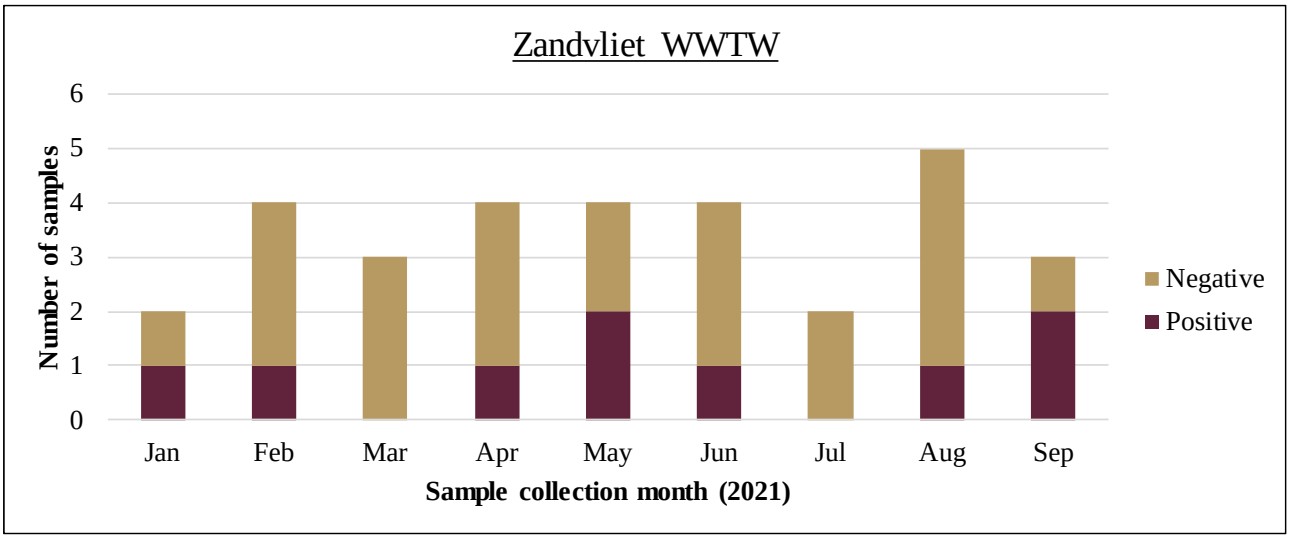

Figure 4.2: The number of positive and negative samples from Zandvliet WWTW

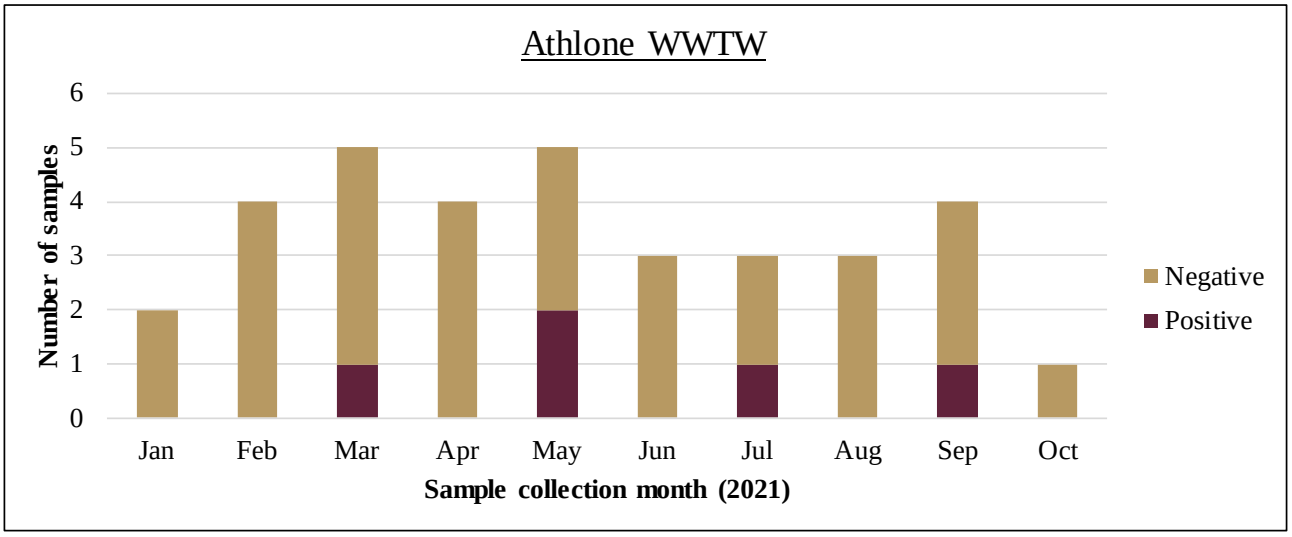

Figure 4.3: The number of positive and negative samples from Athlone WWTW

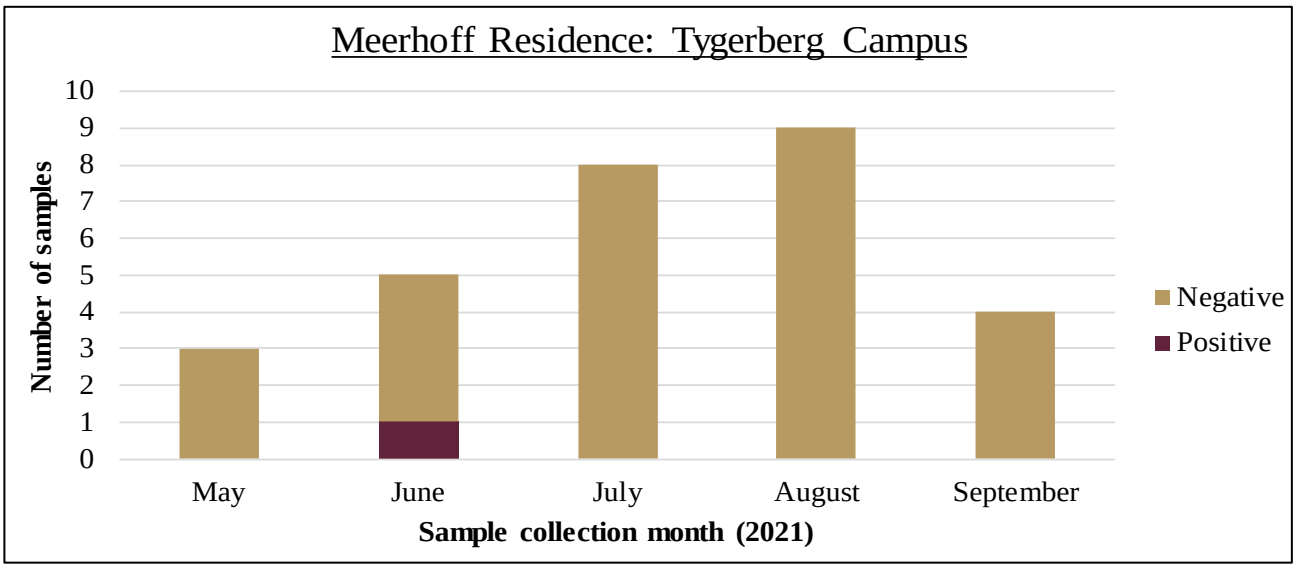

Figure 4.4: The number of positive and negative samples from Meerhoff Residence

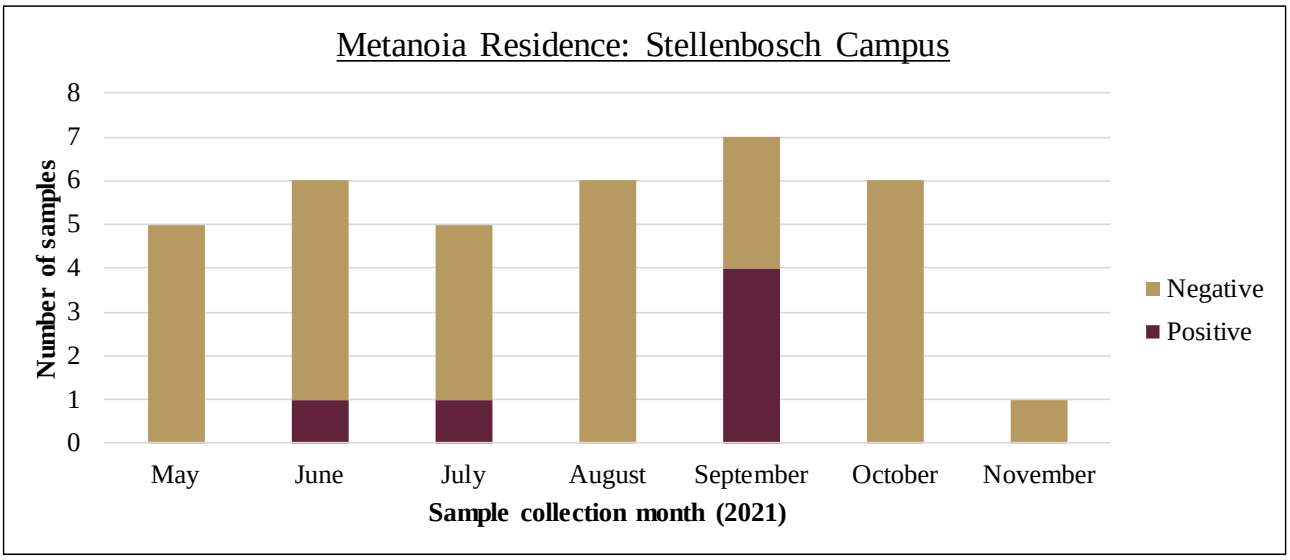

Figure 4.5: The number of positive and negative samples from Metanoia Residence

4.2. Nested RT-PCR

The ORF1, ORF2/3 and ORF2 regions were targeted in all 21 positive samples except where sample volume was depleted, this is detailed in Table 4.2. After purification, samples were quantified on the NanoDrop spectrophotometer (ThermoFisher) which gave concentrations in ng/µL and as measures of purity, 260/280 and 260/230 ratios. The average concentrations and for each sample after purification are shown in Table 4.2. The purity 260/280 and 260/230 ratios can be seen in Addendum D.

Table 4.2: Average concentrations recorded for each sample after purification

	Concentration (ng/µL)		
Sample No.	**ORF1**	**ORF2/3**	**ORF2**
4	16.18	62.79	/
8	20.19	19.86	/
13	23.16	20.07	/
30	27.64	29.51	/
33	21.43	10.5	25.46
35	7.16	8.86	13.95
36	10.21	40.53	28.06
40	19.34	24.56	/
48	24.76	17.89	12.18
51	/	24.93	9.46
58	22.17	7.29	7.48
67	10.93	10.28	10.71
70	/	11.29	19.13
74	/	16.79	8.89
99	4.86	10.2	11.57
155	1.44	/	10.41
163	8.23	10.61	5.1
173	/	41.73	10.67
174	/	25.3	10.14
175	7.41	7.07	11.28
178	11.04	6.93	9.97

Key:
/: Sample volume was depleted

4.3. Sanger sequencing and phylogenetic analysis

The 21 samples which tested positive for HEV RNA by real-time PCR were subjected to Sanger sequencing to determine the HEV genotypes. Following nested RT-PCR, Sanger sequencing of the overlapping ORF2/3 region of the HEV genome was successful in only 4/21 (19.1%) samples. The sample details can be seen in Table 4.3, below. Attempts were made to sequence the nested products of the ORF1 and ORF2 regions, but none were successful. Chromatogram results from sequencing attempts of all 3 regions would often have high levels of noise or be too short for consensus sequences to form. An example of this can be seen in Figure 4.6, this was the sequencing result for Sample 33 where the ORF1 region was targeted.

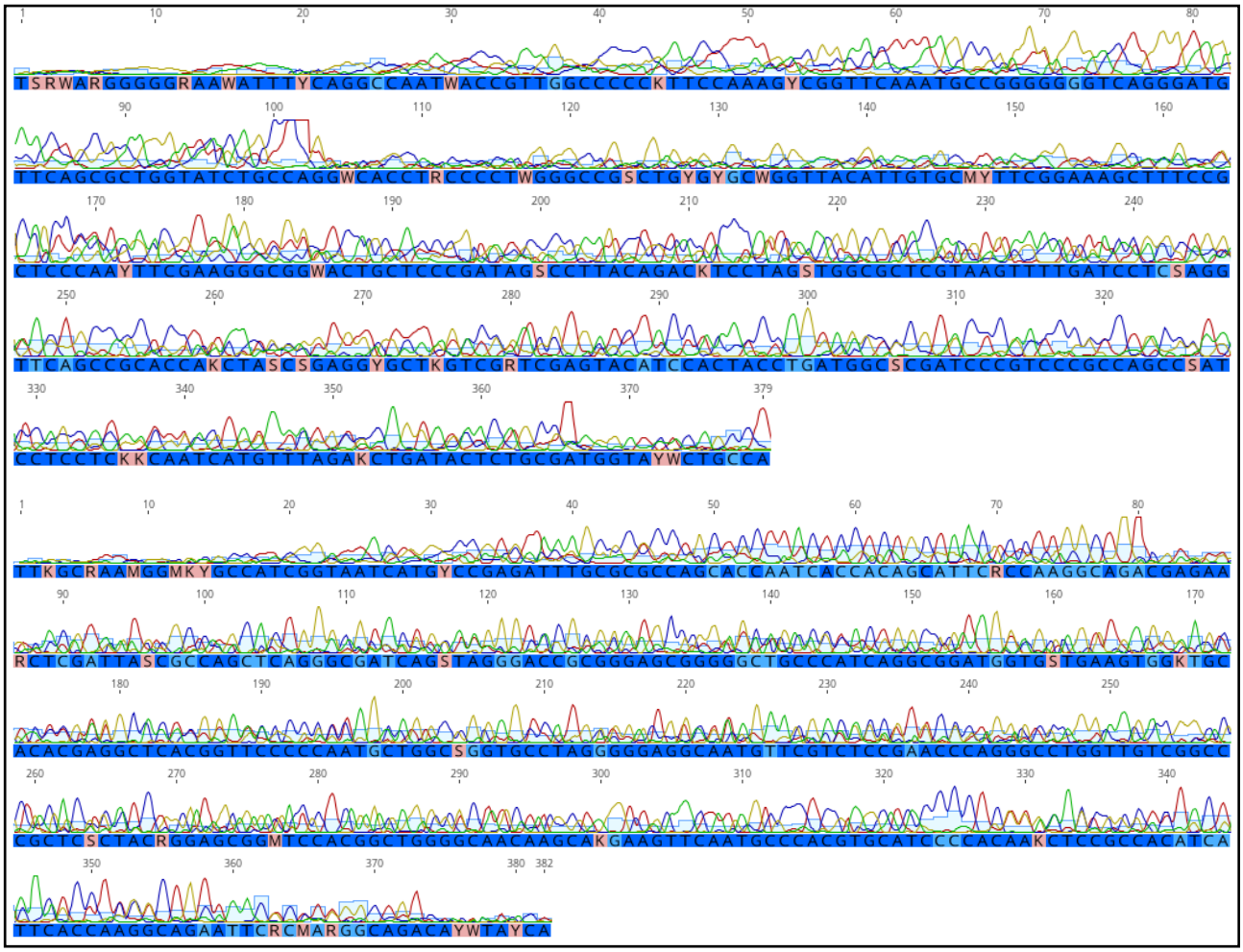

Figure 4.6: Forward and reverse chromatograms generated from Sanger sequencing the amplified ORF1 region of Sample 33. The top sequence is the forward, and the bottom sequence is the reverse. High levels of noise and low sequence quality can be seen in both sequences. These chromatograms were viewed in Geneious Prime 2023.2.1.

Table 4.3: Collection and sequence details of sequenced samples

Sample name	Collection location	Collection date	Consensus sequence length
36ZVC	Zandvliet WWTW	10 May 2021	139 bp
51ATC	Athlone WWTW	15 July 2021	128 bp
173MET	Metanoia Residence: Stellenbosch Campus	8 September 2021	140 bp
174MET	Metanoia Residence: Stellenbosch Campus	15 September 2021	125 bp

The phylogenetic tree shown in Figure 4.7 below was generated after multiple sequence alignment with selected reference sequences representative of HEV genotypes 1, 2, 3, 4, 5, 6, 7, and 8. The fragment size was 126 bp.

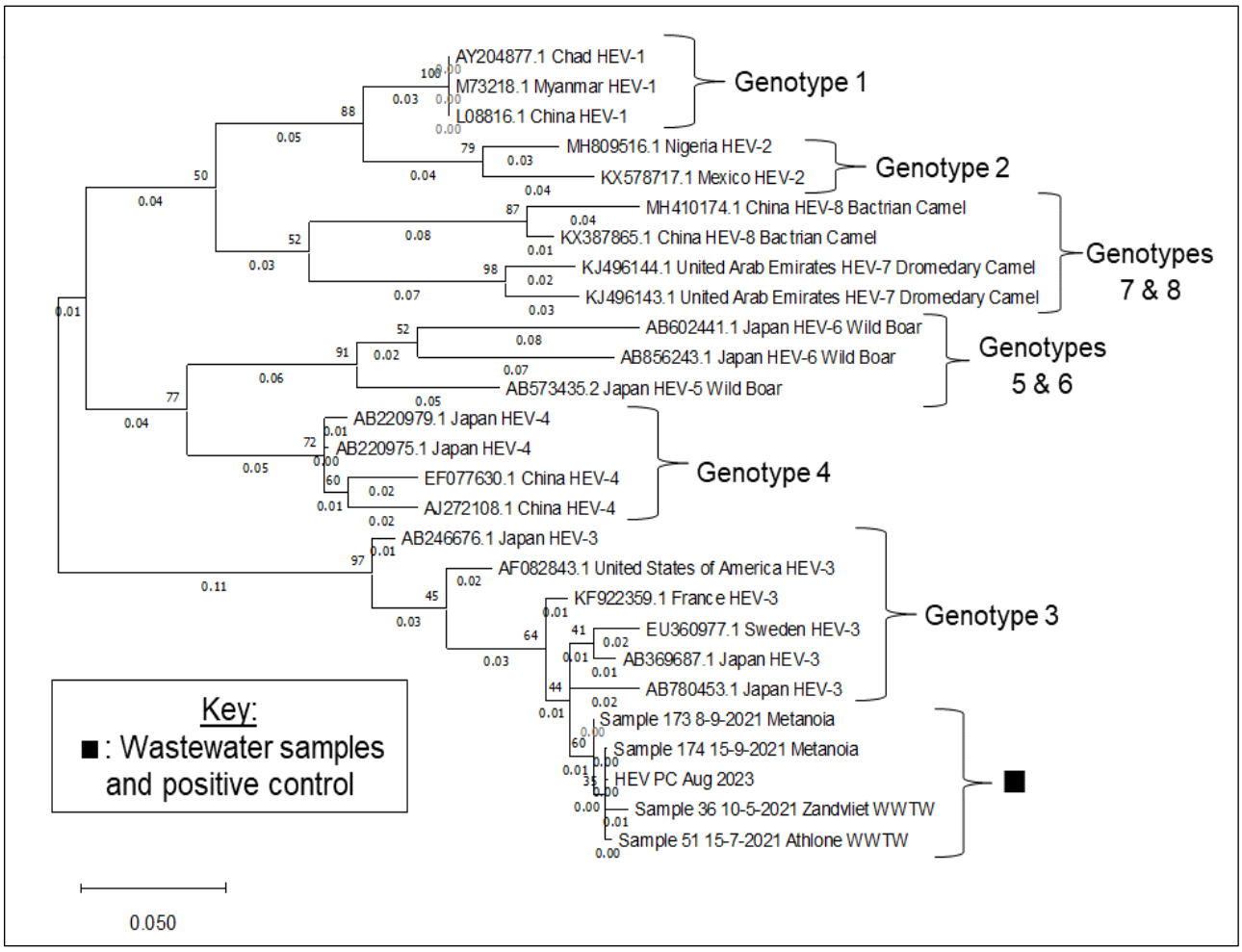

Figure 4.7: Phylogenetic tree generated using the Maximum-Likelihood method and Tamura-Nei model (Felsenstein, 1985; Tamura and Nei, 1993; Tamura et al., 2021). The analysis used 22 reference sequences downloaded from GenBank. The reference sequences are labelled by their accession number, country of origin and genotype. References of the same genotypes which clustered together are labelled with brackets. The percentage of trees in which the associated taxa clustered together is shown at branch nodes. The tree is drawn to scale, branch lengths are measured in the number of substitutions per site and are shown above and below the branches as values less than 1.

Chapter 5: Discussion

HEV infection is usually asymptomatic, but fulminant and chronic hepatitis can occur. In recent years, reports of extrahepatic manifestations of the infection have been increasing. The disease has a global distribution and is considered emerging (Webb and Dalton, 2019). HEV molecular epidemiology differs across regions, and genotypes differ in routes of transmission and their potential morbidity and mortality outcomes. In Sub-Saharan Africa specifically, studies have focused on serology and molecular methods have rarely been applied (Bagulo et al., 2020). This has a left a gap in the molecular epidemiology of HEV in Sub-Saharan African nations, including in South Africa. WBE has been used in multiple developed nations to determine the circulation of HEV and prevalent HEV strains among populations (Béji-Hamza et al., 2015; Smith et al., 2016; Beyer et al., 2020; Iaconelli et al., 2020). Real-time PCR, nested RT-PCR, and Sanger sequencing have all been previously applied to RNA extracts from wastewater to determine the detection rate of HEV. This data was then compared with known clinical data to gain a comprehensive picture of the relevance of HEV to the public.

The main aim of this study was to determine whether HEV was circulating at the time of sampling in selected collection sites within the Western Cape via the use of WBE. WBE had already been applied to these samples for SARS-CoV-2 as part of the SAMRC wastewater surveillance activities (Johnson et al., 2022; Mangwana et al., 2022). In this project, 143 wastewater extracts collected for these wastewater surveillance activities in 2021 were tested for the presence of HEV RNA using a real-time PCR assay. Four sequences were obtained from 4 of 21 samples which tested positive for HEV RNA.

5.1. Determining the limit of detection of the real-time PCR assay

While it would have been ideal to quantify the exact viral loads present in each HEV positive wastewater extract, in practice, the HEV RNA concentrations were too low to be determined accurately. Hence, we came to the decision that we should determine the real-time PCR assay's limit of detection. It was determined that an HEV RNA concentration of 10 IU/mL was detected by the real-time assay consistently. This limit falls within the lower end of the reported range for clinical real-time PCR assays even though inhibitors from the wastewater sample may have been present (Lhomme et al., 2020). Nevertheless, each sample makeup was unique and heterogenous, it is possible that samples which contained HEV RNA were not detected due to low concentration or higher levels of inhibition resulting in reduced amplification. The spiking experiment results also confirmed that the assay was able detect HEV RNA in extracts from wastewater samples despite inhibition which may have been caused by environmental contaminants.

5.2. Real-time PCR (detection of HEV RNA in wastewater extracts)

To the best of knowledge, no studies have been conducted on the circulation of HEV in wastewater in South Africa, except for one MSc study from the University of Pretoria in 2004 (Williams, 2004). In the 2004 study, 199 wastewater extracts from different regions in the Gauteng province were tested for the presence of HEV RNA by amplifying a section of the ORF2 region with RT-PCR and Sanger sequencing. They detected HEV RNA in 9/199 (4.5%) using nested RT-PCR and confirmed 2/199 (1.0%) of these results by sequencing. With HEV cases often being subclinical and mostly asymptomatic, it is difficult to assess the true prevalence of infection by referring to hospital records. This is why countries have made use of WBE to assess HEV circulation and characterise the prevalent strains. In the rest of this section the prevalence of HEV in the Western Cape according to the real-time PCR results will be discussed.

In this study, 21/130 (16.2%) of the wastewater extracts tested positive for the presence of HEV RNA. This indicates that individuals were infected with HEV and shedding the viral particles to the extent that it could be detected in twenty-one wastewater samples. Zandvliet WWTW which represented the largest population had the highest number of positive samples out of the four collection sites. Zandvliet WWTW serves multiple areas including parts of Kuils River, Delft, Blackheath, Blackheath Industria, Blue Downs, Eerste River, De Wijnlanden, Thembokwezi, Mxolisi Phetani, and Khayelitsha (Zutari, 2020). The catchment area has been described as fast-growing, which warranted improvements to expand its wastewater treatment capacity from 72Ml/day to 90 Ml/day by September 2023 (City of Cape Town, Media Office, 2022). The areas included range in population density from 1 171 to 13 715 persons/km^2 with a mean value of 6 727 persons/km^2 (Department: Statistics South Africa, 2011). Khayelitsha and Delft are the most densely populated at 13 715 and 10 120 persons/km^2 respectively. The collection sites vary in socio-economic status, Khayelitsha and Delft high levels of unemployment and low household income levels. Kuils River has the highest percentage of households in formal dwellings at 97.5%. Blue Downs has 76.8% percent of households in formal dwellings while Eerste River has 93.5%. Blackheath has 73.2% of households in formal dwellings but as of 2011, 84.1% of the population had access to a flush toilet connected to sewage (Department: Statistics South Africa, 2011). At the time of writing, Census 2022 data is available for provinces and metros but not individual areas. It should be noted that the populations densities at the time of sampling were likely higher than those reflected by the 2011 Census, considering the growth of the City of Cape Town district from 3 740 031 in 2011 to 4 772 846 in 2022 (Department: Statistics South Africa, 2023).

In future it would be interesting to sample from manholes in each area and compare the frequency of HEV RNA detection, to determine how each area contributed to the HEV RNA detected. Meerhoff student residence had only one positive sample, the least out of the collection sites. It represented the smallest population group and had the least number of samples compared to the other three collection sites, which could have resulted in less frequent HEV RNA detection. Meerhoff being a residence on a medical campus may also have been a factor, as medical students may be more aware and avoidant of common transmission routes of foodborne and faecal-oral diseases.

In terms of the COVID-19 restrictions in place at the time, South Africa moved from adjusted alert level 3 (26 July to 12 September 2021) to adjusted alert level 2 (13 September to 1 October 2021) (South African Government, 2021). This could have resulted in increased economic activity and contact between people, but the connection to increased detection of HEV RNA is unclear. Taking the alert level restrictions into consideration, it is possible that students returned to their residence accommodation in September 2021 due to South Africa moving to adjusted alert level 2, and thus a larger number of students contributed to the wastewater samples in September (South African Government, 2021).

The 16 positive samples had high Ct values, with only one sample having a Ct under 33. We cannot infer an exact concentration since the real-time PCR assay was semi-quantitative, but we can consider these Ct values as relatively high and indicating a low concentration of HEV RNA. The first factor which may explain these values is the environmental nature of the extracts. During troubleshooting, the IC assay and HEV assay had to be separated as the ICs were not amplifying with each run. This is noted in section 3.3.2. and the reaction compositions for the combined and separated assays are seen in Table 3,3 and Table 3.4. The separation improved the performance of the IC amplification, allowing the run results to be validated. A clinical sample representing one patient contains other RNA molecules besides the HEV RNA, but this is to a lesser extent than an environmental sample representing many people.

The overall detection rate of 16.2% is higher than the detection rate found in the Gauteng study of 1.0% (Williams, 2004). This could be due to the different techniques used in this study. This may be due to HEV RNA going undetected in some samples because of inhibitors present in the original environmental samples. Repetition of the study with a larger sample size could reveal prevalence rates more in line with recent literature. It should also be noted that HEV prevalence has been observed to vary greatly within the same country within different regions, and this could also have influenced the observed prevalences (Mansuy et al., 2015; Iaconelli et al., 2020). Nonetheless, HEV may have a different prevalence in the Cape Town region compared to the Pretoria region. This can be seen in the lower recorded seroprevalence of HEV in pregnant women in Pretoria based on a recent study compared to those recorded from the Western Cape province (Simani et al., 2022).

The proportion of positive samples in this study was higher compared to the results of WBE studies on HEV in other African nations. A 2011 study in Cairo, Egypt detected no HEV RNA from 76 wastewater samples (Kamel et al., 2011). More recent studies in Tunisia and Cameroon detected HEV RNA in 3/150 (2%) and 3/157 (1.9%) wastewater samples, respectively (Béji-Hamza et al., 2015; Fatawou et al., 2022). The consumption of pork is low in several African countries due to religious reasons (for example, Tunisia), and where HEV-3 is detected in these countries, zoonotic sources outside of swine and wild boar should be investigated (Béji-Hamza et al., 2015). By comparing these studies which use WBE, it may appear that HEV is circulating to a greater extent in South Africa compared to these nations, but detection in wastewater alone cannot confirm this. The methodology across these studies also varies, both seroprevalence and further

environmental surveillance studies would be required to confirm that HEV is more common in South Africa than in these nations.

5.3. Sanger sequencing

Sanger sequencing was attempted on 16 samples for ORF1, 20 samples for ORF2/3, and 16 samples for the ORF2 HEV genome region. Sequencing was only successful in the ORF2/3 region in four samples. Out of the three regions, ORF2/3 was the shortest targeted region at 126 bp. It is possible that the ORF1 and ORF2 regions were less likely to be intact due to their slightly longer length. In the case of the ORF2 region, the annealing temperature for the reaction is low, compared to the other targets that were attempted on clinical samples over time in the Division of Medical Virology and proven to be effective. The use of the lower annealing temperature combined with the heterogenous nature of the environmental sample likely resulted in non-specific amplification (Lorenz, 2012; Clark et al., 2019). The region targeted by Williams (2004) was a 197 bp region from ORF2 which does not overlap with ORF3 so it could be not compared directly to the sequences obtained in this study. Across all sequencing attempts, chromatograms with high levels of noise were often generated. An example of this is seen in Figure 4.6. This may have been caused by the heterogenous make-up of the wastewater samples. Since the exact RNA make-up in each sample was unknown, it is possible that the nested RT-PCR did not amplify just the target region. It was due to this noise in initial sequencing results that gel purification was selected, to excise only bands that aligned to the target. The nested products for sequenced samples 51 and 174 were purified using this gel excision method. Nested products for sequenced samples 36, 173 and the working positive control were purified using the column purification method. Meerhoff was the only collection site where a sequence could not be obtained. Interestingly, 2 sequences were obtained from Metanoia which has a smaller population while only 1 sample could be sequenced from both Athlone WWTW and Zandvliet WWTW which serve the largest populations. The fact that HEV sequences could be obtained from 4/21 (19.1%) extracts increases confidence in the real-time PCR results. The nested RT-PCR also targeted a different region to the real-time PCR assay which further confirms that the real-time PCR results were unlikely to be false positive amplification.

For genotyping of the acquired sequences, two phylogenetic trees were generated. The first tree used reference sequencing for genotypes HEV-1 to HEV-4 while the second included reference sequences for genotypes HEV-1 to HEV-8. The wastewater extract sample sequences were most closely related to each other and to the working positive control. The samples being closely related indicates that the same if not a very similar strain of HEV is circulating across the three relevant collection sites. However, the target sequence is from a short and highly conserved region, and one would not expect much variation in this region (Nan and Zhang, 2016; Cancela et al., 2022). The positive control was extracted from a faecal sample of a patient who was identified as HEV-infected through clinical testing in the Division of Medical Virology.

The clustering with wastewater samples could indicate that the same genotype observed in clinical cases is circulating in communities, but this would require investigation on a larger scale. The study sequences clustered with the HEV-3 reference sequences, and most closely with AB780453.1. This sequence was

obtained from a Japanese wild boar. Sequence AB780453.1 is classified as HEV-3 subtype 3e, and this subtype has been reported previously in acute hepatitis patients, blood donors and swine (Nakano et al., 2013).

Based on the clustering seen in both phylogenetic trees, the sequences from the wastewater extracts are likely HEV-3. All the sequences clustering with HEV-3 indicates a zoonotic rather than faecal-oral mode of transmission, and points to food as the most likely source of infection. Madden et al. (2016) found that pork consumption was a significant risk factor for HEV IgG seropositivity among 1 161 individuals in the Western Cape. In 2014, HEV RNA was detected in two pork liver spread samples in Cape Town and a high-quality sequence clustering with HEV genotype 3e was obtained (Korsman et al., 2019). In studies conducted in other African countries, HEV-1 and HEV-3 were detected in wastewater in Tunisia, while HEV-3 was detected in a wastewater sample in Cameroon (Béji-Hamza et al., 2015; Fatawou et al., 2022). The detection of HEV-3 in wastewater samples from Egypt, Tunisia, Cameroon, and now South Africa, may indicate that HEV-3 is more widespread across the continent than historically believed (Kmush et al., 2013; Kim et al., 2014). Many reports regarding HEV in Africa are focused on outbreaks and epidemics linked to the water-borne HEV-1 or HEV-2, while infections due to HEV-3 are likely underreported as they are often asymptomatic. This is a possibility that requires further investigation, with studies of both clinical and environmental nature. In the study conducted in Gauteng, the strains detected in wastewater were determined to likely be HEV-1, which differs from the result of this study (Williams, 2004). It is possible that different genotypes or strains of HEV circulate in Gauteng compared to the Western Cape and that HEV is prevalent at different levels in the provinces, further studies are necessary to confirm this. It is also possible that the results are different due to the differing methodology used in this study and the Gauteng study. Aside from methodology, the detected proportion of HEV RNA positive samples and genotypes could differ from 2004 and 2021 as there is a 17-year time difference in sample collection.

HEV-3 can infect humans, swine, wild boar, rabbits, and deer among other mammals (Primadharsini et al., 2021). This opens the possibility of animal reservoirs for the detected HEV RNA in wastewater extracts. This could occur through run-off from pig farms or other domestic animals. Run-off being rainwater that does not get absorbed into soil, but rather washes over farms and land, carrying pesticides, fertilisers, bacteria, and viruses into sewage systems (United States Environmental Protection Agency, 2015; Treagus et al., 2021). Publications on HEV in clinical cases and from animal sources in the Western Cape have reported an overlap in genotype and even subtype of HEV. A 2017 study tested porcine serum samples from a single abattoir in Cape Town for total antibodies to HEV (Van Helden et al., 2017). Out of 947 samples, 575 (60.7%) were positive for HEV total antibody. They also tested 45 of the serum samples for the presence of HEV RNA, 3/45 (6.7%) samples were positive, and the resultant sequences clustered with HEV- 3 subtype 3e. The presence of HEV RNA in pork meat products and swine in Cape Town indicates circulation of HEV-3 among pigs in Cape Town. Detection of HEV-3 has been observed in multiple case studies of chronic HEV infection in South Africa. One example is in an HIV-positive male who developed chronic hepatitis E (Andersson et al., 2013). A second example being a woman with a history of liver disease who had undergone a renal transplant and afterwards developed chronic hepatitis E (Andersson et al., 2015). In 2014, a 54-year-old male in Cape Town

presented with acute liver failure. It was confirmed that he was HEV IgM positive and infected with HEV-3 subtype 3e (Madden et al., 2016). The detection of HEV-3 in South African clinical cases together with the observed genotype circulating in swine in the Western Cape supports that the same strains circulating in pigs are infecting human beings and thus could be shed and detected in wastewater. However, wastewater samples from the Metanoia university residence are less likely to be affected by animal contamination from pigs, which gives further credibility to the detected HEV-3 sequences being shed from humans rather than contamination from a zoonotic reservoir.

September 2021 had the highest proportion of samples positive for HEV RNA. While it has been reported that outbreaks or increased HEV infection occur after rainy or monsoon season, this is applicable to HEV-1 and HEV-2 which are transmitted faecal-orally (Kmush et al., 2013). The rainy season in the Western Cape is generally from June to August (World Bank Organisation, 2021). It is possible that HEV-1 or HEV-2 was present in the positive samples and was not sequenced, as only four sequences were obtained from the 21 positive samples. However, since HEV-3 was the only genotype detected, there is not a clear connection between the increased rainfall and detected positive proportion of HEV RNA positive samples seen in September.

5.4. Environmental contamination due to WWTWs in South Africa

Previous studies have compared HEV RNA levels in influent and effluent wastewater to determine whether surface water contamination is occurring in developed nations (Masclaux et al., 2013; Beyer et al., 2020). A Swiss study detected low concentrations of HEV RNA in influent samples (ranging from $1x10^4$ genome equivalent copies/L to $1x10^5$ genome equivalent copies/L) and no HEV RNA in effluent samples from a wastewater treatment plant (Masclaux et al., 2013). They stated that a HEV RNA may have been present in effluent samples, but the concentrations may have been too low to be detected using their methods. In one German study, HEV RNA was detected in 93/111 (84.8%) of influent samples and in 26/83 (31.3%) of effluent samples from the same wastewater treatment plant (Beyer et al., 2020). The authors determined that treatment of the influent samples reduced the concentration of HEV RNA present in wastewater samples. The study from Germany also tested river water samples where effluents would be released and concluded that the detected HEV RNA concentrations were low enough that they did not present an environmental health risk although this can only be definitely confirmed by determining lack of infectivity of the detected virus.

Media reports have highlighted that raw or under-treated wastewater is being released into water bodies in South Africa due to untrained staff and the lack of maintenance at wastewater treatment facilities across the country (Herbig, 2019; Kretzmann et al., 2021; Masweneng, 2021). In the 2021 Blue and Green Drop Project conducted by AfriForum, they found that 122/142 (85.9%) WWTWs assessed in South Africa did not comply with basic quality standards (De Klerk, 2021). The lack of maintenance of WWTWs has resulted in raw or insufficiently treated wastewater being released into the environment (De Klerk et al., 2021; Kretzmann et al., 2021). Dr Jo Barnes, a senior lecturer emeritus in the Division of Community Health at SU, has warned that the level of *Escherichia coli* present in environmental water was an indicator of other pathogens (Kretzmann, 2021). These pathogens may include HEV, Rotavirus, Norwalk, coxsackie virus, *Vibrio cholera*, and *Toxoplasma gondii* (Chahal et al., 2016; Kretzmann, 2021). Based on these reports it is possible that animals and people could be further exposed to HEV through wastewater contamination. To better quantify this risk, effluent wastewater samples would have to be tested for the presence of HEV RNA. However, the detection of HEV RNA in influent samples in this study combined with the known poor status of infrastructure of WWTWs in South Africa could be indicators of serious risk to communities and the environment.

5.5. Strengths of study

Where clinical samples represent a single patient each, a wastewater extract provides data from a larger population. This gives representation on a population level at a relatively low cost compared to clinical studies. Environmental samples also reduce bias due to human behaviour (Kilaru et al., 2021). Not all people in a population have access to healthcare facilities and people would not seek medical care for infections which are asymptomatic or self-limiting (which HEV infection often is). Wastewater surveillance is also non-invasive method to determine the significance of a pathogen based on their circulation in a population. Although we detected HEV-3 in our samples, HEV-1 and HEV-2 are faecal-orally transmitted, and detection of these genotypes in wastewater would provide early warning that outbreaks could occur.

The RNA samples used in this study were already extracted and collected by collaborators at the SAMRC, allowing more time and resources for real-time PCR testing and sequencing. The samples had already been tested for SARS-CoV-2, showing that RNA was successfully extracted and that detection of viral RNA within the extracts was possible. The protocols for HEV RNA testing of clinical sample using real-time PCR were already established in the Division of Medical Virology and NHLS laboratories. Specifically, the cycling conditions and HEV probe were optimised for real-time PCR. While WBE has been applied to wastewater in developed nations, this is not the case for HEV in South Africa. At the time of writing, only one master's study from the University of Pretoria had done a similar project, and that was almost 20 years ago (Williams, 2004). Molecular methods and available kits have improved since this time. Due to the methods being applied in other nations, it was possible to refer to their protocols and insights apply this to the South African context.

5.6. Limitations of study

Although environmental samples could give non-invasive insights into HEV circulation in communities, there are interferents in the samples which inhibit PCR applications and give sequencing results with high levels of noise. Raw wastewater is an environmental sample, and any RNA present must contend with naturally present RNases, humic substances (organic compounds formed when plant and animal matter decompose), organic and inorganic material (Schrader et al., 2012; Ore et al., 2023). This meant that troubleshooting on the real-time PCR assay and nested RT-PCR purification methods were necessary.

The RNA extracts had to be diluted due to use in multiple studies, this decreased the concentration of HEV RNA present in each sample, making it more difficult for the RT-PCR assay to detect despite its relatively low limit of detection. One could argue that dilution of the samples should have reduced the inhibiting effects of contaminants, but also would have had a negative effect on the assay's detection of HEV RNA (Schrader et al., 2012). For these reasons it is more likely that false negative than false positive results may have occurred with real-time PCR testing. Also due to sample sharing, a small volume of each sample was available, making it difficult to repeat testing and sequencing attempts. As the samples were being used after their initial function, it is unknown how many freeze-thaw cycles they went through before their use in this project. Multiple freeze-thaw cycles may compromise the integrity of viral RNA, thus it is possible that HEV RNA which was initially present in certain samples degraded before testing in this study.

In future, it would be ideal to have an equal number of samples from each location which span across a full year. Due to sample availability and retrospective collection, we were unable to compare positive proportions across locations or observe temporal trends in detection in a statistically significant manner. An increased number of samples overall and inclusion of more WWTWs not only in metros which represent urban areas, but also municipalities in rural areas would also have made the study results more representative of the province. Communities which are not serviced by sewers, manholes and WWTWs were not represented in this study, and future applications should incorporate non-centralised sampling strategies to better represent the country's population (Singer et al., 2023).

When determining the limit of detection, raw wastewater from Klipheuwel WWTW was used. The Klipheuwel WWTW serves a much smaller population and was thought to be less likely to contain high levels of viral RNA. The extraction process uses expensive kits and is labour-intensive, and one would only know if the raw sample contained traces of viral RNA after extraction and real-time RT-PCR testing. To make effective use of the available resources, a raw wastewater sample was selected from this smaller wastewater treatment plant based on the assumption that it was unlikely to contain high levels of viral RNA. The sample was HEV RNA negative, and the real-time RT-PCR data from the experiment was used to determine the LOD. In future, it would be ideal to use a raw wastewater sample from each selected location site to determine a LOD accurate to each site.

Due to the broad host range of HEV, and the zoonotic hosts of HEV-3 specifically, one cannot rule out zoonotic contamination of the wastewater samples. Run-off from piggeries, abattoirs, and farms could have introduced animal sources of HEV into the wastewater samples (Treagus et al., 2021). This is less likely to have occurred with the university residence samples, which generated two of the four HEV RNA sequences obtained, but should still be noted.

5.7. Future research

In future, it would be ideal to begin this type of study from the point of collection and extraction. Selection of an extraction method best suited to HEV may yield better real-time PCR and sequencing results. Inclusion of the extraction would also allow for access to the whole undiluted RNA extract. The samples could be tested at multiple dilutions, to assess the trade-off between reduction of PCR inhibition and RNA concentration. It would be ideal to test the samples before their storage at -80°C, as freeze-thaw cycles may lead to RNA degradation, impacting the ability to amplify and sequence the HEV genome. Since HEV-3 was detected in this study, a future study of this nature should include testing water run-off from piggeries or other farms close to the collection sites, to compare these to the sequences found in wastewater.

It would also be beneficial to optimise the amplification of the ORF2 region in wastewater extracts, as it is commonly used for genotyping and would make the study results comparable to HEV sequences obtained in other South African studies. Regarding genomic sequencing, the application of next generation sequencing (NGS) technologies, particularly those based on short reads, may be able to address the issue of sequencing noise encountered in this study and enable genome assembly from HEV-specific sequence reads. An example of this is a 2020 study done in Italy, where PCR amplicons generated from HEV RNA present in wastewater were unable to be sequenced with Sanger sequencing (Iaconelli et al., 2020). The amplicons were sequenced using ultra-deep sequencing on a MiSeq platform (Illumina) and sequences from 74 positive samples were characterised as HEV-3 and HEV-1. Oxford Nanopore Technology, which generates long reads of nucleic acid sequences, has also been used for the detection of antimicrobial resistance genes in wastewater, and to analyse regions of the SARS-CoV-2 genomes from wastewater samples (Dai et al., 2022; Tamáš et al., 2022). The long-read technology makes it possible to generate a full HEV genome sequence if an intact virus is present in a sample.

Chapter 6: Conclusion

The aim of this study was to determine whether HEV was circulating in communities in the Western Cape, by testing for the presence of HEV RNA in wastewater extracts. Our secondary aim was to determine the prevalent genotype among positive samples.

This study was able to detect HEV RNA in wastewater samples, confirming that the virus was present in wastewater at the time of testing and thus was likely being excreted from infected individuals at the selected sites. HEV RNA was detected in at least one sample from every collection site and in nine out of the eleven months covered by samples. This outcome is in alignment with the findings of serological studies conducted in the Western Cape, which determined that exposure to HEV infection was high (Madden et al., 2016; Lopes et al., 2017; Maponga et al., 2020). Furthermore, we were able to obtain sequence of the ORF2/3 region of the HEV genome from four wastewater extracts. Phylogenetic analysis determined that these likely belonged to HEV-3. This is the same genotype reported in case studies of acute and chronic HEV infection in South African patients and has been detected in swine serum and porcine food products in Cape Town (Andersson et al., 2013, 2015; Madden et al., 2016; Van Helden et al., 2017; Korsman et al., 2019). Historically, hepatitis E infections in Africa have been associated with HEV-1 and HEV-2. The detection of HEV-3 in this study further supports that hepatitis E transmission patterns may be evolving, and perhaps zoonotic infections are commonly occurring silently between the epidemics driven by water-borne transmissions associated with HEV-1 and HEV-2. Therefore. it may not be accurate to assume that only HEV-1 and HEV-2 are dominant across a continent.

The study was able to successfully use HEV RNA as a marker and supplement the existing clinical studies on hepatitis E in South Africa. The circulation of the virus among the population should be communicated to clinicians and healthcare workers, so that HEV is not overlooked as a possible cause of acute hepatitis. Hepatitis E should especially be considered in the case of vulnerable groups such as pregnant women, people living with HIV, solid organ transplant recipients, and patients undergoing cancer treatment. The outcomes of this study also contribute to the knowledge of molecular epidemiology of HEV in Sub-Saharan Africa.

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Addenda

Addendum A: Ethics approval from REC:BES

UNIVERSITEIT
STELLENBOSCH
UNIVERSITY

REC: Biological and Environmental Safety

Feedback Letter: *Approved*

11 April 2022

PI: Miss BL Roberts

REC: BES Reference #: BEE-2022-24569

Title: The application of wastewater epidemiology to Hepatitis E virus in South Africa

Dear Miss BL Roberts

Your New Application, with BEE-2022-24569 was reviewed on 31 March 2022 by the Research Ethics Committee: Biological and Environmental Safety **(REC: BES)** via committee review procedures and was approved. Please note that this clearance is only valid for a period of twelve months. Ethics clearance of protocols spanning more than one year must be renewed annually through submission of a progress report, up to a maximum of three years.

Approval Date: **11 April 2022 - 10 April 2023**

Note: The committee wishes to commend the applicant on the quality of the submitted application!

Please remember to use your REC: BES reference number: # BEE-2022-24569 on any documents or correspondence with the REC: BES concerning your research protocol.

If you have any questions or need further help, please contact the REC: BES office at 021 808 9003.

Visit the Division for Research Developments website www.sun.ac.za/research for documentation on REC: BES policy and procedures.

Sincerely,

Mr. Winston BeukesCoordinator: Research Ethics (Biosafety)
E: applyethics@sun.ac.za (Ethics Help-Desk)

Addendum B: Ct values of and details of the samples which tested positively for HEV RNA

Sample Number:	Epidemiological Week:	Collection Date:	Sample Location:	Ct value:
4	4	25-Jan-21	ZV	37.17
8	6	08-Feb-21	ZV	36.74
13	9	01-Mar-21	AT	34.52
30	18	26-Apr-21	ZV	39.60
33	19	10-May-21	AT	41.33
35	20	10-May-21	AT	38.42
36	**20**	**10-May-21**	**ZV**	**37.00**
40	22	24-May-21	ZV	35.97
99	23	08-Jun-21	MH	29.64
155	24	14-Jun-21	MET	40.23
48	26	28-Jun-21	ZV	42.65
51	**28**	**15-Jul-21**	**AT**	**38.41**
163	29	21-Jul-21	MET	38.53
58	31	02-Aug-21	ZV	39.71
67	36	08-Sep-21	AT	40.16
173	**36**	**08-Sep-21**	**MET**	**33.83**
70	37	13-Sep-21	ZV	37.65
174	**37**	**15-Sep-21**	**MET**	**36.41**
175	38	21-Sep-21	MET	37.30
74	39	27-Sep-21	ZV	38.93
178	39	29-Sep-21	MET	40.73

Sample Location Key:
ZV: Zandvliet WWTW
AT: Athlone WWTW
MH: Meerhof Residence
MET: Metanoia Residence

Addendum C: Complete table showing the sample details for every sample which was tested. Samples which gave invalid results are highlighted in orange. Samples which tested positively are highlighted in blue, and their Ct values are shown.

Sample	Collection Week	Collection Date	Collection Site	Test outcome	Ct values (if positive)
1	3	18-Jan-21	Athlone WWTW	Negative	
3	4	25-Jan-21	Athlone WWTW	Negative	
5	5	01-Feb-21	Athlone WWTW	Negative	
7	6	08-Feb-21	Athlone WWTW	Negative	
9	7	15-Feb-21	Athlone WWTW	Negative	
11	8	22-Feb-21	Athlone WWTW	Negative	
13	9	01-Mar-21	Athlone WWTW	Positive	34.52
15	10	08-Mar-21	Athlone WWTW	Negative	
17	11	15-Mar-21	Athlone WWTW	Negative	
19	12	22-Mar-21	Athlone WWTW	Negative	
21	13	29-Mar-21	Athlone WWTW	Negative	
23	14	05-Apr-21	Athlone WWTW	Negative	
25	15	12-Apr-21	Athlone WWTW	Negative	
27	16	19-Apr-21	Athlone WWTW	Negative	
29	17	26-Apr-21	Athlone WWTW	Negative	
31	18	03-May-21	Athlone WWTW	Negative	
33	19	10-May-21	Athlone WWTW	Positive	41.33
35	20	10-May-21	Athlone WWTW	Positive	38.42
37	21	17-May-21	Athlone WWTW	Negative	
39	22	24-May-21	Athlone WWTW	Negative	
41	23	07-Jun-21	Athlone WWTW	Negative	
43	24	14-Jun-21	Athlone WWTW	Invalid	
45	25	21-Jun-21	Athlone WWTW	Negative	
47	26	28-Jun-21	Athlone WWTW	Negative	
49	27	05-Jul-21	Athlone WWTW	Invalid	
51	28	15-Jul-21	Athlone WWTW	Positive	38.41
53	29	19-Jul-21	Athlone WWTW	Negative	
55	30	26-Jul-21	Athlone WWTW	Negative	
57	31	02-Aug-21	Athlone WWTW	Negative	
59	32	09-Aug-21	Athlone WWTW	Negative	
202	33	betw. 15-21/8/21	Athlone WWTW	Negative	
61	33	16-Aug-21	Athlone WWTW	Invalid	
63	34	23-Aug-21	Athlone WWTW	Invalid	
65	35	30-Aug-21	Athlone WWTW	Invalid	
67	36	08-Sep-21	Athlone WWTW	Positive	40.16
69	37	13-Sep-21	Athlone WWTW	Negative	
71	38	20-Sep-21	Athlone WWTW	Negative	
73	39	27-Sep-21	Athlone WWTW	Negative	
75	40	04-Oct-21	Athlone WWTW	Negative	
2	3	18-Jan-21	Zandvliet WWTW	Negative	
4	4	25-Jan-21	Zandvliet WWTW	Positive	37.17

Sample	Collection Week	Collection Date	Collection Site	Test outcome	Ct values (if positive)
6	5	01-Feb-21	Zandvliet WWTW	Negative	
8	6	08-Feb-21	Zandvliet WWTW	Positive	36.74
10	7	15-Feb-21	Zandvliet WWTW	Negative	
12	8	22-Feb-21	Zandvliet WWTW	Negative	
14	9	01-Mar-21	Zandvliet WWTW	Negative	
18	11	15-Mar-21	Zandvliet WWTW	Negative	
20	12	22-Mar-21	Zandvliet WWTW	Invalid	
22	13	29-Mar-21	Zandvliet WWTW	Negative	
24	14	05-Apr-21	Zandvliet WWTW	Negative	
26	15	12-Apr-21	Zandvliet WWTW	Negative	
28	16	19-Apr-21	Zandvliet WWTW	Negative	
30	17	26-Apr-21	Zandvliet WWTW	Positive	39.6
32	18	03-May-21	Zandvliet WWTW	Negative	
34	19	10-May-21	Zandvliet WWTW	Negative	
36	20	10-May-21	Zandvliet WWTW	Positive	37.00
38	21	17-May-21	Zandvliet WWTW	Invalid	
40	22	24-May-21	Zandvliet WWTW	Positive	35.97
42	23	07-Jun-21	Zandvliet WWTW	Negative	
44	24	14-Jun-21	Zandvliet WWTW	Negative	
46	25	21-Jun-21	Zandvliet WWTW	Negative	
48	26	28-Jun-21	Zandvliet WWTW	Positive	42.65
50	27	05-Jul-21	Zandvliet WWTW	Negative	
52	28	15-Jul-21	Zandvliet WWTW	Negative	
54	29	19-Jul-21	Zandvliet WWTW	Invalid	
56	30	26-Jul-21	Zandvliet WWTW	Invalid	
58	31	02-Aug-21	Zandvliet WWTW	Positive	39.71
60	32	09-Aug-21	Zandvliet WWTW	Negative	
203	33	betw. 15-21/8/21	Zandvliet WWTW	Negative	
62	33	16-Aug-21	Zandvliet WWTW	Negative	
64	34	23-Aug-21	Zandvliet WWTW	Negative	
66	35	30-Aug-21	Zandvliet WWTW	Invalid	
68	36	08-Sep-21	Zandvliet WWTW	Invalid	
70	37	13-Sep-21	Zandvliet WWTW	Positive	37.65
72	38	20-Sep-21	Zandvliet WWTW	Negative	
74	39	27-Sep-21	Zandvliet WWTW	Positive	38.93
92	19	12-May-21	Meerhoff Residence	Negative	
93	20	19-May-21	Meerhoff Residence	Negative	
94	21	26-May-21	Meerhoff Residence	Negative	
95	21	26-May-21	Meerhoff Residence	Invalid	
98	23	08-Jun-21	Meerhoff Residence	Negative	
99	23	08-Jun-21	Meerhoff Residence	Positive	29.64
102	25	22-Jun-21	Meerhoff Residence	Negative	
103	25	22-Jun-21	Meerhoff Residence	Negative	
104	26	29-Jun-21	Meerhoff Residence	Invalid	
105	26	29-Jun-21	Meerhoff Residence	Negative	

Sample	Collection Week	Collection Date	Collection Site	Test outcome	Ct values (if positive)
106	27	07-Jul-21	Meerhoff Residence	Negative	
107	27	07-Jul-21	Meerhoff Residence	Negative	
108	28	14-Jul-21	Meerhoff Residence	Negative	
109	28	14-Jul-21	Meerhoff Residence	Negative	
189	28	14-Jul-21	Meerhoff Residence	Negative	
190	28	14-Jul-21	Meerhoff Residence	Negative	
110	29	21-Jul-21	Meerhoff Residence	Negative	
111	29	21-Jul-21	Meerhoff Residence	Negative	
114	31	04-Aug-21	Meerhoff Residence	Negative	
115	31	04-Aug-21	Meerhoff Residence	Negative	
116	32	11-Aug-21	Meerhoff Residence	Negative	
117	32	11-Aug-21	Meerhoff Residence	Negative	
191	33	18-Aug-21	Meerhoff Residence	Negative	
192	33	18-Aug-21	Meerhoff Residence	Negative	
200	34	24-Aug-21	Meerhoff Residence	Negative	
120	34	25-Aug-21	Meerhoff Residence	Negative	
121	34	25-Aug-21	Meerhoff Residence	Negative	
124	36	08-Sep-21	Meerhoff Residence	Negative	
125	36	08-Sep-21	Meerhoff Residence	Negative	
126	37	15-Sep-21	Meerhoff Residence	Negative	
127	37	15-Sep-21	Meerhoff Residence	Negative	
147	18	04-May-21	Metanoia Residence	Negative	
148	19	12-May-21	Metanoia Residence	Negative	
149	20	19-May-21	Metanoia Residence	Negative	
150	21	26-May-21	Metanoia Residence	Negative	
151	22	31-May-21	Metanoia Residence	Negative	
153	23	07-Jun-21	Metanoia Residence	Negative	
155	24	14-Jun-21	Metanoia Residence	Positive	40.23
156	24	16-Jun-21	Metanoia Residence	Negative	
157	25	23-Jun-21	Metanoia Residence	Negative	
158	26	28-Jun-21	Metanoia Residence	Negative	
159	26	30-Jun-21	Metanoia Residence	Negative	
160	27	05-Jul-21	Metanoia Residence	Negative	
161	27	07-Jul-21	Metanoia Residence	Negative	
162	28	12-Jul-21	Metanoia Residence	Negative	
163	29	21-Jul-21	Metanoia Residence	Positive	38.53
164	30	28-Jul-21	Metanoia Residence	Negative	
166	31	04-Aug-21	Metanoia Residence	Negative	
167	32	11-Aug-21	Metanoia Residence	Negative	
169	33	18-Aug-21	Metanoia Residence	Negative	
170	34	24-Aug-21	Metanoia Residence	Negative	
201	34	25-Aug-21	Metanoia Residence	Negative	
171	35	31-Aug-21	Metanoia Residence	Negative	
193	35	01-Sep-21	Metanoia Residence	Negative	
173	36	08-Sep-21	Metanoia Residence	Positive	33.83

Sample	Collection Week	Collection Date	Collection Site	Test outcome	Ct values (if positive)
174	37	15-Sep-21	Metanoia Residence	Positive	36.41
175	38	21-Sep-21	Metanoia Residence	Positive	37.3
176	38	22-Sep-21	Metanoia Residence	Negative	
177	39	28-Sep-21	Metanoia Residence	Negative	
178	39	29-Sep-21	Metanoia Residence	Positive	40.73
180	41	12-Oct-21	Metanoia Residence	Negative	
181	41	13-Oct-21	Metanoia Residence	Negative	
182	42	19-Oct-21	Metanoia Residence	Negative	
183	42	20-Oct-21	Metanoia Residence	Negative	
184	43	26-Oct-21	Metanoia Residence	Negative	
185	43	27-Oct-21	Metanoia Residence	Negative	
187	46	16-Nov-21	Metanoia Residence	Negative	

Addendum D: Table showing the average concentrations and purity ratios of nested RT-PCR products after purification

	ORF1			ORF2/3			ORF2		
No.	**Conc. (ng/µL)**	**260/280 ratio**	**260/230 ratio**	**Conc. (ng/µL)**	**260/280 ratio**	**260/230 ratio**	**Conc. (ng/µL)**	**260/280 ratio**	**260/230 ratio**
4	16.18	2.39	3.92	62.79	4.49	3.79	/		
8	20.19	1.70	2.33	19.86	1.75	2.27	/		
13	23.16	2.99	4.70	20.07	3.54	2.69	/		
30	27.64	2.06	2.62	29.51	2.23	2.77	/		
33	21.43	1.80	1.09	10.50	1.75	0.47	25.46	1.87	1.14
35	7.16	1.52	0.15	8.86	1.62	0.43	13.95	1.80	0.40
36*	10.21	1.63	0.30	40.53	1.79	1.03	28.06	1.83	1.13
40	19.34	2.19	2.07	24.56	2.22	2.48	/		
48	24.76	2.00	1.15	17.89	1.62	1.00	12.18	1.64	0.60
51*	/			24.93	1.64	0.81	9.46	1.59	0.46
58	22.17	1.98	1.29	7.29	1.26	0.32	7.48	1.53	0.27
67	10.93	1.53	0.10	10.28	1.39	0.23	10.71	1.57	0.41
70	/			11.29	1.49	0.83	19.13	1.79	0.43
74	/			16.79	1.81	1.07	8.89	1.75	0.57
99	4.86	1.19	0.07	10.20	1.87	0.36	11.57	1.97	0.69
155	1.44	1.02	0.04	/			10.41	1.90	0.72
163	8.23	1.57	0.27	10.61	1.74	0.91	5.10	2.13	0.50
173*	/			41.73	1.90	1.47	10.67	2.09	0.70
174*	/			25.30	1.86	0.98	10.14	2.17	0.54
175	7.41	1.64	0.06	7.07	1.78	0.18	11.28	2.00	0.63
178	11.04	1.74	0.20	6.93	2.11	0.25	9.97	1.91	0.24

Key:

***: *ORF2/3 sequences were obtained from these samples*

/: Nested RT-PCR could not be performed as sample volume was depleted