A Real-Time Reverse Transcription Polymerase Chain Reaction For The Sensitive Detection Of Hepatitis A Virus In Various Clinical And Environmental Samples.

by
Nothukela Mpazi

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Supervisor: Dr. Nokwazi Nkosi
Co-supervisor: Prof. Corena de Beer

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Abstract

Hepatitis A virus (HAV) is a growing public health concern worldwide due to its contribution to acute viral hepatitis. In South Africa, HAV data has been accounted for in clinical and environmental studies separately. Simultaneous detection of the HAV in clinical and environmental samples in the Western Cape province has not yet been explored. The current serological method of HAV detection has been linked to cross-reactivity between antibodies resulting in false positive results. Supplemental use of real-time reverse transcription (RT) polymerase chain reaction (PCR) may have the benefit of limiting cross-reactivity and serving as an early detection method in outbreak settings as it detects HAV ribonucleic acid (RNA) two weeks prior to seroconversion.

The aim was to assess the presence of HAV (RNA) in various clinical and environmental wastewater samples using an in-house established real-time RT-PCR assay and to describe circulating HAV genotype(s). The objectives were to identify serologically tested HAV residual serum, plasma and stool samples referred to the National Health Laboratory Service (NHLS) Medical Virology Laboratories at Tygerberg Hospital (TBH), Groote Schuur Hospitals (GSH); and Western Cape Blood Service (WCBS); to screen untreated wastewater environmental samples for HAV presence from the South African Medical Research Council (SAMRC); to analyse data by age, sex, liver function enzyme parameters and location of retrieval using Microsoft Excel and to assess circulating genotype (s) through Sanger sequencing.

Two different primers and probes were evaluated for HAV RNA detection in 353 samples comprising of 179 clinical (serum, stool, and plasma) and 174 environmental (untreated wastewater RNA eluates) samples obtained through convenience sampling from TBH; GSH NHLS serology laboratories; WCBS and SAMRC (from five Stellenbosch University residences and two Cape Town areas). BioEdit software; Genome detectives Krisp genotyping tool version 2.43 and Mega 11 software were used to assess HAV genotype(s) on selected clinical samples only.

HAV RNA was positive in 43.6% (78/179) clinical samples and 32.7% (57/174) environmental samples. In the ages younger 15 years HAV RNA positive samples were higher in males in compared to females. In contrast age groups >16 years HAV RNA positive samples were higher in females compared to males. The City of Cape Town areas had a higher detection of HAV RNA positive environmental samples (>40%), while a 28% detection was recorded from the Stellenbosch University student residence samples. The identified circulating HAV genotype was HAV IB.

Using the two primer and probe sets this assay successfully detected HAV RNA in various clinical and environmental untreated wastewater samples offering a 92% and 100% sensitivity for the first and second primer set, respectively. Specificity was 100% and 75% respectively. Performance characteristics of second primer set evaluated limited by sample number evaluated. Using assays with 100% specificity might be used in diagnostic settings as a supplemental confirmatory technique. This study was semi-qualitative in nature, future studies may assess the quantitative aspect and cost related to the implementation of the assay in diagnostic settings. [Words:483]

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Opsomming

Hepatitis A-virus (HAV) is 'n groeiende openbare gesondheidsorg entiteit wêreldwyd as gevolg van sy bydrae tot akute virale hepatitis. In Suid-Afrika is HAV-data afsonderlik in kliniese en omgewingstudies verreken. Gelyktydige opsporing van die HAV in kliniese en omgewingsmonsters in die Wes-Kaap provinsie is nog nie ondersoek nie. Die huidige serologiese metode van HAV-opsporing is gekoppel aan kruisreaktiwiteit tussen teenliggaampies wat lei tot vals positiewe resultate met 'n toetsherhalingsperiode van twee weke. Aanvullende gebruik van intydse omgekeerde transkripsie polimerase kettingreaksie (RT-PCR) kan die ernstig siektes bevoordeel deur die herhalingsperiode van twee weke te beperk, kruisreaktiwiteit te beperk en te dien as 'n vroeë opsporingsmetode in uitbraak-instellings aangesien dit HAV RNA twee weke voor opspoor tot seroomskakeling.

Die doel was om die teenwoordigheid van HAV-ribonukleïensuur (RNA) in verskeie kliniese en omgewingsafvalwatermonsters te bepaal deur gebruik te maak van 'n in-huis gevestigde intydse RT-PCR-toets en om sirkulerende HAV-genotipe(s) te beskryf. Die doelwitte was om serologies-getoetste HAV-serum-, plasma- en stoelmonsters te identifiseer wat verwys is na die Nasionale Gesondheids Laboratorium Dienste (NGLD) Mediese Virologie Laboratoria by Tygerberg-hospitaal (TBH), Groote Schuur-hospitaal (GSH); en Wes-Kaapse Bloeddiens (WKB); om onbehandelde afvalwater-omgewingsmonsters vir HAV teenwoordigheid van die Suid-Afrikaanse Mediese Navorsingsraad (SAMNR) te verkry en te sift; om data volgens ouderdom, geslag, lewerfunksie-ensiemparameters en ligging van herwinning te ontleed met behulp van Microsoft Excel en om sirkulerende genotipe(s) deur middel van Sanger-volgordebepaling te assesseer.

In-huis-intydse RT-PCR-toetse vir HAV-RNA-opsporing is opgestel vir 353 monsters wat bestaan uit 179 kliniese (serum, stoelgang en plasma) en 174 omgewings- (onbehandelde afvalwater-RNA eluate) monsters wat verkry is deur middel van geriefsmonsters van TBH; GSH NHLS serologie laboratoriums; WKBS en SAMNR (vanaf vyf Universiteit Stellenbosch-koshuise en twee Kaapstad areas). Om genotipe(s) en volgordebelynings te assesseer Sanger-volgordebepaling, BioEdit sagteware; Genoom Speurders Krisp genotipering instrument weergawe 2.43 en Mega 11 sagteware is slegs op geselekteerde kliniese monsters gebruik.

HAV RNA was positief in 43.6% (78/179) kliniese monsters en 32.73% (57/174) omgewingsmonsters. HAV RNA positiewe monsters was hoër by mans in ouderdomsgroep 1 tot 15 jaar in vergelyking met vroue; in ouderdomsgroepe >16 jaar was hoër vroulik in vergelyking met mans. Verspreiding van positiewe monsters volgens ouderdom, geslag en lewerfunksie-ensieme is nie vir die omgewingsmonsters geassesseer nie, maar die meeste HAV RNA-positiewe monsters was van die Kaapstad-gebiede (>40%) in vergelyking met die Universiteit Stellenbosch-koshuise (28%). Die geïdentifiseerde genotipe was die HAV IB genotipe wat slegs op kliniese monsters geassesseer is. Die toets het 100% spesifisiteit gebied.

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Hierdie studie was in staat om HAV RNA in verskeie kliniese en omgewings onbehandelde afvalwatermonsters op te spoor deur gebruik te maak van 'n in-huis gevestigde intydse RT-PCR-toets. Sensitiwiteit was 92% en 100% tussen die twee reaksies, onderskeidelik. Die spesifiteit was 100% en 75% onderskeidelik. Prestasie eienskappe van die tweede reaksie geëvalueer beperk deur monster beskikbaarheid.Hierdie toets was egter kwalitatief van aard, toekomstige studies kan die kwantitatiewe aspek assesseer. In diagnostiese laboratorium implementering het hoë kliniese spesifisiteit geskiktheid vir gebruik en aanvulling tot bestaande serologiese tegnieke aangedui.

[Totale woorde:489]

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List of Abbreviations

ABI	Applied Biosystems
ALP	Alkaline phosphatase
ALT	Alanine transaminase
AST	Aspartate transaminase
CDC	Centre for Disease Control and Prevention
CI	Confidence interval
CLIA	Chemiluminescence immunoassay
COVID-19	Coronavirus disease 2019
CRE	Cis-acting replicating element
Ct	Cycle threshold
ddNTP	Dideoxynucleoside triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
ECLIA	Electrochemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EPI-SA	Expanded Programme on Immunisation of South Africa
EQA	External Quality Assessment
GDP	Gross domestic product
GSH	Groote Schuur Hospital
HAM	Hepatitis A Immunoglobulin M
HATA	Hepatitis A total antibodies
HAV	Hepatitis A Virus
HBV	Hepatitis B Virus
HBsAg	Hepatitis B surface antigen
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus

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IC	Internal control
ID	Identifier
Ig	Immunoglobulin
IQR	Interquartile range
IRES	Internal ribosome entry site
LoD	Limit of detection
MEGA	Molecular Evolutionary Genetic Analysis
MS	Microsoft
MSM	Men who have sex with men
NCBI	National Centre for Biotechnology Information
NCR	Non-coding region
NHLS	National Health Laboratory Service
NIBSC	National Institute for Biological Standards and Control
NICD	National Institute For Communicable Diseases
NPV	Negative predictive value
NTC	Non-template control
ORF	Open reading frame
PAHO	Pan American Health Organisation
PC	Positive control
PCR	Polymerase chain reaction
PPV	Positive predictive value
QCMD	Quality Control for Molecular Diagnostics
RIA	Radioimmunoassay
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Reverse transcription
SAMRC	South African Medical Research Council
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
TAE	Tris acetate

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TBH	Tygerberg Hospital
UNFPA	United Nations Population Fund
USA	United States of America
UTR	Untranslated region
UV	Ultraviolet
VP	Viral protein
WBE	Water-based Epidemiology
WCBS	Western Cape Blood Service
WHO	World Health Organisation

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Chapter 1: INTRODUCTION

Hepatitis A Virus (HAV), a member of the *Picornaviridae* family, is of key interest to public health for its contribution to acute viral hepatitis disease burden, a growing health concern worldwide. HAV causes the highly infectious self-limiting viral disease, Hepatitis A. Estimated at 1.4 million cases by the World Health Organisation (WHO), HAV disease contributes to 0.5% of mortality rates recorded annually globally (PAHO, 2016; WHO, 2016). In South Africa, HAV disease contributes to approximately 0.3% deaths yearly and the severity of the disease increases with age (South African Department of Health, 2019). Faecal-oral transmission of HAV often leads to an asymptomatic infection rendering it difficult to detect and account for in epidemiological data. Resultantly, the true burden of HAV disease may not be fully understood due to under-reporting. Global knowledge of the full impact of HAV on public health remains a work in progress.

HAV endemicity is dependent upon a country's socioeconomic status and the state of its sanitary conditions. Higher socioeconomic status and improved sanitary conditions are associated with a lower disease prevalence, while lower socioeconomic status and poorer sanitary conditions lead to a higher HAV prevalence (WHO, 2016). South Africa is a lower middle-income country and is transitioning from hyper-endemicity to intermediate endemicity for HAV (Enoch et al., 2019, Mazanderani et al., 2019). Improved sanitary conditions over the years reduced early childhood HAV infections, while other parts of the population remain susceptible. HAV incidence in South Africa is based on geographical epidemiological studies on clinical and environmental samples as separate studies (Chigor and Okoh, 2012; Mazanderani et al., 2019, Rachida and Taylor, 2020).

The Western Cape (WC) is described as the province with the highest outbreak incidence and low testing rates compared to other provinces in South Africa (Prabdial-Sing et al., 2021). The detection of HAV involves mostly serological testing techniques on clinical samples (Enoch et al., 2019). Simultaneous detection of HAV in clinical and untreated environmental wastewater samples in the WC has not yet been explored using a molecular technique such as real-time reverse transcription (RT) polymerase chain reaction (PCR).

Enzyme-linked immunosorbent assay (ELISA) is a commonly used serological technique for anti-HAV immunoglobulin (Ig) G and IgM (anti-HAV IgG and anti-HAV IgM) which uses an indirect method to detect and measure the presence of anti-HAV antibodies generated by the infected host. The test measures antibody responses against a specific HAV antigen; some serum or plasma samples used in the test contain a mixture of target and non-target

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antigens. The non-target antigens compete with the target antigens and may cause cross-reactivity and lead to false positive results (Nainan et al., 2006). Other potential causes of false positive anti-HAV IgM results include factors such as strong immune responses, cross-reactive antibodies, heterologous reactions to similar viruses and subclinical reactivation of latent viruses or interfering substances including naturally occurring IgM antibodies (Tan et al., 2021). All these might contribute to the limitations of the assay used for HAV detection.

In 6 – 7% of symptomatic patients, anti-HAV IgM ELISA tests may be negative during initial HAV infection before liver enzymes reach peak levels, a period when the infected is highly infectious, and the ELISA test may therefore need to be repeated after two weeks for accuracy (Lee et al., 2008; Shin et al., 2010, Hyun et al., 2012). It is imperative that alternative methods such as real-time RT-PCR are used to limit the need for repeat tests and increase accuracy, especially in severe cases. Detection of HAV in environmental samples, such as wastewater, enables the identification of the virus in cases unaccounted for by laboratory data and the tracking of patterns of disease in specific locations in the province.

Real-time RT-PCR is a molecular technique that combines reverse transcription of ribonucleic acid (RNA) into deoxyribonucleic acid (DNA) with amplification of specified DNA targets in real-time. It detects the viral nucleic acid genome and not the viral antigen, thereby limiting the chances of cross -reactivity. It is a technique that eliminates post-amplification handling and has high sensitivity and a fast turnaround time in the analysis of various sample types. HAV RNA can be detected two to three weeks before the onset of symptoms, therefore real-time RT-PCR is important in diagnostic settings as this enables HAV detection during the window period of infection (Costafreda et. al., 2006). It is essential to understand exposure, the development of clinical symptoms, contact tracing, and the application of fundamental epidemiological principles while employing this technique.

It is important that diagnostic pathology centres or services are able to assess different serological and molecular methods for sensitive detection of the viruses responsible for diseases. The Tygerberg Hospital (TBH) Medical Virology laboratory of the National Health Laboratory Service (NHLS) forms part of the largest diagnostic pathology services in South Africa but has yet to include the detection of HAV using real-time RT-PCR as a routine diagnostic test. This study is proposed to establish the feasibility of incorporating the assay for use within the NHLS Medical Virology laboratory as a diagnostic tool for HAV detection. Additionally, it is aimed at creating awareness of outbreaks by earlier detection.

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To contribute towards the limited information on the impact of HAV in the WC, a small-scale laboratory-based study using a real-time RT-PCR technique for the detection of HAV in multiple clinical and environmental samples from areas in the WC, South Africa, was performed.

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Chapter 2: LITERATURE REVIEW

A brief literature review relevant to the study is given below. This includes a review of the history and origin of HAV, structure and infection, as well as the epidemiology with regards to HAV endemicity globally, extrapolated from human clinical serological and molecular data. An overview of past and current methods of HAV detection and diagnostic techniques, prevalent genotypes globally and HAV treatment is presented culminating in the presentation of the research question, hypothesis, aims and objectives of the study.

2.1 History of HAV

Identification of HAV dates to the time of Hippocrates, though only suspected to be a causative agent for catarrhal jaundice at the time (Cockayne, 1912). The 1700s to 1800s epidemiological studies by Findlay et al. (1932) on the widely spread epidemic jaundice concluded that a virus was the cause of the disease, and it was named viral hepatitis. These observations were based on other known but dissimilar viruses at the time such as rubella and herpes zoster virus.

The spread of viral hepatitis, including HAV, caused high morbidity and mortality during World War I and World War II (Haven, 1944; Neefe et al., 1944). Volunteers were purposely infected with the virus to better understand the epidemiological studies conducted. This unethical approach also had the potential to introduce volunteer bias, as during these experiments volunteers were accepted without prior knowledge of their immune status. The inability of the researchers to determine an immune volunteer prior to infecting them with the virus distorted interpretation of results (MacCallum et al., 1951; Dohmen, 1952). These studies paved way for understanding the incubation period of HAV before its characterisation.

Viral hepatitis was assumed to occur in two forms, "infectious hepatitis form" with a shorter incubation period and "homologous serum hepatitis form" with a longer incubation period (MacCallum, 1953; Krugman et al., 1967). After the identification of the Australian or HM175 strain (175 laboratory accession number) isolated from an infected faecal sample, the "infectious hepatitis form" HAV and the "homologous serum form" Hepatitis B Virus (HBV) were distinguished (Blumberg et al., 1965). HAV was confirmed as an etiological agent of HAV disease in the mid-20th century. Viral isolation enabled HAV electron microscopy characterisation (Feinstone et al., 1973), followed by the molecular description of the HAV genome and structure (Ticehurst, 1983; Sherlock, 1984; Gust et al., 1985).

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2.2 HAV Genome and Structure

The HAV genome aligns with the characteristics of the *Picornaviridae* family in genome size, transmission routes and replication sites (Ticehurst et al., 1983, Cohen et al., 1987, Cuthbert, 2001). Due to specific differences in the genome, HAV was classified as the only species under the *Hepatovirus* genus known to infect humans. The capsid structure, genome sequences, unusual codon usage and physiochemical properties of HAV did not properly correspond with other picornaviruses in the family (King et al., 2012; Wang et al., 2015; Pinto et al., 2018; Stuart et al., 2019). HAV has one serotype and seven genotypes. HAV disease can be caused by three genotypes I, II and III in humans, which can be further classified into sub-genotypes A and B with the most globally reported genotypes I and III (McKnight and Lemon, 2018; Mariojoules et al., 2019).

The HM175 strain has extensively been used to study the biophysical properties and replicative strategies of HAV (Blumberg et al., 1965, Brown et al., 1991, Feng et al., 2013). This strain has a long single open reading frame (ORF) with an internal complex stem-loop which functions as a cis-acting replicating element (*cre*). The ORF is bound to a 5' untranslated region (UTR) segment which is approximately 735 nucleotides long and forms most of the genome which contains around 7500 nucleotides. It also contains an internal ribosome entry site (IRES), as well as a 3'UTR segment which is 63 nucleotides long. Termination is linked to a virally encoded protein associated with RNA synthesis, known as VPg, as well as an elongated poly-A tail (Brown et al., 1991).

These segments have secondary structures which have been linked to host binding, but there is currently insufficient evidence to support this link. The carboxyl-terminal extension of the VP1 forms part of the non-structural section of the genome where the 67 residue polypeptides in the HAV capsid attach. This is one of the differences between HAV and other picornaviruses. This virus lacks antigenic variation which leads to unusual use of conserved codons compared to other picornaviruses (Stuart et al., 2019). Figure 2.1 illustrates the HAV genome organisation, indicating the 5' covalently linked VPg protein, 5'UTR, 3'UTR, and 3' poly (A) tail. The structural proteins from VP4 to VPI under P1 and non-structural proteins from 2B and 3D under P2 and P3.

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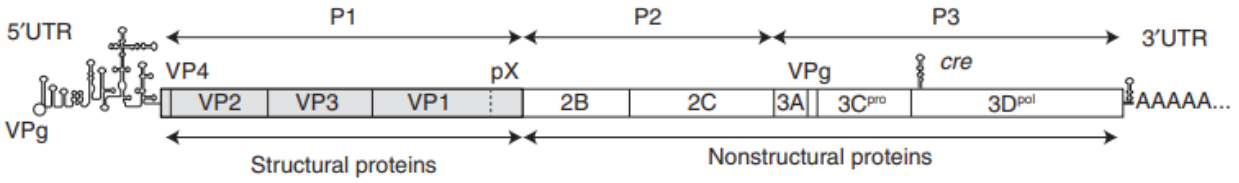

Figure 2.1: HAV genome structure (adapted from Nainan et al., 2006)

HAV has a membranous capsid structure called a quasi-envelope (e-HAV) found only in serum samples compared to the non-enveloped structure identified in stool samples, eHAV might have enabled HAV to evade the immune system and contributed to its genetic and physical stability compared to other picornaviruses (Feng et al., 2013). As there was no other evidence to support these claims, this was the only study reporting on e-HAV conducted only on HAV and no other picornaviruses thus comparable entities might have been missed. The structure of HAV has been used as a model for vaccine development, but there are still a few structural differences between HAV and other picornaviruses in terms of host binding resulting in a continuous lack of in-depth HAV understanding despite availability of active vaccines (Wang et al., 2015).

2.3 Epidemiology of HAV

HAV infects approximately 1.4 million people yearly globally through ingestion of contaminated foods and water or direct contact with an infected person (PAHO, 2016). There is a significant association between the rate of infection and sanitary conditions as well as socioeconomic indicators. High-income countries have incredibly low HAV endemicity (less than 50% of the population seropositive for anti-HAV IgG), whereas low-income regions have high endemicity (more than 90% of the population seropositive for anti-HAV IgG). Both moderate and low degrees of endemicity exist in middle-income countries (WHO, 2016). Most children in highly endemic regions get infected at an early age, usually with asymptomatic infections, but develop lifetime immunity following resolved infection. Some children and adults in low-endemic nations are vulnerable to symptomatic infections (Hutin et al., 2017).

South Africa is classified as a lower middle-income country based on the Gross Domestic Product (GDP) and slow economic growth, high unemployment rates and overall inequality in the country. Population in South Africa has increased by 12% since 1994 (~41 million) with a high rate of rural to urban migration. About 40% of the population lives in urban areas contributing to overcrowding and increasing the chances of being infected with HAV. Rural

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populations tend to be more vulnerable to HAV infection due to poor sanitation which is a problem in South Africa and most parts of the WC province (UNFPA, 2021).

2.3.1 HAV endemicity in the world

Majority of the population in highly endemic countries, such as those in Africa, South America, and parts of Asia, has immunity to HAV infection, and HAV disease related epidemics are thus infrequent compares to low endemic countries in North America, Europe, and Australia (Hutin et al., 2017). In Sub-Saharan Africa, where approximately all the population develops HAV immunity, the prevalence of HAV is highest (Franco et al., 2012; Jefferies et al., 2018). One of the most serious concerns linked with the high HAV prevalence is that individuals in affected regions are unaware of HAV infections and are poorly informed about treatment and prevention strategies (Jefferies et al., 2018).

2.3.1.1 HAV in Asia

HAV epidemiological studies in some South-East Asian countries still lack; with Thailand and Lao People's Republic being the exception. Cross-sectional studies indicate these countries to be transitioning from intermediate to low endemicity for HAV with more than 90% of those aged 60 years and older exposed, and about 50% of the population by age 20 years being anti-HAV IgG seropositive (Sa-nguanmo et al., 2016; Posuwan et al., 2019; Khounvisith et al., 2020). This is in direct contrast to previous cross-sectional studies done two decades ago indicating transition from low to high endemicity for HAV. Under-reporting still remains a limitation in determining the true burden of HAV in these countries (Juffrie et al., 2000; Poovorawan et al., 2009; Posuwan et al., 2019). Singapore, Indonesia, and Malaysia initially reported low endemicity with 50% HAV exposure in individuals aged 25 years and older (Luksamijarulkul et al., 2003; Venugopalan et al., 2004). Over ten years ago a shift from low to highly endemic for HAV was reported by Lee et al. (2011) and Juniastuti et al. (2019). Under-reporting on HAV endemicity in Asia is still problematic as these are the only recently published studies, with occasional reports in the entire continent, this could hinder the WHO's plan for eradicating viral hepatitis by 2030 (Hernandez-Suarez et al., 2021).

2.3.1.2 HAV in South America

An epidemiological study investigating the seroprevalence of anti-HAV IgG antibody and incidence of HAV found countries such as Bolivia, Belize, and El Salvador to have a high to intermediate HAV incidence (Bryan et al., 2001; Campolmi et al., 2013). Low prevalence

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and incidence were observed in countries such as Argentina, Brazil, Panama, many of which had implemented the HAV vaccine in the expanded immunisation programmes over the past two decades (Yanez et al., 2014; Nunes et al., 2017; Souto et al., 2019, Andani et al., 2020). This confirms that active vaccines help lower the infection rates and lower HAV incidence.

2.3.1.3 HAV in Europe, North America, and Australia

In countries with improved sanitary conditions such as parts of North America, Europe, Australia, and parts of Asia, the highest prevalence of HAV disease has been observed mainly among men who have sex with men (MSM), injecting drug users, homeless individuals, and individuals ingesting HAV contaminated foods (Mariojoules et al., 2019; Smith et al., 2019; Webb et al., 2020). This is due to the possibility that the adult and young adult populations may be more susceptible to HAV infection having avoided exposure during childhood (Pisano et al., 2021). In the United States of America (USA) following a 96% decline during the period 1996 to 2011, HAV infection cases increased significantly in 2016 to 2018 following an increase in the number of reported HAV infection cases in the homeless, in injecting drug users and in MSM (Webb et al., 2020).

2.3.1.4 HAV in Africa

Initially regarded as the continent with the highest HAV endemicity, some countries in Africa are transitioning from high to intermediate endemicity for HAV, as numerous countries have made great progress in improving water, sanitation, and socioeconomic growth. As a result, the typical age of initial HAV exposure and infection, as well as the prevalence of HAV infection, are likely to shift because of these advancements (Patterson et al., 2019).

With exceptions from Egypt, Gabon, Nigeria, Somalia, Tunisia, and South Africa most studies on HAV in Africa are from two decades ago (Enoch et al., 2019; Talaat et al., 2019, Ayouni et al., 2021). Multiple cohorts and cross-sectional studies have been conducted to determine the seroprevalence of HAV in parts of the African continent across targeted age groups be they children , adolescent, adult, or combined age groups (Sathar et al., 1994; Ndlovu et al., 2019; Patterson et al., 2019). These studies have been conducted in countries such as Algeria, Cameroon, Egypt, Kenya, Nigeria, Morocco, Tunisia, and Somalia (Forbi et al., 2012; Afegbue et al., 2013). The approach used in these studies was mostly serological assays such as ELISA. Less than five studies utilised real-time RT-PCR and the findings showed a transition in HAV endemicity from high to intermediate with exceptions such as Somalia (Hassan-Kadle et al., 2018). The shortcomings of the studies were the

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failure to extrapolate on the entire populations of these respective countries. Only a few countries have recent peer-reviewed published data, thus implying that the African continent may have a large under-reporting which could also influence the WHO's plan of viral hepatitis eradication in the year 2030.Futhermore vaccinations for HAV in most parts of the African continent is still not part of the expanded immunisation programmes dues to the presumed high HAV endemicity by the WHO (WHO, 2016)

2.3.2 HAV in Wastewater

Wastewater surveillance particularly in South Africa has always been an important tool towards establishing the epidemiology of HAV within different geographical locations in the country. This is supported by the data of studies done from selected sewage areas, faecal-contaminated water, and wastewater treatment plants with varying outcomes indicative of HAV seasonal detection (Adefisoye et al., 2016; Rashida and Taylor, 2020).

Supporting data from several local studies suggested an increasingly high HAV incidence from wastewater in general (treated and untreated) with high HAV detection rates in the winter and spring months (Chigor and Okoh, 2012; Adefisoye et al., 2016). In untreated wastewater effluent HAV has been detected in more than 43% of the samples with viral titres ranging between 1.34×10^5 to 3.70×10^{10} genome copies/L (gc/l) with the circulating genotype IB (Rashida and Taylor, 2020). Other African countries such as Kenya, a country with low or intermediate HAV incidence, reported 88-100% enteric virus detection including HAV with low concentrations detected in rainy than dry seasons (van Zyl et al., 2019). Tunisia, one of the highly HAV endemic regions reported more than 54% HAV detection in untreated wastewater with varying seasonal detection (Kazmi et al., 2021). The mentioned studies account for recent published data on untreated wastewater in Africa. Similar studies have been conducted in other countries including a quantitative HAV study done in Brazil documented that in 54% of the samples HAV was detected particularly in spring and summer seasons (Prado et al., 2012).

In comparison to countries reporting low endemicity such as parts of Europe, the United Kingdom (North Wales), the latter explored HAV wastewater surveillance from wastewater treatment plants, within the study no HAV was detected (Farkas et al., 2018). In a quantitative study done in France, HAV was detected in 59% of samples, with circulating genotype HAV IA (Bisseux et al., 2018).

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This renewed interest in HAV in wastewater-based epidemiology (WBE) stems from the WBE research done on severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) worldwide in countries such as USA, Australia, and France (Ahmed et al., 2020; Wurtzer et al., 2020). There is strong indication of SARS-CoV-2 presence in wastewater, and this creates an awareness of the possible infected population. HAV RNA detection in wastewater may yield favourable results towards early HAV detection within the WC.

2.4 Methods of HAV Detection and Diagnosis

Identifying a virus as the source of an infection or disease is critical as viral infections have similar clinical symptoms, enabling viruses with severe outcomes to be detected earlier for effective treatment. The phase of infection plays a role in determining which diagnostic test to be used (Nainan et al., 2006). During an acute HAV infection, a person's viral load is highest, but as the virus clears, it may be reduced to low or undetectable levels. Anti-HAV antibodies may require weeks to be produced during the primary response to the virus. As a result, the diagnostic test chosen is determined by the patient's infection stage (Costafreda et al., 2003, Nainan et al., 2006). Tests for the virus genome are best performed during the onset of symptoms. Levels of anti-HAV IgM vs anti-HAV IgG against the virus can be used to detect if an infection occurred recently or in the past respectively, although none of these antibodies may be present at the start of a primary infection requiring alternative techniques of detection to be implemented (Hyun et al., 2012).

2.4.1 HAV clinical manifestation and diagnosis

Prior to molecular detection, a clinical diagnosis which is the identification of a cause of a disease by observing displayed clinical symptoms, is required. HAV symptom manifestations usually occur two to six weeks after the incubation period and may persist for more than two months (Nainan et al., 2006; Tom, 2009). As an enteric virus, HAV replicates in the hepatocytes and epithelial cells of the gastrointestinal tract. The virions are then released into the blood and bile, shedding in the stool of an infected patient. Viral shedding of HAV in stool prior to symptom onset increases the risk of transmission during this period (Shin and Jeong, 2018).

Clinical signs of HAV infection or disease include fever, malaise, nausea or vomiting, abdominal pain, dark urine, and jaundice (Armstrong and Bell, 2002). Symptoms such as pruritus, diarrhoea, arthralgia, and skin rash are less common. These are also common

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clinical presentations amongst other viral hepatitis patients and specific HAV diagnostic confirmation is required (Armstrong and Bell, 2002; Shin and Jeong, 2018).

Upon determining clinical symptoms, diagnosis is usually made after serological and biochemical screening tests for assessing liver function by measuring liver enzymes such as alanine aminotransaminase (ALT), aspartate aminotransaminase (AST), alkaline phosphatase (ALP) and bilirubin, total protein albumin and anti-HAV IgM and anti-HAV IgG antibodies.

Normal liver enzyme levels are 7–56 U/L (ALT), 0-25 U/L (AST), 41-133 U/L (ALP) and 2-21 µmol/L (bilirubin) (Nainan et al., 2006). Patients potentially infected with HAV show elevated ALT levels (>500 U/L), AST (>81 U/L), ALP (>133 U/L) and bilirubin levels (>24 µmol/L) (Tom, 2009).

Nainan et al. (2006) indicated that children younger than 6 years old are usually asymptomatic and thus infections may be unaccounted for in epidemiological data. This is supported by finding for the CDC (2020) in which HAV infection rates dropped in all age categories between 2005 and 2009, and they stayed stable from 2010 outbreaks started to be recorded in 2016. Except for those under the age of 20, practically all age groups showed a significant increase in hepatitis A rates between 2017 and 2019. This is consistent with the launch of the hepatitis A vaccine in 1996 and the gradual expansion to a universal childhood immunization recommendation in 2006. The rates of confirmed cases of hepatitis A declined across the board in 2020 compared to rates in 2019, with those aged 30-39 seeing the greatest rates all based on USA study population possibly indicating a need for assessing the role played by age in HAV infections.

2.4.2 Serology and viral culture HAV detection

Serology, the examination of protein antibodies present in blood synthesised by the immune system against diseases, was used in the early attempts to identify individual viruses such as HAV. In the first half of the 20th century, tissue culture, a technique used to grow tissues and cells outside of a living organism in a controlled medium, was developed and refined (Robbin and Enders, 1950; Feinstone et al., 1973). As a result, viruses such as HAV were grown using cell culture, and their pathogenic effects on cells were discovered (Nainan et al., 2006). Serology and tissue culture have both been modified, enhanced, and upgraded over the years and are still important diagnostic tools for viral infections (Nainan et al., 2006; Wine et al., 2015; Louten, 2016). Anti-HAV IgM can be detected from 5-10 days prior to

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symptom onset and can remain positive for up to 6 months. Anti-HAV IgG is then found to persist thereafter, providing immunity for longer (Nainan et al, 2006, Louten. 2016).

Radioimmunoassay (RIA), complement fixation and haemagglutination assays are some of the serological strategies that have been used to detect anti-HAV antibodies historically (Anderson et al., 1986). Most research laboratories currently use commercial enzyme immune assays (EIAs), and assays conducted on random access automated systems to identify anti-HAV antibodies instead due to ease of use and increased accuracy (Ambrosch et al., 1994, Wine et al., 2015). Most recently used assays include ELISA as well as chemiluminescent immunoassays (CLIAs) (Saeed et al., 2020).

In cases where patients have other viral infections such as HBV there is a risk of false positive results on a serological test such as ELISA and studies have shown ELISA tests to be prone to cross-reactivity (CDC, 2005; Valota et al., 2019).Using the example of patients with acute HAV infection, Sakiani et al. (2014) described the phenomena of false-positive antibodies. It was suggested that the most probable cause may be an unusually robust immunological response with polyclonal B-cell activation creating inappropriate antibodies. Other reactive species such as cytomegalovirus and Epstein Barr virus have also been reported to contribute to false-positive results (Valota et al., 2019). Often up to 7% of symptomatic patients display no IgM antibodies when tested using the ELISA test during the initial stage of infection due to viral, host and immunological factors leading to potentially false-negative results (Jung et al., 2010; Lee et al., 2015). All these factors contribute to the limitations that serological tests have in detecting HAV indicating a need for supplemental detection methods for increased sensitivity and accuracy.

2.4.3 Real-time RT-PCR HAV detection

Nucleic acid-based methods have been used to detect HAV RNA and real-time RT-PCR viral RNA amplification is presently the most sensitive method for HAV RNA detection. The conceptual basis of measuring DNA amplification in real-time by fluorescence monitoring was the most significant milestone in PCR use. Fluorescence is monitored after each cycle in real-time during the amplification process, the intensity of the fluorescent signal reflects the instantaneous amount of DNA amplicons in the sample at that exact time (Jean et al., 2001; Costafreda et al., 2006).

Real-time RT-PCR requires no post-PCR processing as all the constituents are included in a single PCR tube and amplicons are measured in real-time, thereby reducing the risk of

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contamination, and increasing the turnaround time significantly. It also offers fast detection and quantification of target DNA sequences as compared to antigen detection in serological assays. The simultaneous amplification and detection of newly produced DNA amplicons allows for a shorter amplification time. Real-time RT-PCR detects HAV RNA in serum from two weeks before and one to two months after symptom onset and this can assist with earlier detection in outbreak settings (Costafreda et al., 2006). A known limitation of real-time RT-PCR is the inability to distinguish between viable or active and inactive viral infections which is why it is essential to understand exposure, the development of clinical symptoms, contact tracing, and the application of fundamental epidemiological principles while employing this technique (Chigor and Okoh, 2012).

Considering the benefits, the use of real-time RT-PCR will be beneficial in the identification of HAV RNA in clinical and environmental samples of diverse types. The method has also been recommended to determine the actual cause of an infection should HAV be suspected. Hiramoto et al. (2021) makes this argument on HBV, Saeed et al. (2016) on Hepatitis E virus and Maki et al. (2020) on Human Immunodeficiency Virus (HIV) coinfections, as using this technique reduces the detrimental and possibly lethal repercussions associated with these viruses during a co-infection.

2.5 HAV Genotyping

Molecular characterisation to determine the circulating HAV strain in various parts of the world has been recorded (Artika et al., 2020). Genotype I sub-genotype A (IA) has been identified in the eastern parts of Asia (Jeong et al., 2021) and IIIA found in the southern parts (Hernandez-Suarez et al., 2021). In Europe, reports of genotype IA correspond with circulating genotype in parts of the South American continent (Bruni et al., 2017; Ndumbi et al., 2018; Mariojoules et al., 2019). According to outbreak reports between 2016 and 2018, in the USA, genotype IB was found primarily in the homeless and injecting drug user population groups (Webb, 2020; Schoch et al., 2021). Although having been linked to genotype IIA like some countries in Europe, most of the South American continent has been classified with genotype IB prevalence especially in MSM cases (Ndumbi et al., 2018).

In Africa, over the past two decades, the pre-existing genotype has been reported as genotype IB across the continent (Patterson et al., 2019). A shift was evident with the identification of new genotype IB strain, which is potentially highly infectious, especially in endemically transitioning countries such as South Africa and Tunisia (Rachida and Taylor,

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2020; Ayouni et al., 2021). The newly circulating genotype IIIA identified in Gabon is hypothesised to attribute to HAV being highly infectious (Abe et al., 2020).

2.6 Prevention and Treatment of HAV

There is no specific antiviral therapy available for HAV. Supportive treatment, including rest, improved sanitation measures, nutritional support, sufficient hydration, avoidance of hepatotoxins and symptomatic treatment of fever is used (Desai et al., 2020). Prevention is the best way to limit HAV infections and inactivated single antigen vaccines have been available since 1992. Vaccines contain purified, formalin-inactivated virions found in cell culture, which are incorporated into an adjuvant of aluminium hydroxide (Nainan et al., 2006). The vaccine triggers a high immune response with an efficiency ranging from 95-98% (CDC, 2005). The HAV vaccine is given from 2 years to older age groups (Prabdial-Sing et al., 2018). The vaccine is administered in two doses, with the second dose after 6 months and it can provide an estimated immunity duration of 20 years (Nelson et al., 2018; Shin & Jeong, 2018).

In South Africa, the HAV vaccine is currently only available in the private health sector and does not form part of the national Expanded Programme on Immunisation in South Africa (EPI-SA) (Lopes et al., 2017).The WHO had classified South Africa as a region that was highly endemic for HAV in the past and thus required no vaccination (WHO, 2016). In other parts of the world vaccinations are focused on at-risk groups such as MSM, drug users (injection and non-injection), the homeless and individuals with chronic illnesses. The latter might cause severe complications with HAV co-infection.

Approved vaccines for preventing HAV amongst these at-risk individuals are Havrix® and Vaqta® which are single antigen activated vaccines (Nelson et al., 2018; Desai et al., 2020). To improve management of viral hepatitis, the WHO in 2016 adopted a sustainable development strategy to help in the global elimination of viral hepatitis by year 2030 (WHO, 2016). However, the strategy seems limited to only HBV and hepatitis C Virus (HCV) with no accounting for HAV (WHO, 2021). Therefore, studies indicating the incidence of HAV in parts of the country are essential in understanding the true burden of HAV.

2.7 Research Question

Is an in-house real-time RT-PCR assay an effective diagnostic method for detecting HAV RNA in various clinical and environmental samples and does detection differ across different age groups, sex, and liver function enzymes?

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2.8 Hypothesis

A sensitive real-time RT-PCR-assay is an effective diagnostic method for HAV RNA detection in various clinical and environmental samples.

2.9 Aims and Objectives

2.9.1 Aim

The aim of this study is to detect the presence of HAV RNA in various clinical and environmental wastewater samples using an in-house real-time RT-PCR assay and describe the circulating HAV genotype(s) in the WC, South Africa.

2.9.2 Objectives

The specific objectives of this study are:

- To identify and collect serologically tested HAV residual serum, plasma and stool samples referred to the NHLS Medical Virology Laboratories at TBH and Groote Schuur Hospitals (GSH) and Western Cape Blood Service (WCBS).
- To develop an in-house semi-qualitative real-time RT-PCR assay to detect the presence of HAV RNA in the identified samples.
- To retrieve and screen South African Medical Research Council (SAMRC) environmental untreated wastewater RNA eluate samples for HAV presence using the assay.
- To analyse and compare the data based on age, sex and liver function enzyme parameters and location using Microsoft (MS) Excel software; and
- To determine circulating genotype(s) in clinical samples through Sanger sequencing technology.

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Chapter 3: MATERIALS AND METHODS

3.1 Ethical Considerations

This project was approved by the Health Research Ethics Committee of Stellenbosch University, South Africa. Reference number S21/02/026 (Addendum A)

3.2 Study Design

This was a laboratory-based cross-sectional study aimed at detecting the presence of HAV RNA in various clinical samples using an in-house real-time RT-PCR assay. Detection of HAV RNA enabled hypothesis testing and addressed the research question.

The cross-sectional study design was selected because data analysis is at a single time point and convenience sampling was used for collection. In contrast to alternative prospective study design such as case-control or cohort study designs which occur over a longer period in terms of data analysis, this study design required no retrospective or prospective following up over time (Wang et al., 2020).

A semi-quantitative approach was used, where the qualitative (demographics, HAV total antibodies [HATA], HAV IgM [HAM] results) and quantitative data (real-time RT-PCR Ct values and liver function enzyme results) were collected and determined at similar time frames.

3.3 Methodology

The methodology was divided into three main stages: sample collection, laboratory analyses and data analyses. Below is a schematic diagram (Figure 3.1) outlining an overview of the steps implemented in this project to achieve the aims and objectives of this study.

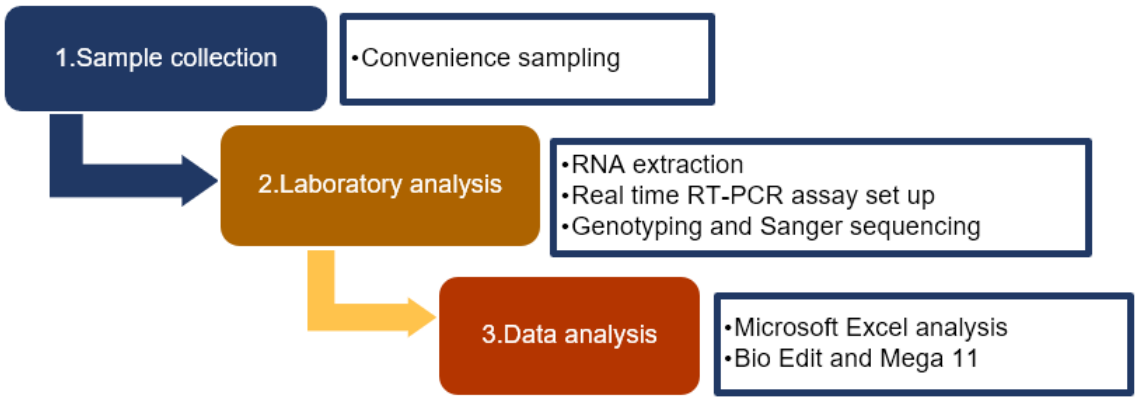

Figure 3.1: Schematic representation of the methodology

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3.3.1 Sample Collection and Sample size

3.3.1.1 Sampling Method

Sample selection is an important aspect of conducting credible research, the generation of research with great validity requires a thorough understanding of sampling design principles used (Moser et al., 2018). The method used for sample collection for the project was non-probability convenience sampling, a sampling method which employs the first primary sampling source accessible without any additional requirements; that is collection of samples anywhere they can be found practically or conveniently where no inclusion criteria is described. This sampling technique enabled easier access to available samples.

3.3.1.2 Sample Collection and Processing of Clinical Samples.

Clinical serum and stool samples collected from two diagnostic laboratories in the WC Province, TBH NHLS Virology laboratory Cape Town, GSH NHLS Virology laboratory Cape Town and plasma samples from the WCBS Pinelands, Cape Town, were selected and included in the study. TBH and GSH Virology laboratories are bio-safety level 2 diagnostic laboratories conducting testing of samples from patients within the surrounding areas in WC, the majority of which are patients at TBH, GSH and Red Cross Hospital and referral district level hospitals.

A total of 107 [TBH (69); GSH (38)] serum and stool samples received from these locations were serologically evaluated using ECLIA. Samples that tested positive for HAM were identified and selected from the NHLS Trak Care database. As part of routine testing the Roche Cobas e601 analyser was used to qualitatively detect the presence of both anti-HAV IgM and anti-HAV IgG. Collection of samples from the NHLS serology laboratory at TBH upon receipt of HAM positive sample list from the NHLS Trak Care system was conducted weekly from May 2021 until June 2022 based on sample availability. Upon collection, samples were stored at -20°C at the Stellenbosch University (SU), Tygerberg Campus Virology laboratory until RNA extraction.

The WCBS is a non-profit, independent organisation operating throughout WC that supplies safe blood and blood products to all communities in WC. Plasma samples tested negative for HIV, HBV and HCV were collected and received in a 72-sample batch and stored at -20°C. The selected samples were unscreened healthy blood donor samples, and as such, their primary goal was to screen for HAV RNA and ensure that the assay could detect HAV even in presumed healthy individuals.

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3.3.1.3 Sample Collection and Processing of Environmental wastewater samples

A total of 174 environmental untreated wastewater samples were collected from the SAMRC. These samples were residual RNA eluates collected from various locations around the City of Cape Town sampling sites and at SU student residences as part of the SAMRC COVID-19 Wastewater studies. The samples were extracted between January and November 2021 at the SAMRC. Upon receipt of available sample sheets, samples from the SAMRC's -80°C storage freezer were collected. 20 µl (5 µl sample diluted in 15 µl nuclease free water [NFW]) retrieved samples from locations (Zandvliet, Athlone, as well as Meerhof, Hippocrates, Metanoia and 3 Susters student residences) were aliquoted into 2 ml centrifuge tubes and transported in ice to the -20°C freezer at SU Medical Virology laboratory until HAV detection was conducted on a weekly basis from August 2022 until September 2022.

3.3.2 Assay Verification and Validation

Diagnostic and analytical sensitivity were determined using HAV Quality Control for Molecular Diagnostics (QCMD) External Quality Assurance (EQA) plasma samples Challenges 1 and 2 (2019) and Challenge 1 (2022), National Institute for Biological Standards and Control (NIBSC) HAV standard, and stool samples from patients with clinical acute HAV infection confirmed by serological testing. Table 3.1 indicates other measures used to verify and validate this assay, such as precision (inter- and intra-assay) accuracy, and specificity for primer sets adapted from Chigor and Okoh (2012) and Saïd et al. (2014). The criteria indicated in Table 3.1 is in reference to criteria used by Rabenau et al. (2007) and Newman and Maritz (2017).

A spike and recovery experiment was done on two environmental samples for validation purposes to determine whether analyte detection is affected by differences between the diluent used to prepare the standard curve and the biological matrix. The procedure involved spiking 100 µl of the NIBSC HAV standard of 26 300 IU/ml (C_t value 33) into 5ml of an HAV unscreened untreated wastewater sample and extracted using the Qiagen RNeasy® PowerSoil® Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. An un-spiked control sample was also included. The extracted samples were then subjected to real-time RT-PCR amplification as will be indicated in Section 3.3.3.2. The recovery was calculated as copies of the sample recovered (C_t values)/ copies spiked (C_t values) ×100 as this was a semi-qualitative approach. This was not done in replicates but as a single real-time RT-PCR run. Table 3.2 illustrates the components in the experiment.

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Table 3.1: Assay verification and validation criteria

Verification and Validation	Calibrator (Specimen)	Sample type	Method of analysis	Samples required for a semi-qualitative assay	Number of samples used in this study
Accuracy (Chigor and Okoh. [2012] primers)	EQA and NFW	Plasma and NFW	% of pos or neg samples	9 (3 pos, 3 low pos, 3 neg) in triplicates for 5 days	3 (3 pos, 0 low pos, 0 neg) in triplicates for 3 days
Intra-assay precision (Chigor and Okoh. [2012] primers)	EQA	Plasma	Mean, standard deviation and % Coefficient of variation	2 (1 pos and 1 low pos) in triplicates for 5 days	3 (3 pos, 0 low pos, 0 neg) in triplicates for 3 days
Inter-assay precision (Chigor and Okoh. [2012] primers)	EQA	Plasma	Mean, standard deviation and % Coefficient of variation	2 (1 pos and 1 low pos) in triplicates for 5 days	3 (3pos and 0 low pos, 0 neg) in triplicates for 3 days
Diagnostic sensitivity					
Chigor and Okoh. (2012) primers	EQA and patient sample	Plasma, Serum, and Stool	% of pos samples	20 (10 pos, 10 low pos)	15 (15 pos, 0 low pos)
Saïd et al., (2014) primers	EQA	Plasma			7 (7 pos, 0 low pos)
Specificity					
Chigor and Okoh. (2012) primers	Patient samples	Plasma, Serum, and Stool	% of neg samples	20 (neg)	15 (neg)
Saïd et al., (2014) primers					3 (neg)
Verification and Validation	**Calibrator (Specimen)**	**Sample type**	**Method of analysis**	**Samples required for a semi-qualitative assay**	**Number of samples used in this study**
LoD Both Primers	HAV NIBSC standard	Serum	Probit analysis	5 concentrations 5 replicates	5 concentrations with 3 replicates

pos- positive and neg- negative, NFW

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Table 3.2: Environmental samples recovery experiment design

Component		Volume (concentration IU/ml)
Spiking sample	NIBSC HAV standard	100 µl (26 300 IU/ml)
Diluent samples	Untreated wastewater sample	5 ml (HAV RNA not detected)

3.3.3 Laboratory Analysis

To generate positive controls and assess the real-time RT-PCR assay performance against a well-established benchmark, QCMD EQA samples for HAV from the 2019 challenges 1, 2 and 2022 Challenge 1 were tested from the TBH NHLS laboratory. The NIBSC HAV-positive standard was later acquired and was used as a positive control. The non-template control (NTC) used was NFW. The QCMD and NIBSC HAV standard samples were stored at -20°C and -80°C respectively until analysis. RNA extractions were performed at room temperature in a Class II Bio-safety Laminaire® cabinet.

3.3.3.1 RNA Extraction

RNA was extracted from 174 (64 serum and 72 plasma) samples using the QIAamp® MinElute® Virus Spin kit according to the manufacturer's instructions (Qiagen®, Hilden, Germany). RNA was also extracted from the QCMD EQA and NIBSC HAV-positive standard samples. Prior to extraction, samples were thawed and centrifuged at 3 000 revolutions per minute (rpm) for three minutes using an Eppendorf centrifuge 5415D (Merck Laboratory Supplies Pty Ltd). The bio-safety cabinets were disinfected with 10% bleach and 70% ethanol prior to use.

RNA extractions were as follows: 200 µl of serum/plasma patient sample was added to 25 µl Qiagen protease in a 1.5 ml microcentrifuge tube for cell lysis. Then, 200 µl of Buffer AL containing 28 µg/ml of carrier RNA was added to the mixture, pulse vortexed for approximately 15 seconds and incubated at 56°C for 15 minutes using a Digital dry bath (Accublock™, Labnet International Inc.). The mixture was briefly centrifuged after the time lapsed, then pulse-vortexed for 15 seconds, after which 250 µl absolute ethanol was added and left at room temperature for five minutes followed by another brief centrifuge. The lysate mixture was added to a QIAamp MinElute spin column placed in a collection tube and centrifuged at 8 000 rpm for one minute. AW1 and AW2 wash buffers (500 µl each) were added to the spin columns sequentially, separated by centrifugation at 8 000 rpm for one

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minute. After AW2 was added, 500 µl of ethanol was added and the spin columns were centrifuged for one minute at 8 000 rpm and subsequently centrifugation for three minutes at 13 200 rpm. The membranes of the columns were dried on the digital dry bath for three minutes. The viral RNA was eluted with 50 µl of Buffer AVE. Extracted RNA was stored at -20°C.

Five stool samples were extracted using the automated NucliSENS® easyMag® (bioMérieux Inc.) instrument to extract the sufficient concentration of the virus from the samples. The NucliSENS® easyMag® benchtop instrument is a second-generation system for automated isolation of nucleic acids from clinical samples based upon silica extraction technology. This extraction method was selected as there was no established extraction protocol for stool samples using the QIAamp® MinElute® Virus spin kit. The apparatus offers a high-quality nucleic acid eluate free of amplification inhibitors and other substances that might affect enzyme substrates, with optimal target recovery. The yield of the extracted samples was 25 µl. In total 179 samples were extracted overall and stored at -20°C until quality assessment and real-time RT-PCR.

The 174 untreated wastewater environmental sample RNA eluates retrieved from the SAMRC premises were extracted using the Qiagen RNeasy® PowerSoil® Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Prior to extraction, untreated wastewater samples were retrieved in a 50 ml centrifuge tube from the torpedo-shaped passive samplers after a weekly wastewater sampling process from selected sampling areas. The samples were then centrifuged at 4 700 rpm for 20 minutes to produce a pellet used for extraction as described by Mangwana et.al. (2022).

The concentration and quality of the RNA extracted from all clinical samples were assessed using the NanoDrop™ ND-1000 spectrophotometer (Thermofisher Scientific, USA). Measuring pedestals were cleaned, and the nucleic acid option in ND-1000 computer software was used. The spectrophotometer was blanked using one µl of elution buffer AVE from the QIAamp® MinElute® Virus spin kit and the pedestal was cleaned before sample loading, then after loading the option for RNA analysis was selected on the software, one µl of the sample was added.

The measure option on the software was selected to read the samples and the concentration in ng/µl, and 260/280 and 260/230 ratios were recorded for each sample. The concentration in ng/µl represents the amount of RNA extracted, 260/280 ratio determined the RNA though measuring light absorption at the wavelengths of 260 nm and 280 nm. A 260/280 ratio of

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1.8 - 2.0 was pure RNA. The 260/230 ratio is a secondary measure of nucleic acid purity that corresponds to light absorption at a wavelength of 260 nm and 230 nm. Acceptable A260/230 ratio is between 2.0 - 2.2; a lower ratio suggests the presence of impurities. After concentration readings the samples were stored at -20°C until downstream processing and the RNA readings recorded in a Microsoft Excel 365 file (Microsoft Corporation, Redmond, Seattle, USA).

3.3.3.2 Real-time RT-PCR Assay

Real-time RT-PCR has fully established itself as an advanced research and diagnostics tool for the detection and quantification of RNA targets. Through use of fluorescent reporter molecules to measure the yield of amplicons during each cycle, as well as the integration of the DNA amplification and detection steps into a single integrated assay, eliminates the need for post-PCR processing and helps generate reliable and repeatable results.

A well-established tool for detecting HAV are serology assays such as the ECLIA format. During the primary response to the infection, however, antibodies can take weeks to develop. As a result, the diagnostic test chosen will be based on the patient's infection stage, and virus testing is frequently performed during the onset of symptoms. This illustrates the advantage of PCR methods, and according to Costafreda et al. (2006) real-time RT-PCR can detect viral RNA two to three weeks before symptoms appear.

In this project an in-house established real-time RT-PCR protocol for HAV detection from clinical and environmental wastewater samples was set up for a total of 353 samples (179 clinical and 174 untreated wastewater eluates). For testing clinical samples, adapted primers and probes based on a peer-reviewed article by Chigor and Okoh (2012) were used. The HAV primers selected were based on a fragment of the highly conserved 5’ noncoding region (5’ NCR), because of its functional structure in the translation and replication processes and a less than 5% maximum nucleotide divergence, this is the most stable region in the genome. As a result, the 5′ NCR was a suitable region for developing an accurate real-time PCR technique for HAV. The Qiagen QuantiFast Pathogen RT-PCR and IC amplification kit (Qiagen, Germany) was utilised in this one-step real-time RT-PCR assay. Figure 3.2 indicates the HAV genome region used for amplification as described by Chigor and Okoh (2012). and Table 3.3 indicate the probe and primers for the clinical samples with F- forward, R- reverse, P- probe.

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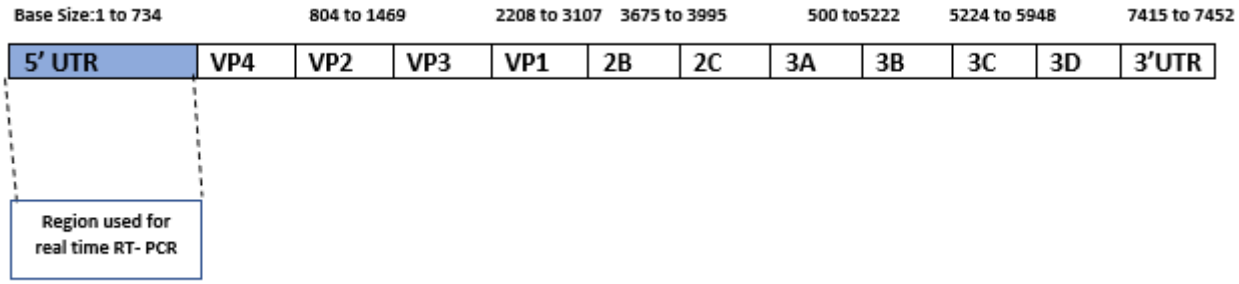

Figure 3.2: Genome region of real-time RT-PCR amplification

Environmental wastewater samples were amplified using primers and probes as indicated in Table 3.4 from a peer-reviewed work by Saïd et al. (2014) targeting the region indicated by the positions on the table.

Table 3.3: HAV Primers and Probes used on clinical sample amplification

Virus	Primers and labelled TaqMan probe	References
HAV	HAV68 (F): 5′-TCA CCG TTT GCC TAG-3′	Chigor VN, Okoh AI. Int J Environ Res Public Health. 2012 Nov 5;9(11):4017-32. doi: 10.3390/ijerph9114017. PMID: 23202829; PMCID: PMC3524610.
	HAV240 (R): 5′-GGA GAG CCC TGG AAG AAA G-3′	
	HAV150 (P): 5′-FAM-CCT GAA CCT GCA GGA ATT AA-TAMSp-3′	

Table 3.4: HAV primers and probes used on environmental sample amplification

Virus	Primers and labelled TaqMan probe	Position	References
HAV	HAV68 (F): TCA CCG TTT GCG TAG	68-85	Saïd R et al. Water Sci Technol. 2014;69(5):923-33. doi: 10.2166/wst.2013.799. PMID: 24622538.
	HAV240 (R): GGA GAG CCC TGG AAG AAA G	222-240	
	HAV150 (P): 5′-FAM-TTA ATT CCT GCA GGT TCA GG-BHQ™	150-169	

The volume of the RT-PCR master mix was calculated according to the number of samples to be used on a specific reaction. Final concentrations were 400 nM for the forward and reverse primers, 250 nM for the probe as adapted from Chigor and Okoh (2012). Stock concentrations were 40 µM for the primers 10 µM for the probe as per manufacture recommendations for QuantiFast Kit. The reactions were 25 µl and were prepared in 2 ml Eppendorf centrifuge tubes with reagents supplied in the amplification kit.

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The clinical samples were initially amplified using the following thermal cycling conditions: RT for three minutes at 50°C, Taq activation for 10 minutes at 95°C with 45 cycles of denaturation for 15 seconds at 95°C, annealing for one minute at 60°C and extension for one minute at 70°C as recommended by Chigor and Okoh (2012). To fit the optimal conditions for the amplification kit used the following conditions were adjusted as indicated in Table 3.5 based on manufacturer's instructions.

The environmental wastewater samples were amplified using optimised thermal cycling conditions by Saïd et al. (2014). The Table 3.5 below indicates the optimised conditions used on amplifying environmental wastewater samples.

All samples were amplified on the Bio-Rad thermal cycler C1000 and connector system for clinical and environmental samples, respectively. Clinical samples were amplified for one hour 45 minutes while the environmental samples were amplified for two hours 42 minutes. The resulting data files were stored and saved on OneDrive until analysis.

Table 3.5: Optimised thermal cycling conditions used for clinical and environmental samples

Cycling conditions	***RT***	***Taq-activation***	***Denaturation***	***Annealing and Extension***
Clinical samples 45 cycles	50°C 30 minutes	95°C 5 minutes	95°C 15 seconds	60°C 30 seconds
Environmental samples 50 cycles	50°C 45 minutes	95°C 15 minutes	95°C 5 seconds	60°C 60 seconds

3.3.3.3 Genotyping and Sanger sequencing

Genotyping was performed on the positive samples to characterise the circulating genotypes. Molecular characterisation was performed using Sanger sequencing technology and the National Centre for Biotechnology Information (NCBI) database. Genotyping viruses is crucial for the effective management of highly infectious viral infections, such as HAV disease. HAV genotypes were subtyped using phylogenetic analysis to differentiate them from one another by comparing recently discovered strains to previously known strains worldwide.

Before genotyping, only clinical samples with real- time RT-PCR amplification result C_t values less than 30 units were selected to increase the chances of detection by agarose gel

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electrophoresis following conventional nested RT-PCR processing. Primers used on the nested RT-PCR targeted the VP1 to P2B junction, shaded in blue in Figure 3.3, and Table 3.6 outlines the primers used according to Hutin et al. (1999).

One-step nested RT-PCR reactions were prepared using the Promega® Access RT-PCR System (Promega®, USA) with the pre-nested step in a 50 µl reaction, a final concentration of 300 nM and 25 µl reactions for the nested step, and a final concentration of 300 nM for the primers.

The thermal cycling conditions for the pre-nested step were as follows: RT step at 50°C for 40 min, Taq activation at 95°C for 15 minutes, followed by 45 cycles of denaturation for 15 s at 95°C, 20 seconds at 50°C for annealing, and extension for 40 seconds at 72°C. The thermal cycling parameters for the nested step included an activation step for 15 minutes at 95°C, followed by 40 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 55°C, and extension for 30 seconds at 72°C. A Simpli Amp thermal cycler (Applied Biosystems, USA) was used to amplify the samples for two hours 30 minutes and one hour 45 minutes for the pre-nested and nested reactions, respectively. The specific instructions are outlined in Addendum B.

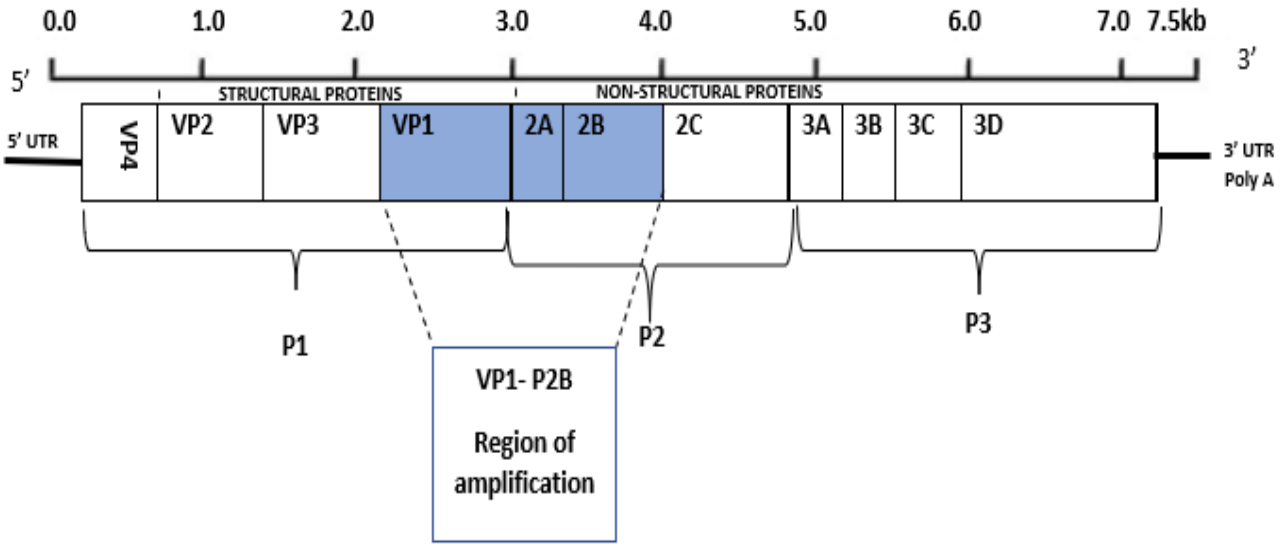

Figure 3.3: RT-PCR target region for clinical samples

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Table 3.6: Primers used for RT-PCR on clinical samples

Genomic region	Oligonucleotides	Position	Sequences
VP1–P2B junction	External forward (+)	2870	5'GACAGATTCTACATTTGGATTGGT3'
	External reverse (-)	3381	5'C CATTTCAAGAGTCCACACACT3'
	Internal forward (+)	2897	5'CTATTCAGATTGCAAATTACAAT3'
	Internal reverse (-)	3288	5'AACTTCATTATTTCATGCTCCT3'

3.3.3.3.1 Agarose gel electrophoresis

Post-amplification, the nested reaction products were subjected to agarose gel electrophoresis using the Enduro™ electrophoresis system. A 2% gel was prepared using Seakem® LE agarose (Lonza, Rockland, Maine, USA) powder into 1X TAE (0.04M tris-acetate; 0.001M EDTA). One part of novel juice, used for both DNA visualization under ultraviolet (UV) light and DNA tracking, was combined with 10 µL of the nested PCR products and loaded on the gel, and a 1 kb DNA ladder (Promega©, Madison, Wisconsin USA) was used to measure the expected 361 bp molecular size of the nested products. All the samples were loaded and run on 1X TAE buffer at 110 V for 45 minutes, visualised using the Bio-Rad Chemi Doc (Bio-Rad Inc., California USA) transilluminator. Specific instructions on gel preparation found in Addendum C.

3.3.3.3.2 Sequencing

DNA sequencing determines the sequence of nucleotides in a DNA sequence. Sequence determination of viral genomes is most performed using the dideoxy-chain-termination technology, also known as the "Sanger Method". DNA templates, primers, DNA polymerase, common deoxynucleotide triphosphate (dNTPs), and dideoxynucleoside triphosphate (ddNTPs) are needed for this technique. Only one of the four dideoxynucleotides (ddATP, ddGTP, ddCTP, or ddTTP) is added to each sequencing reaction created from viral DNA samples to stop DNA elongation. Heat is used to denature the viral DNA into single strands before sequencing. Subsequently, one of the template strands is annealed to a primer. The 3′ ends of this primer are deliberately positioned such that they are near the desired DNA sequence (Artika et al., 2020).

Samples selected to be sequenced were those with detectable visible bands on agarose gel electrophoresis visualised and measured to 361 bp as per the expected amplicon size of

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the nested products. Before sequencing, excess dNTPs, primers, enzymes, and buffers used previously on the samples during RT-PCR were removed to prevent inhibitors during sequencing. The NucleoSpin Gel and PCR Clean-up kit (Macherey Nagel, Germany) was used to clean up the nested PCR products following the manufacturer's instructions. Bidirectional Sanger sequencing was conducted on the ABI 3500XL genetic analyser using an in-house protocol from the Tygerberg NHLS Medical Virology laboratory.

3.3.4 Sequence analysis

Following Sanger sequencing, the trace data files from the sequencer were received in ABI format and exported to Bio-Edit software for chromatogram viewing, trimming and sequence alignment. Files were then saved in FASTA format from Bio-Edit and exported to the NCBI nucleotide basic alignment tool (BLAST) database to find sequences in GenBank that aligned well with HAV strains worldwide. Genotyping was conducted using the Krisp genotyping tool (Genome Detective Virus Tool version 2.43) to ascertain the genetic relatedness of HAV sequence variants and amplicon lengths. A phylogenetic tree was then constructed using Molecular Evolutionary Genetics Analysis (MEGA) 11 software with sequence alignment using Clustal W to establish an evolutionary relationship between the different HAV strains from GenBank.

3.3.5 Quality Control

Several quality control procedures were used to ensure the validity of the experiments. All the clinical and environmental samples were transported in properly sealed tubes on dry ice from their locations and stored until processing at Medical Virology Stellenbosch University. Sample collection was performed weekly, real-time RT-PCR runs were performed weekly, and the amplified samples were stored at -20°C and then moved to -80°C until genotyping and sequencing. To limit contamination, all the working spaces were meticulously cleaned with 10% bleach, followed by 70% ethanol at the start and end of each experiment. Six monthly calibrated pipettes were used, and sterile tips were used throughout. The thermal amplification cycler instruments used were calibrated every six months. The working spaces for RNA extraction, PCR preparation, template addition and gel preparation were all separated from one another. A unidirectional workflow was adhered to.

In addition to ensuring that the extraction and amplification kits worked as intended and the experiments were valid, positive and NTCs were included to ensure that there was no contamination or carryover during the extraction and amplification process. The positive control was initially HAV QCMD EQA from 2019 challenges 1 and 2 (HAVRNA19-QCMD

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samples), which were later replaced with the NIBSC HAV standard. NFW was used as the NTC.

The collection of samples from the NHLS TBH and GSH laboratories was performed by accessing the NHLS TrakCare database and identifying anti-HAV IgM positive results. Demographic data and liver function enzyme results for all samples were extracted from TrakCare and transcribed to an MS Excel 365 spreadsheet (Microsoft Corporation, Redmond, Seattle, USA). Demographic data from the WCBS was provided in a MS Excel 365 spreadsheet file. All patient names were anonymised, and samples were assigned unique identifiers (ID) to ensure confidentiality. The same was performed for the unscreened samples from the WCBS. The IDs also indicated the location of the sample collection site for easier tracking. TBH samples were denoted by a ‘T,’ GSH a ‘G,’ and WCBS by a ‘W.’ The environmental samples were named on the overall data files by location and date of collection in DD/MM/YY (e.g., MET010221) and in the results by location only.

All absorbance results from extractions and real-time RT-PCR results were stored in a MS Excel 365 spreadsheet, which was backed up on OneDrive, an online storage platform accessible to the investigator. Agarose gel electrophoresis images and sequencing files were also stored on OneDrive.

3.3.5.1 Statistical analysis

Descriptive statistics were performed, and all analyses were conducted on MS Excel.

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Chapter 4: RESULTS

The purpose of the study was to assess whether an in-house established real-time RT-PCR method would be able to detect HAV RNA in 353 samples (179 clinical samples and 174 untreated wastewater environmental samples). The aim was to contribute towards the limited information on the detection of HAV in the WC.

Results of the study are given below, including clinical and environmental demographic and epidemiological data respectively; assay verification; overall data on samples HAV RNA detection on clinical and environmental samples across age groups and sexes; distribution of liver function enzymes across age groups on positive clinical samples and results on clinical samples genotyped including the phylogenetic tree analysis.

4.1 Demographic Information

A total of 179 samples were obtained from the NHLS Medical Virology laboratories at TBH, GSH, and WCBS between April 2021 and June 2022, comprising 101 females (56.4%) and 78 males (43.57%) with a median age of 27 [IQR 12- 37] years. A summary of the demographic information is given in Table 4.1, including age, sex, and location of sample retrieval.

Table 4.1: Clinical samples demographic information

Characteristics		Location of sample retrieval		
		TBH (n=73)	GSH (n=34)	WCBS (n=72)
Sex	Female	42 (57.5%)	19 (55.9%)	40 (55.6%)
	Male	31 (42.5%)	15 (44.1%)	32 (44.4%)
Age (years)	1-15	37 (50.6%)	18 (52.9%)	0 (00.0%)
	16-35	25 (34.3%)	14 (41.2%)	27 (37.5.%)
	36-75	11 (15.1%)	2 (05.9%)	45 (62.5%)

A total of 174 untreated environmental wastewater samples from the SAMRC were screened for the presence of HAV RNA. The samples were originally collected from the following locations in Cape Town: Athlone (ATC), Zandvliet (ZTV), and Stellenbosch University student residences Metanoia (MET), Meerhof (MH1 and MH2), Hippocrates (HIPPO), and 3 Susters (3SUS). Table 4.2 outlines the original locations of the samples received and the total number used in this study.

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Table 4.2: Environmental samples across different locations

Locations		Total
Stellenbosch University	3SUS	3
	HIPPO	3
	MH 1	40
	MH 2	33
	MET	15
City of Cape Town	ATC	41
	ZTV	39

4.2 Assay verification and validation

Accuracy, precision, analytical sensitivity/limit of detection (LoD), diagnostic sensitivity and specificity are the performance criteria which were examined for the verification or validation of this assay. Table 4.3 below indicates the results with the percentage of precision; mean; standard deviation and coefficient of variation for intra- and inter-assay precision, efficiency value (E), correlation coefficient (R^2) and slope for analytical sensitivity, and positive predictive value (PPV) and negative predictive value (NPV) for the diagnostic sensitivity and specificity respectively with analysis on both the adapted Chigor and Okoh (2012) and Saïd et al. (2014) primers. Additional data on the calculations and formulae are indicated in addendum D.

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Table 4.3:Assay verification and validation results

Verification/Validation variables	Measure for analysis	Results
Accuracy Chigor and Okoh. (2012)	% of pos (pos/total n)	59.3 (16/27)
	% of neg (neg/total n)	40.7 (11/27)
Precision Chigor and Okoh. (2012)	**Inter-assay:** Mean SD %CV	 1.7 1.3 79.0
	Intra-assay: Mean SD %CV	 1.6 0.4 26.4
Analytical specificity / LoD	E value	327.7% (Chigor and Okoh, 2012)
		93.2% (Saïd et al., 2014)
	R^2	0.3 (Chigor and Okoh, 2012)
		0.9 (Saïd et al., 2014)
	Slope	-1.6 (Chigor and Okoh, 2012)
		-3.4 (Saïd et al., 2014)
	LoD	26.0 IU/ml (Chigor and Okoh, 2012)
		263.0 IU/ml (Saïd et al., 2014)
Diagnostic sensitivity	% positivity	92.3% (Chigor and Okoh, 2012)
		100.0% (Saïd et al., 2014)
	PPV	100.0% (Chigor and Okoh, 2012)
		85.7% (Saïd et al., 2014)
Specificity	% of negativity	100.0% (Chigor and Okoh, 2012)
		75.0% (Saïd et al., 2014)
	NPV	100.0% (Chigor and Okoh, 2012)
		94.4% (Saïd et al., 2014)

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Table 4.4: Recovery experiments real-time RT-PCR results

Sample	Concentration (IU/ml)	Ct value
Spiked sample	26 300 IU/ml (100 µl)	32 to 33
Recovered sample	Not measured	34
Control sample	HAV RNA not detected	0

For the recovery experiments the recovery rate was qualitatively estimated to a 100.00% based on the Ct values which were closer to the Ct values of the spiked sample. Addendum E has the spike recovery amplification charts.

4.3 HAV RNA Detection

4.3.1 RNA Extractions: RNA Quality

The NanoDrop spectrophotometer recorded absorption readings for clinical samples, which were later added to the MS Excel spreadsheet. Absorbance readings with A260/280 and A260/230 ratios within 1.8 - 2.0 indicate pure RNA extracted. Ratios less than 1.8 - 2.2 would suggest the presence of impurities in the extracted RNA sample. Clinical samples had RNA concentration levels ranging from 20.03 ng/l to 206.8 ng/l. Samples had good purity values > 1.8 (260/280), with some exceptions. All data are shown in Addendum F. Environmental sample absorbance data was unavailable for use.

4.3.2 Real-time RT-PCR HAV RNA Detection

In the evaluation of real-time RT-PCR amplification runs, acceptable criteria for HAV RNA positive samples were Ct values between $0<Ct\leq45$ units with internal controls (ICs) Ct values of 30±3 units. HAV RNA negative samples with Ct value of zero and IC values of 30±3 units were acceptable. A valid real-time RT-PCR run was considered when the positive control (PC) was positive and the non-template control (NTC) was negative, an invalid run had a negative positive control (PC) and positive NTC and or absent NTC, invalid runs were repeated and only the repeated runs were included in results. The internal control is an added measure to ensure correct interpretation of results and exclude the presence of PCR inhibitors An example of a valid real-time RT-PCR run is shown in the Figure 4.4 indicating the amplification plot with the relative fluorescent units (RFU), cycle number and amplification plots. Successfully amplified samples with the positive HAV RNA target (in

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blue) and ICs (green) and an indication of the threshold and negative samples. Overall data on the Ct values of all the samples in the study are recorded in Addendum G.

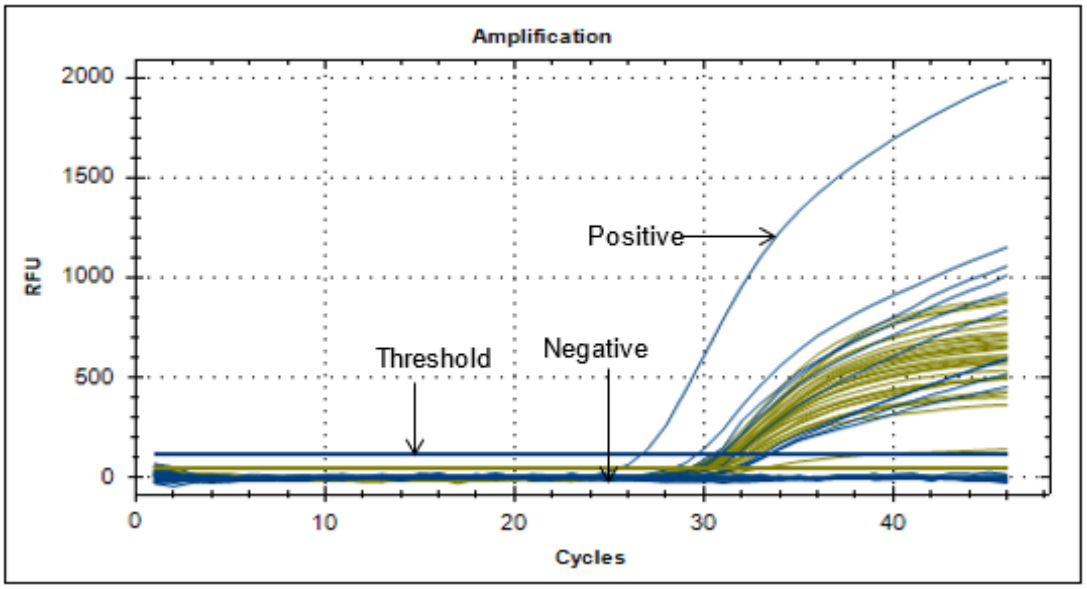

Figure 4.1: An example of a valid real- time RT-PCR run using criteria above

4.3.2.1 HAV RNA Detection on Clinical Samples

HAV RNA was positive in 78/179 (43.6%) clinical samples. Within the HAV RNA positive sample category, 45/78 (58.0%) were females and 33/78 (42.0%) males with the median age of 14 (IQR 7-28] years. 53/78 (68.0%) HAV RNA positive samples were retrieved from TBH and 25/78 (32.1%) from GSH. No HAV RNA was detected from WCBS samples.

HAV RNA was negative in 101/179 (56.4%) of samples. Within the HAV RNA negative sample category, 56/101 (55.4 %) were females and 45/101 (44.6%) were males. 20/101 (19.8%) HAV RNA negative samples were retrieved from TBH, 9/101 (8.9%) from GSH and 72/101 (71.3%) from WCBS. The summary of the overall distribution of HAV RNA positive and negative clinical samples utilising the 179 data set based on sample type and demographics such as sex, age, location, and the p values are given in Table 4.5 below.

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Table 4.5: HAV RNA detection outcomes across sex, age, and location

Variables		HAV RNA positive/Total (%)	HAV RNA Negative/Total (%)	~~P~~
Sample type	Serum	73/102 (71.6)	29/102 (28.4)	~~0.5~~
	Stool	5/5 (100.0)	0/5 (00.0)	
	Plasma	0/72 (00.0)	72/72 (100.0)	
Sex	Females	45/101 (44.6)	56/101 (55.4)	~~0.1~~
	Males	33/78 (42.3)	45/78 (57.7)	
Age (years):	1 to15	41/55 (74.5)	14/55 (25.5)	~~0.1~~
	16 to 35	28/66 (42.4)	38/66 (57.6)	
	36 to 75	9/58 (15.5)	49/58 (84.5)	
Location	TBH	53/73 (72.6)	20/73 (27.4)	~~0.2~~
	GSH	25/34 (73.5)	9/34 (26.5)	
	WCBS	0/72 (00.0)	72/72 (100.0)	

The percentage of HAV RNA positive samples are illustrated in Figure 4.2 with indication of the positive samples between the sexes and across different age groups.

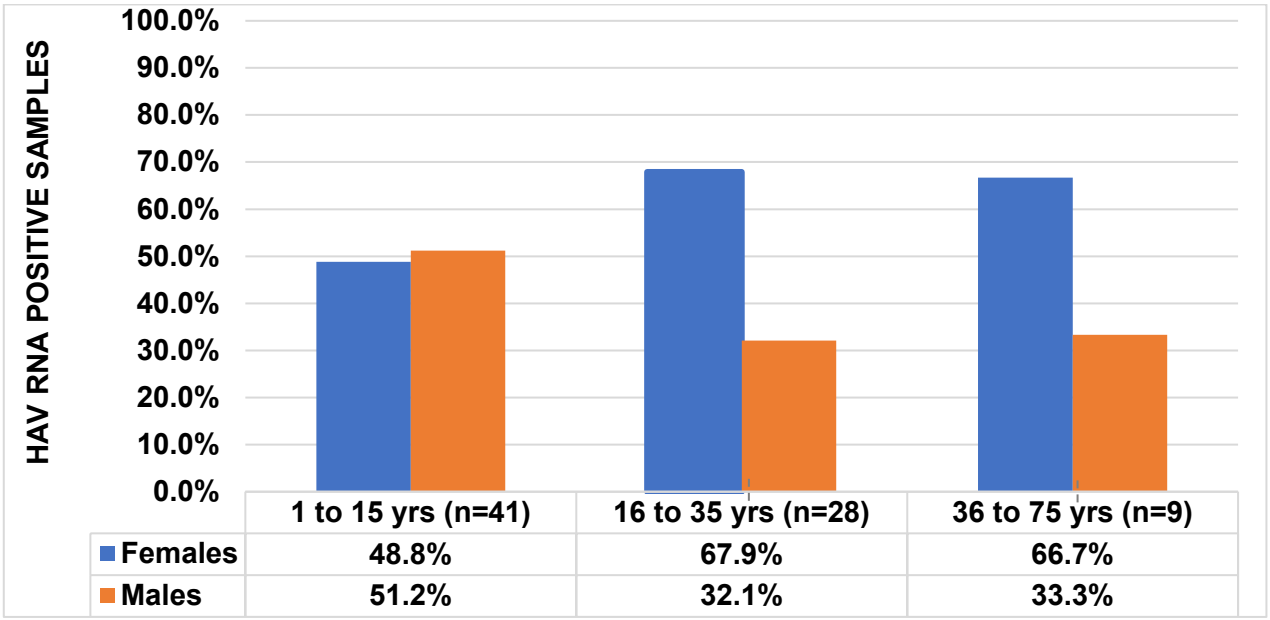

Figure 4.2: HAV RNA positive samples by age and sex

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Liver function enzyme levels data was assessed on HAV RNA positive samples (n=78). ALT, AST and ALP recorded levels were assessed across all ages. Figure 4.3 below illustrates the distribution of liver function enzyme levels by age.

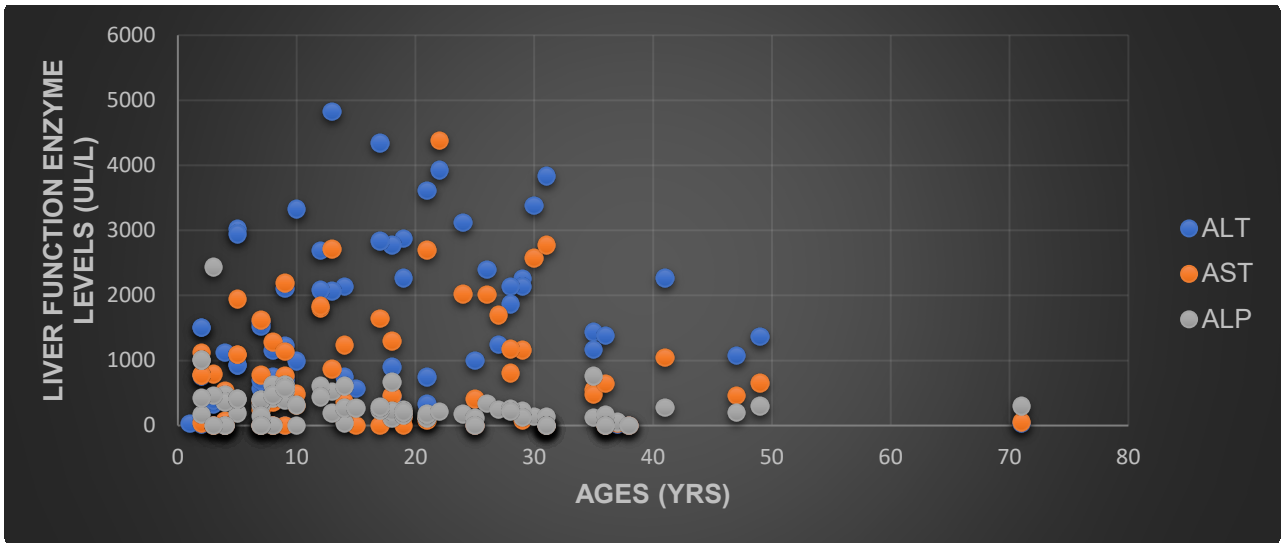

Figure 4.3: Distribution of liver function enzymes ALT(blue), AST (orange) and ALP (g) across all ages in HAV RNA positive samples

4.3.2.2 HAV RNA Detection on Environmental Wastewater Samples

A total number of 174 wastewater samples were screened for HAV presence from locations indicated in Table 4.6 over a one-year period including the summary of the distribution of all HAV RNA positive and negative results. A total of 57/174 (33.0%) samples were HAV RNA positive, and 117 (67.0%) samples were HAV RNA negative. Figure 4.4 below indicates the percentage distribution of the HAV RNA positive samples based on location of retrieval.

Table 4.6: HAV RNA detection in environmental samples across different locations

Location	HAV RNA positive/Total (%)	HAV RNA negative/Total (%)
3SUS	1/3 (33.3)	2/3 (66.7)
HIPPO	1/3 (33.3)	2/3 (66.7)
MH1	11/40 (27.5)	29/40 (72.5)
MH2	7/33 (21.2)	26/33 (78.8)
MET	4/15 (26.7)	11/15 (73.3)
ATC	16/41 (39.0)	25/41 (61.0)
ZTV	17/39 (43.6)	22/39 (56.4)

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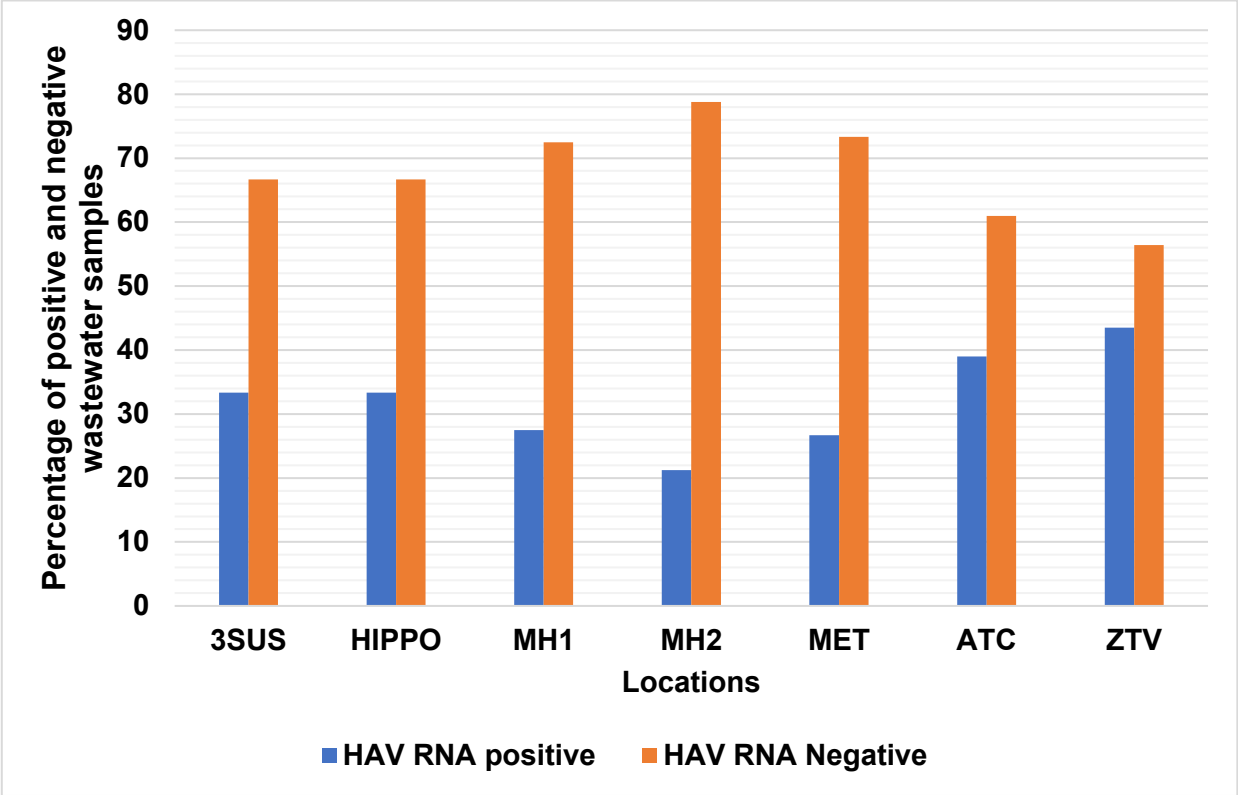

Figure 4.4: Percentage of HAV RNA positive environmental samples by retrieval location

Further distribution of the HAV RNA positive environmental samples (excluding 3 SUS) is illustrated in the epidemiological spot map below indicating areas in and around the City of Cape Town (Figure 4.5).

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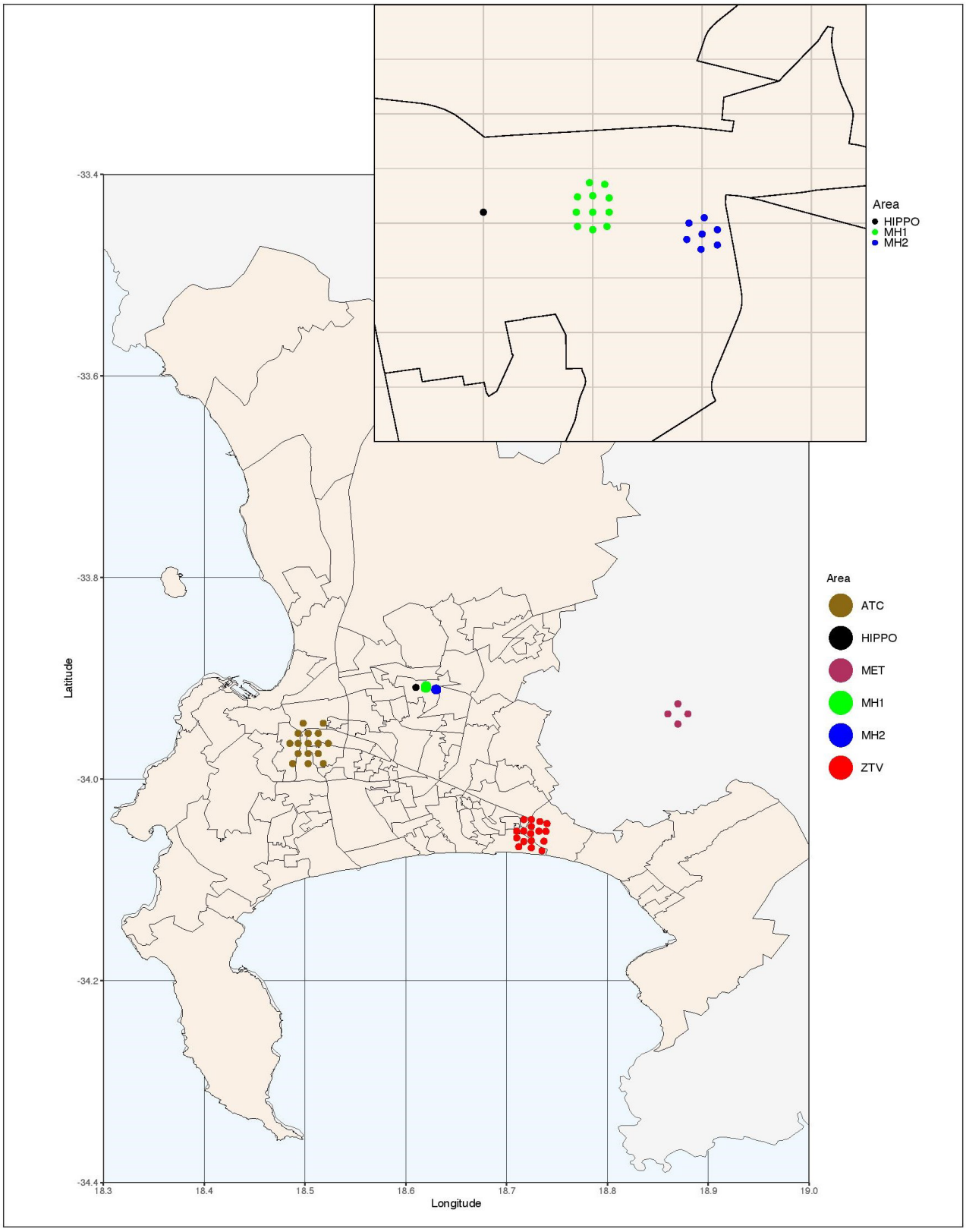

Figure 4.5: Epidemiological spot map for environmental HAV RNA positive samples drawn to scale using codeafrica.com

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4.4 Genotyping

Conventional RT-PCR for HAV detection amplifying target region VP1-P2B (Figure 3.3) was conducted in only 50 of the 78 positive clinical samples. Fifty samples were selected with RT-PCR Ct values less than or equal to 30 to increase the likelihood of successful batch amplification. Following agarose gel electrophoresis, 40/50 (80%) of the amplified samples were positive, as shown in Figure 4.6. Some of the gel images are compiled and presented in Addendum H.

Of the 40 successfully amplified samples, six samples were successfully sequenced and genotyped using the KRISP genotyping tool (Genome Detective Virus Tool version 2.34). Table 4.7 below indicates the HAV genotypes of sequenced samples with bootstrap support. Refer to NanoDrop readings, absorbance values and KRISP genotyping results (Addendum I).

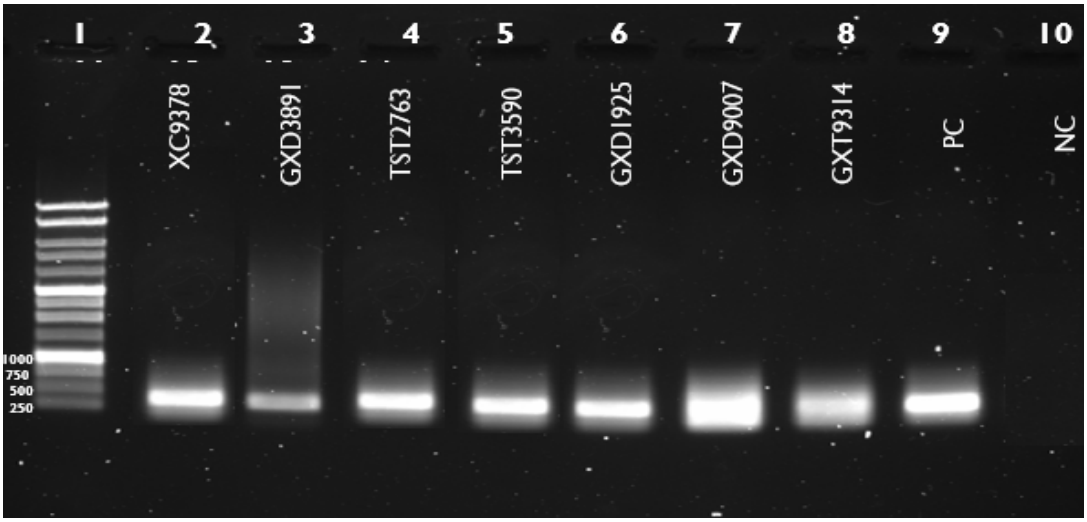

Figure 4.6: Clinical samples RT-PCR amplicon visualisation

Table 4.7: Genotyping outcomes for clinical samples

Sample ID	*Genotype assignment*	*Bootstrap support*	*Reference sequence*
TXG9563	HAV I. B	74.0	NC_001489.1
GSG2839	HAV I B	78.0	
TST0533	HAV I. B	72.0	
TST9201	HAV I. B	83.0	
TXN5226	HAV I. B	73.0	
GXD5547	HAV I. B	83.0	

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4.5 Phylogenetic analysis

The six sequenced samples were aligned and compared with available sequences in GenBank, 32 (more than 97% similar) sequences were matched and aligned. Evolutionary history was inferred using the neighbour-joining method. The optimal tree is shown in Figure 4.7.

The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1 000 replicates) is shown next to the branches. The tree was drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura 3-parameter method and are in the units of the number of base substitutions per site. This analysis included 26 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion). Evolutionary analyses were conducted using MEGA11.

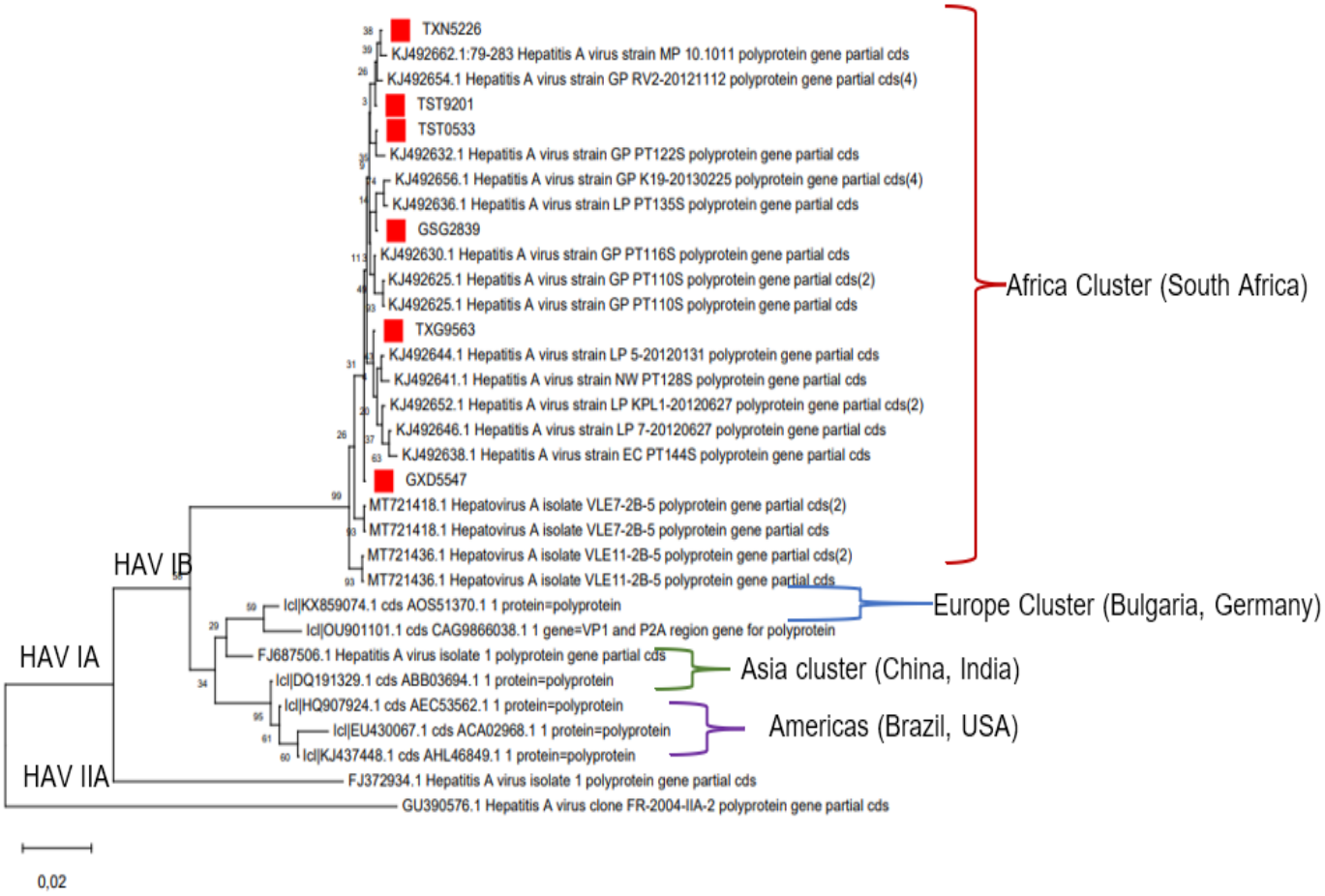

Figure 4.7: Phylogenetic representation of circulating HAV strain on genotyped clinical samples

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4.6 Quality control

The use of QCMD HAVRNA EQA samples from challenges 1, 2 (2019) and HAVRNA challenge 1 (2022), NIBSC HAV standard and their detection is outline in Table 4.8. The table further indicates study performance against the QCMD reference panel sample composition and HAV RNA genotype.

Table 4.8: EQA and standard detection using real-time RT- PCR assay

EQA Challenge/ Standard (Primers used)	Sample names	QCMD panel report	Detection in this study (Positive or Negative)
1 Adapted (Chigor and Okoh, 2012)	HAVRNA19 C1-01	Positive, Genotype IIA, core sample	Positive
	HAVRNA19 C1-02:	Positive Genotype IB-educational sample (dilution of HAVRNA C1-03)	Positive
	HAVRNA19 C1-03:	Positive, genotype IB, core sample	Positive
	HAVRNA19 C1-04:	Negative, core sample.	Negative
2 Adapted (Chigor and Okoh, 2012)	HAVRNA19 C2-03:	Positive, Genotype IB, core samples	Positive
	HAVRNA19 C2-04:	Positive, genotype IIA, core sample	Negative
3 Adapted Chigor and Okoh, 2012)	HAVRNA22 C1-01:	Negative, core sample	Negative
	HAVRNA22 C1-02:	Positive, genotype IB, core sample	Positive
	HAVRNA22 C1-03:	Positive, genotype IB, core sample	Positive
	HAVRNA22 C1-04:	Positive, genotype IB, core sample	Positive
1 (Saïd et al., 2014)	HAVRNA22 C1-01:	Negative, core sample	Positive
	HAVRNA22 C1-02:	Positive, genotype IB, core sample	Positive
	HAVRNA22 C1-03:	Positive, genotype IB, core sample	Positive
	HAVRNA22 C1-04:	Positive, genotype IB, core sample	Positive
NIBSC HAV standard (both primers)	Standard	Genotype IB	Positive

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Chapter 5: DISCUSSION

HAV disease is one of the main causes of public health concern in South Africa, thus testing laboratories and clinicians must notify the South African National Institute for Communicable Diseases (NICD). These diagnostic public laboratories comprise the NHLS, which provides services to almost 80% of South African residents. South Africa's national surveillance of HAV infections is crucial for identifying outbreaks, tracking age-patterns in disease frequency, and intervening to interrupt transmission chains. In this study, an in-house real-time RT-PCR assay was used to detect the presence of HAV RNA in various clinical and untreated wastewater environmental samples. This was performed to assess the suitability of the assay for use within the SU Medical Virology NHLS laboratory. Assessment of frequency of HAV in terms distribution of the tested samples by sex, age, liver function enzymes, various locations and the circulating genotype(s) additionally contribute towards the body of knowledge with regards to HAV incidence in the WC.

Real-time RT-PCR has the added advantage of accurately determining the presence of the HAV RNA target, compared to serologic ELISA tests, which are prone to cross-reactivity and only detect antibody responses (Nainan et al., 2006; Tan et al., 2021). Since viral RNA can be found in patients with HAV disease 17 days before and up to 79 days after liver enzymes reach peak levels (Costafreda et al., 2006), real-time RT-PCR assays can be utilised to supplement serological data.

The results of this study are discussed in this chapter with a focus on assay verification results; HAV RNA detection in clinical samples by age, sex, and liver function enzymes; HAV RNA detection in environmental wastewater samples by location; phylogenetic analysis; study strengths and limitations; and future recommendations.

5.1 Real-time RT-PCR Assay Verification

The suitability of an assay for use after being established, optimised, and standardised is determined through the verification procedure. The samples or methods used should be verified in all diagnostic laboratory assays (Šimundić, 2009). In this study, estimates of the analytical and diagnostic performance parameters of this in-house assay were included in the verification process, including clinical sensitivity, specificity, and analytical sensitivity (LoD), precision and accuracy for two primers sets, the adapted Chigor and Okoh (2012) for clinical samples and Saïd et al. (2014) for environmental samples.

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5.1.1 Diagnostic performance of the assay

Diagnostic sensitivity refers to the percentage of true positive tests detected by the assay in all patient samples with a clinical condition (Šimundić, 2009). Within the conditions of this study, the adapted Chigor and Okoh (2012) primers and probes indicated that these primers were better suited for HAV exclusion than the Saïd et al. (2014) primers and probes because of the 25% higher specificity observed compared to the Saïd et al. (2014) primers and probes, 75% specificity. The lower sensitivity of the adapted Chigor and Okoh (2012) primers might also be attributed to poor primer design due to possible template and primer mismatches (Persson et al., 2019; Gohl et al., 2021).

Differences in sensitivity could also be a result of different amplification regions targeted by the primers and probes, while comparing different HAV primer designs. Costafreda et al. (2006) indicated that differences in the target regions of the primers and probes could influence sensitivity. The adapted Chigor and Okoh (2012) primers and probes indicated a 59.26% accuracy with a similar mean in precision between the inter- and intra-assay comparisons, but with a higher inter-assay coefficient variation in the inter-assay than the intra-assay. This might be due to the insufficient 20 replicate samples required for assessing precision. The simple method for determining accuracy for any given level for evaluating an assay's precision is to do 20 replicate analyses in a single run on a single day. Similar to that, the inter-assay precision is calculated by taking 20 measurements of a sample over several days (Chesher, 2008). Unfortunately, the technique used in this investigation was inadequate and may have underestimated precision as a result of low sample size and reagent limitations accuracy and precision for the Saïd et al. (2012) primers was not included in the study.

Specificity refers to the percentage of true-negative tests detected by the assay for all patient samples without a clinical condition (Šimundić, 2009). Other prevalent hepatitis viruses that can exhibit symptoms comparable to those of acute HAV infection did not cross-react with this assay. The high specificity obtained from both primers sets indicated the ability of the primers to differentiate and detect only HAV RNA among other viruses such as HIV, HCV, and HBV. The WCBS samples did not contain HAV RNA as a result of the source of the samples which are presumably healthy individuals. However, the absence of the clinical history of the individuals from which the samples were retrieved is hindrance on inferring the actual state of health of the individuals.

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The varying high PPV and NPV in both primer sets indicated the ability of the assay to positively predict a positive HAV disease case after a HAV RNA positive test in done and also indicated the minimisation of possible false-positive results when using the assay which is essential for a highly contagious disease, such as HAV disease. The NPV and PPV of an assay may vary among different study populations with different disease prevalence rates. Kozak et al. (2022) in a USA population recorded 100% PPV and 92%% NPV similar to the recorded results on the adapted Chigor and Okoh. (2012) primers. When a population has a high prevalence of the disease, PPV usually increases, while the NPV declines (Trevethan et al., 2017). Thus, the results of the predictive values cannot be generalised across populations with different disease prevalence rates which were not included in this study.

5.1.2 5.1.2 Analytical performance of the assay: LoD

The NIBSC HAV standard was used to determine the analytical sensitivity of the assay, with the LoD being 236 IU/ml and 26 IU/ml using Saïd et al. (2014) and adapted Chigor and Okoh (2012) primers, respectively. The validity of the PCR runs used to determine the LoD should be considered when determining the true LoD among the different primer sets. A valid real-time PCR run should have an efficiency value (E-value) of 90-110%, corresponding to a slope of between -3.33 and -3.6. The correlation coefficient (R^2) was closer to 1, indicating good linear correlation (Larionov et al., 2005). For the Saïd et al. (2014) primers, the efficiency was between 90 and 100%, the R^2-value was closer to 1 (0.99), and the slope was -3.40. This indicated that the LoD obtained from the reaction was valid for use. The adapted Chigor and Okoh (2012) primers had an efficiency greater than 110%, lower R^2 values, and an inaccurate slope, indicating possible pipetting errors and poor precision, which might have interfered with the validity of the PCR run.

However, the obtained LoD from the adapted Chigor and Okoh (2012) primers was considered (26 IU/ml) based on amplification of the NIBSC genotype IB HAV RNA standard, indicated a high analytical sensitivity but with lower efficiency and R^2 value. Costa-Mattioli et al. (2002) in their HAV RNA quantification study obtained through real-time RT-PCR define a LoD of 72 copies/ml. Similarly, Costafreda et al. (2006) optimised the primers used by Costa-Mattioli et al. (2002) and recorded a LoD of 203 copies/ml. In a recent HAV detection study, Kozak et al. (2022) determined the LoD to be 430 copies/ml. Factors such as sample integrity (which may contain PCR inhibitors), PCR primer and probe design (the primers are not optimal), extraction platform and inaccurate pipetting (sample or reagent) could have influenced the efficiency of the adapted Chigor and Okoh (2012) primers.

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5.2 HAV RNA Detection Clinical Samples

HAV RNA was detected in 43.6% of the clinical samples, a higher proportion of HAVRNA was detected in males than in females, however, the difference was not statistically significant ($p>0.05$). These samples comprised plasma samples of unknown anti-HAV IgM status, anti-HAV IgM-positive serum samples and stool samples. HAV RNA was not detected in plasma samples.

Failure to detect HAV RNA in the WCBS plasma samples could have been a result of including presumably healthy blood donor samples, but since serological anti-HAV IgM and anti-HAV IgG identification was not conducted, the argument is still open to interpretation. These samples provide a valid control cohort in this study that provide validity of test method in the assessment of assay specificity.

The first stage in every real-time RT-PCR assay is the collection of the sample and the purification of RNA. The quality of the RNA template or eluate is the most crucial factor in determining the reproducibility and significance of the subsequent real-time RT-PCR results. All issues that impair reproducibility, and thus the usability of PCR results, result from errors during extraction; thus, haemolysed samples were excluded from extractions to prevent interference during amplification.

The measured RNA produced good absorbance values thus confirming that good quality RNA was obtained, and the exceptions did not interfere with the downstream processes as HAV detection was conducted on all samples, despite low RNA quality. The performance of the real-time RT-PCR assay depends on the purity and integrity of RNA. Beginning with low-quality RNA could negatively affect the outcomes of subsequent applications (Fleige et al., 2006).

HAV RNA detection using real-time RT-PCR in the WC or parts of South Africa has been mostly described in environmental RT-qPCR studies; thus, comparison with South African-based real-time RT-PCR studies is limited. One study that determined whether gastroenteritis and other enteric viruses could be detected in faecal specimens collected with Bio-wipes reported HAV RNA in 2.1% (4/190) of samples (Mans et al., 2014). The collection method, sample types, and analysis methods differed from those used in this study. The sample population was not extrapolated to a larger population, as the study was conducted only in the Limpopo province within a single hospital.

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In Rio de Janeiro, South America, De Paula et al. (2004) similarly used real-time RT-PCR to determine the presence of HAV in patients with acute HAV infections and reported a positivity rate of 26% (12/46). The sample size was small and should thus not be extrapolated to the larger population, but the advantages of real-time RT-PCR's ability to detect HAV RNA prior to seroconversion were acknowledged. Higher HAV detection rates of 88% (46/52) (95% CI: 76-95) and 95% (95% CI :87.2–98.6), were reported in an assay using serum and stool samples by Persson et al. (2020) and Kozak et al. (2022). These studies were reported from Sweden and Canada respectively, countries which are considered low-endemic regions with frequent outbreaks, hence the possible high detection of HAV.

Though not using real-time RT-PCR for HAV detection, using serological methods the seroprevalence of HAV in the WC has been recorded closer to the HAV RNA detection percentage in this study; such studies reported a 44.1% and 26% HAV seroprevalence (Enoch et al., 2019; Mazanderani et al., 2019). This detection method differs from that used in this study. Therefore, comparisons are limited with no stratification by gender. Studies in low HAV endemic regions, such as USA and France, Yin et al. (2020) and Tram et al. (2020) reported a 93% HAV RNA positivity rate, which was higher in males than females. The gender distribution varies with this study, which found more females with HAV RNA detection.

HAV RNA detection was highest in children younger than 15 years (75%) and lowest in adults over 36 years of age. 41/78 (53%) HAV RNA positive samples were from children between 1-15 years. This corresponds to reports by Enoch et al. (2019) and Mazanderani et al. (2019) where higher HAV seroprevalence rates were found in individuals between ages four and seven years and 11–13 years, respectively. While establishing the HAV outbreak threshold, Prabdial Sing et al. (2022) reported the WC to have the highest incidence rates compared to other provinces, with regular outbreaks occurring in the age range of two to 60 years, with the highest frequency in 15 to 19-year-olds. The use of serological techniques also limits the effective comparison to the molecular technique used this study.

Normal liver function is determined by biochemical tests such as AST, ALT, and ALP, and any abnormalities in these tests are suggestive of injury to hepatocytes. To illustrate, ALT levels (>500 U/L), AST (>81 U/L), and ALP (>133 U/L) may be indicative of hepatic injury caused by a viral infection such as HAV (Tom, 2009). All liver function enzymes in this study

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indicated hepatic injury and were above normal levels. ALT and ALP levels in the HAV RNA-positive samples were highest within the age group 1 to15 years; AST levels were highest in those aged between 16 and 35 years.

Overall, elevated liver function enzymes ALT, AST and ALP were observed in age groups one to 15 years compared to other age groups. Similarly, the one to 15-year age group had the highest number of HAV RNA detection compared to other age groups. Few samples were found to be from the age group 36 to 75 years due to convenience sampling therefore the distribution of liver function enzyme results in the elderly age group was limited in the study. This may reflect the current epidemiology of HAV in the community served by these sites during time of sampling.

Normann et al. (2004) found HAV RNA detection to be associated with the liver enzyme ALT; as increased ALT and HAV RNA levels were found simultaneously, the study found that low ALT levels corresponded with no HAV RNA presences. A positive correlation between HAV RNA detection and liver function enzymes ALT and AST was recorded, with no mention of the correlation between liver function enzyme and age (Fujiwara et al., 1997; Lee et al., 2015; Tram et al., 2019).

5.3 HAV RNA Detection Environmental samples

The detection of HAV in untreated wastewater would offer a crucial overview of its spread throughout the population, highlighting the emergence of asymptomatic illnesses and movement between the environment and human population in a specific location. The study included more samples from university residences than from local City of Cape Town areas because of sample availability. Sampling was initially done on university residences as these are examples of shared spaces where the overall risk of HAV transmission may be high, however HAV was only detected in 26% of the samples. In determining the recovery rate though similar to the C_t value of the spiking samples and estimated at 100%, the concentration was not measured for the recovered sample due to the nature of the semi-quantitative assay design. Similar studies made no reference to the methods of spike recovery used and its outcomes (Rachida and Taylor, 2020). A SARS-CoV-2 study sampling similar areas had a recovery rate of 10-18% with the use of the following formula (Mangwana et al., 2022):

$$\textit{recovery rate} = \frac{copies\ recoverd\ (gc/\mu l)\times 100}{copies\ spiked\ (\frac{gc}{\mu l})} \times\ 100.$$

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Of the 174 samples, HAV RNA was detected in 33% of the environmental samples. The detection was lower in this study compared to the 80% recorded by Rachida and Taylor (2020) on the molecular characterisation of HAV in various wastewater sources across South Africa. This study accounted for all the untreated wastewater samples included in the study with no specific distribution by location; hence, the actual HAV RNA distribution specific to the WC was not properly demonstrated.

Conversely, Osuolale and Okoh (2015) did not detect any HAV RNA in untreated wastewater samples. Adefisoye et al. (2016) reported 6% HAV RNA detection, accounting for possible low viral shedding, resulting in lower detection. These studies differ geographically in terms of sampling locations, and thus might reflect differences in socioeconomic and sanitary conditions. The geographic distribution pattern of HAV is highly correlated to the socioeconomic level and sanitary conditions, with HAV infections recorded higher in low socioeconomic regions with poor conditions and lower in high-income areas with good sanitary conditions (Enoch et al., 2019).

The Ct values of HAV RNA positive wastewater samples was more than 35 for the majority of samples, possibly indicating lower concentrations of HAV RNA in the sample. Quantification was not assessed therefore that argument cannot be made for this study. Kenya, a country with low or intermediate HAV incidence, reported 88-100% enteric virus detection including HAV with Ct value closer to 30 units detected in rainy than dry seasons (van Zyl et al., 2019). Seasonal distribution was not inferred for the wastewater samples because of the sampling method used.

Ourdani et al. (2016) indicated a 67% HAV detection in untreated wastewater samples in Tunisia, another lower middle-income country with a similar transition from high to intermediate HAV endemicity, as South Africa. One of the major limitations of this study was the inability to extrapolate the results to the larger Tunisian population, due to a sample size of only 271 over 13 months.

Most HAV RNA-positive environmental samples were from local areas, such as ATC (39.00%) and ZTV (49.50%), compared to university residences (26.00%). ZTV recorded the highest detection rate, which may have been due to this area being in a rural part of Cape Town, and the high detection rate could indicate an increase in HAV infections in the area. With improved sanitary conditions of 97% in urban areas, there is still a 13% improvement in the rural populations needed in South Africa (UNFPA, 2021). One serology study recorded a 75% seropositivity in university students and findings were significantly

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correlated to the residences (Sanein et al., 2014). Other factors included family members and drinking water, which were aspects not factored in this study as this assessed overall area detection and not individual cases. Published HAV detection in individual cases or overall area in university residences has not been done in South Africa therefore data is limited.

A recent HAV surveillance study using untreated wastewater in the USA reported that HAV had the highest relative abundance compared with other viruses sampled in the study; this was observed during an outbreak in the USA (McCall et al., 2021; Hellmer et al., 2014). The sampling days depended on the clinical cases reported; thus, no sampling was performed on days without clinical cases, which could have influenced the observed high detection rate. In clinical case reports of HAV, prior studies have revealed negative or low detection rates in wastewater from low-endemic areas. Farkas et al. (2018) used qPCR to assess enteric viruses in London with no HAV detection, despite samples being collected on days without clinical cases. Similarly, Kokkinos et al. (2010) employed RT-PCR and nested PCR and observed less than 10% detection.

The spot map was used as a tool for visualising HAV dissemination in the different areas. A higher proportion of positive samples were detected from the residential areas than in academic facilities. The residential areas formed part of the City of Cape Town district (ATC and ZTV) and the SU samples were part of the Tygerberg (HIPPO, MH1 and MH2) and Cape Winelands districts (3SUS and MET). The 3SUS area was not included in the spot map because of inability to locate the exact location of the residence on the map. This area of the Cape Winelands district was not part of the map due to construction restraints in constructing the map. Mapping out the areas with positive samples enables visualisation of possible vulnerable population in these areas and indicates a need for health interventions. The spot map did not depict disease risk since it did not account for population size.

In this study, there was no relationship found between clinical and environmental samples. The varying sampling locations and methods used may have influenced those outcomes. Limited access to the clinical history of patients and the inability to pinpoint individual cases in environmental samples limit the ability to correlate clinical and environmental samples.

5.4 Phylogenetic analysis

In this study, all genotyped samples were HAV genotype I sub-genotype B, with over 99% similarity to HAV partial strains isolated in South Africa, with the percentage of nucleotide

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identity ranging between 72% and 83% compared to the reference sequence. Similarly, Rachida and Taylor (2020) reported on HAV IB as the circulating genotype in the molecular characterisation of HAV strains in wastewater in South Africa, outlining a possible infectious strain emerging from the sampling areas. In contrast, Ourdani et al. (2016) reported the circulation of the HAV IA genotype in Tunisia, another middle-income country as South Africa, and the co-circulation of HAV IB was reported in 7% of the sequenced samples. Similar findings in middle-income countries such as Brazil indicated a 75% prevalence of genotype IB in the sampled population. It is recognised that the primers used may have been limited to possibly only detecting HAV IB genotypes as comparisons or matches with other genotypes were not found.

Ourdarni et al. (2016) used the VP3-VP1 region with a 261bp size compared to the VP1–P2B 361bp region used in this study and by Rachida and Taylor (2020); hence, the difference in genotypes found. The different geographic regions also influenced the identified genotypes. Kokkinos et al. (2010) identified HAV IA similar to the findings of Ourdani et al. (2016) in a country considered to have low endemicity. In contrast, 83% of HAV detected in this samples, using the same region of amplification as Ourdani et al. (2016) and Kokkinos et al. (2010), HAV IB was identified in Iran, which correlates with other genotypes found in the Middle East region (Nasiri et al., 2022). This indicates that geographical region and other factors may influence the circulating genotype. In low endemic countries in parts of the USA, France, and the UK, the circulation of genotype IA and IIIA had been reported (Ndumbi et al., 2018; Mariojoules et al., 2019; Smith et al., 2019; Webb et al., 2020).

The sequencing of samples was limited to clinical samples as the RT-PCR assay was optimised for HAV detection in clinical samples. Genotyping of environmental samples was not conducted due to limited resources.

5.5 Strengths

This study recorded the detection of HAV in clinical and untreated environmental wastewater samples in parallel, contributing to the understanding of the prevalence of HAV in the WC. The most common approach for identifying acute HAV infection is anti-HAV IgM detection in the patient's serum, which has good clinical benefits but the potential for false negatives caused by low antibody titres, as well as the persistence of antibodies for months after an acute HAV infection, are drawbacks of serology that may limit the ability to evaluate outbreak events.

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This real-time RT-PCR assay using the Saïd et al. (2014) primers detected clinical (plasma, serum, and stool) and environmental samples with high sensitivity and specificity. As viral RNA is present 14 days before seroconversion, this assay may be able to identify asymptomatic cases and aid in contact tracing. It may be useful for surveillance studies in low- and middle-income countries such as South Africa to examine various sample types.

Reports of HAV disease outbreaks have relied on case-based surveillance and the work of local healthcare professionals, who can find epidemiological connections between cases. As a result, opportunities to identify clusters of cases and prevent outbreaks through early diagnosis, infection prevention and control, were lost. When acute HAV infection is suspected, it is possible to use a hybrid strategy that combines serology with molecular testing. To reduce the possibility of missed cases in this circumstance, serological screening may be performed first, and RT-PCR could then assess seronegative patients. This approach may offer an affordable alternative and reduce false-positive cases in patients without acute HAV disease. Clinically, this might lower transmission and reveal individuals who would benefit from post-exposure prophylaxis and treatment. To critically evaluate whether this strategy is workable, laboratories must examine specimen workflow, cost, and local prevalence. Using Saïd et al (2014) primers is recommended for use in diagnostic settings for testing of serum, stool, plasma, and untreated wastewater (e.g., screening in outbreak settings).

5.6 Limitations

The small sample size limited the ability to extrapolate the results to a larger population. Only a few stool samples were available for testing, as this is not frequently collected for HAV diagnosis; thus, additional analysis of more stool samples is needed. Small sample size and reagent unavailability also affected sensitivity and specificity assessment, precision, and accuracy measurements on the Saïd et al.(2012) primers. Other limitations include the limited geographical regions sampled, the exclusion of symptomatic patients that may have been IgM negative, the small sample size for the genotype assessment.

A semi-quantitative real-time RT-PCR analysis of HAV was performed. Thus, the concentrations or viral load copies were not determined, which could have enabled the identification of the severity of HAV infection or disease. Monitoring of viraemia in patients may be beneficial, as viral load is a good predictor of illness severity.

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To improve clinical care and outbreak responses, additional research to identify the specific viral load linked with higher transmission may be helpful. There are currently no established viral load threshold values for HAV management.

Limitations of phylogenetic analysis were observed; a limited number of samples were sequenced and thus genotyped because of the limited time towards thesis project completion and unavailability of monetary resources to optimise the RT-assay further to cater to environmental sample genotype detection, however, detection of genotype IB in serum implies predominance of this genotype in our setting and thus increases likelihood that this genotype will be detected in the environmental samples.

5.7 Future research

Prospective longitudinal studies can monitor HAV prevalence in the WC province on clinical and environmental samples. A larger sample size will enable extrapolating the results to a larger population, Other factors such as costs, reagents user training should also be looked into in future studies. More stool samples should be included for analysis. More extensive phylogenetic analysis should also be done to accurately determine the overall circulating genotype in a larger sample size and various other genotypes in both clinical and environmental samples.

Finally, associations between geographical region(s) and clinical and environmental detection should be investigated. HAV seasonal distribution in environmental samples can also be assessed by prospective studies.

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Chapter 6: CONCLUSION

This study was able to detect HAV in various clinical samples and environmental untreated wastewater using in-house established real-time RT-PCR assays and published primers. In line with the aims and objectives, the distribution of positive clinical samples was more prevalent in females than in males, with an increased HAV RNA detection in ages younger than 15 years, corresponding with previous studies, with liver enzyme distribution indicating similar trends. The method is semi-qualitative in nature and future studies may assess the quantitative aspect. Clinical severity testing can be assessed with quantitative testing.

The environmental sample detection rate was lower than that in corresponding studies nationally. In terms of diagnostic laboratory implementation, high clinical specificity indicates suitability for use combined with serology to reduce the possibility of false-positive or false-negative serologically identified cases, especially in clinically ill patients. Due to the limited size, extrapolation to a larger population is not possible. The sampling areas differed for the environmental and clinical samples; therefore, no correlation could be demonstrated between HAV detection in environmental samples and clinical samples.

Future studies should investigate correlations in the detection of HAV RNA between untreated environmental wastewater and clinical samples from the same geographical area. Considering that this study only conducted molecular characterisation on clinical samples, future studies should also include HAV genotypes circulating in untreated environmental wastewater samples.

The data from this study will contribute to an improved understanding of the epidemiology of HAV in WC. Furthermore, to our knowledge, this is the first study to implement simultaneous HAV detection using a real-time RT-PCR assay in WC. The cost of the assay will be the ultimate deciding factor whether this assay should be included in the routine diagnostic testing for HAV in the Medical Virology NHLS laboratory at TBH.

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Addenda

Addendum A:- Ethical clearance

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forward together
sonke siya phambili
saam vorentoe

Approval Letter
Progress Report

22/04/2022

Project ID: 21690

Ethics Reference No: S21/02/026

Project Title: A real-time reverse transcriptase PCR assay for the detection of suspected acute Hepatitis A Virus infection in serum samples, Tygerberg Virology.

Dear Ms N Mpazi

We refer to your request for an extension/annual renewal of ethics approval received 28/02/2022.

The Health Research Ethics Committee reviewed and **approved** the annual progress report through an expedited review process.

The approval of this project is extended for a further year.

Approval date: 30 April 2022

Expiry date: 29 April 2023

Kindly be reminded to submit progress reports two (2) months before expiry date.

Where to submit any documentation

Kindly note that the HREC uses an electronic ethics review management system, *Infonetica*, to manage ethics applications and ethics review process. To submit any documentation to HREC, please click on the following link: https://applyethics.sun.ac.za.

Please remember to use your Project Id 21690 and ethics reference number S21/02/026 on any documents or correspondence with the HREC concerning your research protocol.

Please note that for studies involving the use of questionnaires, the final copy should be uploaded on Infonetica.

Yours sincerely,

Melody Shana
Coordinator: Health Research Ethics Committee 1

National Health Research Ethics Council (NHREC) Registration Number:
REC-130408-012 (HREC1)•REC-230208-010 (HREC2)

Federal Wide Assurance Number: 00001372
Office of Human Research Protections (OHRP) Institutional Review Board (IRB) Number:
IRB0005240 (HREC1)•IRB0005239 (HREC2)

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Addendum B:- RT-PCR Preparations

For the Pre-nested reaction

All working benches were disinfected with 10% bleach and 70% ethanol and 200 µl (0,2 ml) PCR reaction tubes to contain samples were properly labelled and placed on an ice block. Then two 2 ml centrifuge tubes were labelled " Pre" and "Ne" for the pre -nested and nested master mixes, respectively. The master mixes were prepared in their respective tubes as indicated in the tables 8.2 and 8.3 below.

Master mix components for pre-nested conventional RT-PCR

Master mixture components for pre-nested (first round) amplification step.	
Reagents	**Initial volume per 1 reaction**
NFW	19
dNTP mix	1
AMV/ Tfl Buffer	10
External primer (forward)	2
External primer (reverse)	2
MgSO4	4
AMV / RT	1
Tfl DNA Polymerase	1
RNA template	10
Final volume	40

Master mix components for nested conventional RT-PCR

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Master mixture components for nested (second round) amplification step.	
Reagents	**Initial volume per 1 reaction**
NFW	5.5
GoTaq G2 Flexi buffer	10
dNTP mix	1
Internal primer (forward)	1
Internal primer (reverse)	1
MgCl2	4
GoTaq G2 Flexi DNA Polymerase	0.5
RNA template	5
Final volume	25

Then 40 µl of the pre-nested reaction master mix was added into the labelled appropriate 200µl tubes and into each reaction tube and 20 µl for the master mix from the nested reaction were added into the 200 µl tubes labelled .These preparations were performed in a clean room.

RNA eluates were added in a separate template addition room where the counters were disinfected before use and 10 µl of each sample (extracted RNA) was added into the 200µl labelled for pre-nested reaction These were then loaded into the thermocycler under the parameters described in table below. Reactions were two hours 30 minutes and one-hour 45minutes for the pre-nested and nested reactions, respectively.

The nested reaction tubes were wrapped with paper towel and placed into the -20°C fridge to store prior to template addition of pre-nested PCR reaction products. After the pre-nested reaction was completed, nested reaction 200µl tubes were retrieved from the fridge and 5µl of the pre-nested reaction PCR products were added on the nested reaction tubes and subsequently placed on the thermal cycler. Thermal cycling conditions are indicated in the table below.

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Thermal cycling conditions of both the pre-nested and nested PCR reactions:

Thermal cycling conditions for the pre-nested round (first round product).	**Time**	**Temperature**	**Cycles**
Reverse transcription	40 min	50°C	1
Initial PCR activation step	15 min	95°C	45
Three-step cycling program:	Three-step cycling program:	Three-step cycling program:	Three-step cycling program:
1 Denature 2. Annealing 3. Extension	15s 2. 20s 3. 40s	95°C 2. 50°C 3. 72°C	1
Thermal cycling conditions for the nested round (second round product).			
Activation step	15 min	95°C	1
Three-step cycling program: 1. Denature 4 2. Annealing 3. Extension	 30s 30s 30s	 95°C 2. 55°C 3. 72°C	40

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Addendum C: - Agarose Gel Preparation

Preparations commenced from making of the 50X and 1X TAE buffer used.

Firstly 500 ml EDTA (0.5M) was made by weighing 93.05 g of EDTA and pouring it into a 500 ml flask. Dissolving it in 400 ml of nuclease free water (NFW) and adjusting the pH with Hydrochloric acid and Sodium Hydroxide to obtain a pH of 8.0.EDTA only dissolves at a pH of 8.0. Therefore NFW was added to bring the total volume up to 500 ml. This was used to make 50X TAE as follows: Weighing 242 g of Tris-base and place it into a 1000 ml (1 L) Erlenmeyer flask, dissolving the Tris-base with ~700 ml of NFW. Then adding 57.1 ml of 100% glacial acid/acetic acid and 100 ml of EDTA (pH 8.0; 0.5M).Adjusting the total volume to 1000 ml with NFW.

The 50X TAE buffer was used to make the 1XTAE by adding 20 ml of 50XTAE buffer to a 1000 ml flask and adjusting the total volume to 1000 ml with NFW and this was the TAE used in making the gel and running buffer.

Preparations for 2% agarose gel and sample loading by

Weighing out 2 g of SeaKem LE agarose powder for every 100 ml of 1XTAE buffer. Pouring the powder into a 500 ml flask .Adding the amount of buffer required to make the 2% agarose gel and placing the flask in the microwave for 1-3 minutes. Stirring the flask until the agarose is completely dissolved. Placing a magnetic stir bar into the gel and place the flask on the magnetic stirrer to cool the flask until one can hold the flask. Then place the gel comb in the gel tray and place pour the gel after cooling. After 45 minutes the solidified gel was ready to be loaded and placed on the Labnet gel running matrix and 1XTAE running buffer covered the gel and loading commenced as follows: add 1ul of Novel juice into parafilm tape and mix with 10ul of the RNA eluates and load onto the gel, the same was repeated for all samples and Genetic DNA marker. After loading, samples were held at room temperature for 5 minutes before closing the gel matrix and running the gel at 110 V for 45 minutes. After 45 minutes samples were placed on the ChemiDoc Bio-Rad imaging instrument, analysed, and saved.

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Addendum D: - Assay Verification

Sensitivity and Specificity was assessed using the 2X2 (two by two) table method for both the adapted Chigor and Okoh Primers and the Saiid et al. primers.

TP- True Positive, FN – False negative, FP – False negative, TN – True Negative

Sensitivity = TP/(TP+FN) x100

PPV = TP/(TP+FP) x100

Specificity = TN/(TN+FP) x100

NPV = TN/FN+TN x100

Performance Characteristics for the Chigor and Okoh et al. (2012) primers

30 samples were tested, composed of 10 QCMD EQA samples challenge 1, 2 (2019) and challenge 1 (2022), 5 x stool samples from anti-HAV IgM positive patients, 5 x stool samples from patients without HAV infection and 10 x serum samples from patients who tested anti-HAV-IgM negative but antibody positive for HIV and Hepatitis C virus and HBsAg positive.

		Reference HAV RNA	
		Positive samples (+)	Negative samples (-)
Test HAV RNA	Positive samples (+)	12	0
	Negative samples (-)	1	17

Sensitivity = 92.3%

Specificity = 100.0%

PPV= 100.0%

NPV= 94.4%

Table indicating the information on the samples used for assessment, reference results and obtained results:

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Sample ID	Sample Type	Experiment number	Ct value	Obtained Result (P- Positive and N- Negative)	Expected Result
EQA HAVRNA22 c1-1	Plasma	41	0	N	N
EQA HAVRNA22 c1-2	Plasma	41	33.34	P	P
EQA HAVRNA22 c1-3	Plasma	41	34.22	P	P
EQA HAVRNA22 c1-4	Plasma	41	32.54	P	P
TPA8160	Stool	16	25.79	P	P
TPA8170	Stool	16	33.00	P	P
TST0533	Stool	32	29.22	P	P
TX3898	Stool	32	29.97	P	P
TST1482	Stool	32.36	31.15	P	P
TQF4813	Serum	9	0	N	N
7QF4817	Serum	9	0	N	N
TST8328	Serum	9	0	N	N
TST9197	Serum	9	0	N	N
TXM8390	Serum	9	0	N	N
TSN1679	Stool	40	0	N	N
TSN0050	Stool	40	0	N	N
TSN0902	Stool	40	0	N	N
TSN2777	Stool	40	0	N	N
TSN8161	Stool	40	0	N	N
EQA HAVRNA19 c1-1	Plasma	5	32.71	P	P
EQA HAVRNA19 c1-2	Plasma	3	38.64	P	P
EQA HAVRNA19 c1-3	Plasma	14	32.74	P	P
EQA HAVRNA19 c1-4	Plasma	23	0	N	N
EQA HAVRNA19 c2-3	Plasma	25	27.38	P	P
EQA HAVRNA19 c2-4	Plasma	35	0	N	P
TQF4850	Serum	9	0	N	N
7QF4889	Serum	9	0	N	N
TST8305	Serum	9	0	N	N
TST9106	Serum	9	0	N	N

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Performance Characteristics for the Saïd et al. (2012) primers

Sample n = 10, comprised of EQA challenge 1 HAVRNA 2022 x 4 samples, 3 x samples from HCV RNA positive / anti-HAV IgM negative patients and 3 x samples known HAV RNA positive.

		Reference HAV RNA	
		Positive samples (+)	Negative samples (-)
Test HAV RNA	Positive samples (+)	6	1
	Negative samples (-)	0	3

Sensitivity = 100%

Specificity = 75%

PPV = 85.7.0%

NPV = 100.0%

Samples used in the table below

Sample ID	Sample Type	Experiment number	Ct value	Obtained Result (P-Positive and N-Negative)	Expected Result
EQA HAVRNA22 C1-1	Plasma	72	0.00	N	P
EQA HAVRNA22 C1-2	Plasma	72	0.00	P	P
EQA HAVRNA22 C1-3	Plasma	72	31.83	P	P
EQA HAVRNA22 C1-4	Plasma	72	31.58	P	P
TMG3931	Plasma	71	0.00	N	N
TMG5499	Plasma	71	0.00	N	N
TST0910	Plasma	71	0.00	N	N
GSA0194	Serum	71	30.40	P	P
GST9314	Serum	71	29.59	P	P
TXF7357	Serum	71	30.84	P	P

The data for the accuracy and precision is found in the table below for the adapted Chigor et al primers comprised of 4 x EQA samples form challenge 1 2022,1 HAV NISBC standard

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and 1x NFW. The data represented in this table explains the results obtained in section 4.2 of the results table 4.3

Sample	Day 1 Ct values	HAV detection (P-positive; N-Negative)	Day 2 Ct values	HAV detection (P-positive; N-Negative)	Day 3 Ct Values	HAV detection (P-positive; N-Negative)
EQA 22 C1-2	41.16	P	36.52	P	0.00	N
EQA 22 C1-2	36.43	P	36.49	P	36.20	P
EQA 22 C1-2	32.78	P	34.89	P	37.47	P
EQA 22 C1-3	0.00	N	0.00	N	0.00	N
EQA 22 C1-3	0.00	N	0.00	N	0.00	N
EQA 22 C1-3	0.00	N	0.00	N	0.00	N
EQA 22 C1-4	34.61	P	0.00	N	45.36	P
EQA 22 C1-4	34.63	P	35.54	P	0.00	N
EQA 22 C1-4	34.47	P	42.14	p	38.43	P
HAV RNA Standard	29.81	P	28.82	P	32.04	P
HAV RNA Standard	29.12	P	-	-	-	-
HAV RNA Standard	28.44	P	-	-	-	-
NFW	0.00	N	0.00	N	0.00	N
NFW	0.00		-	-	-	-
NFW	0.00		-	-	-	-

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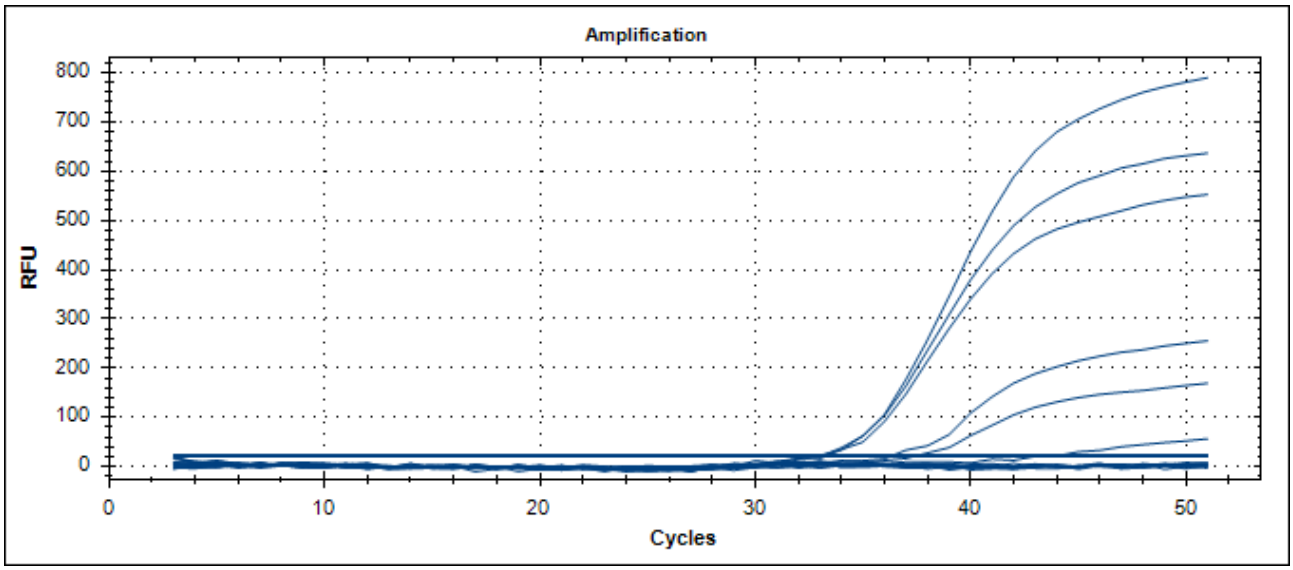
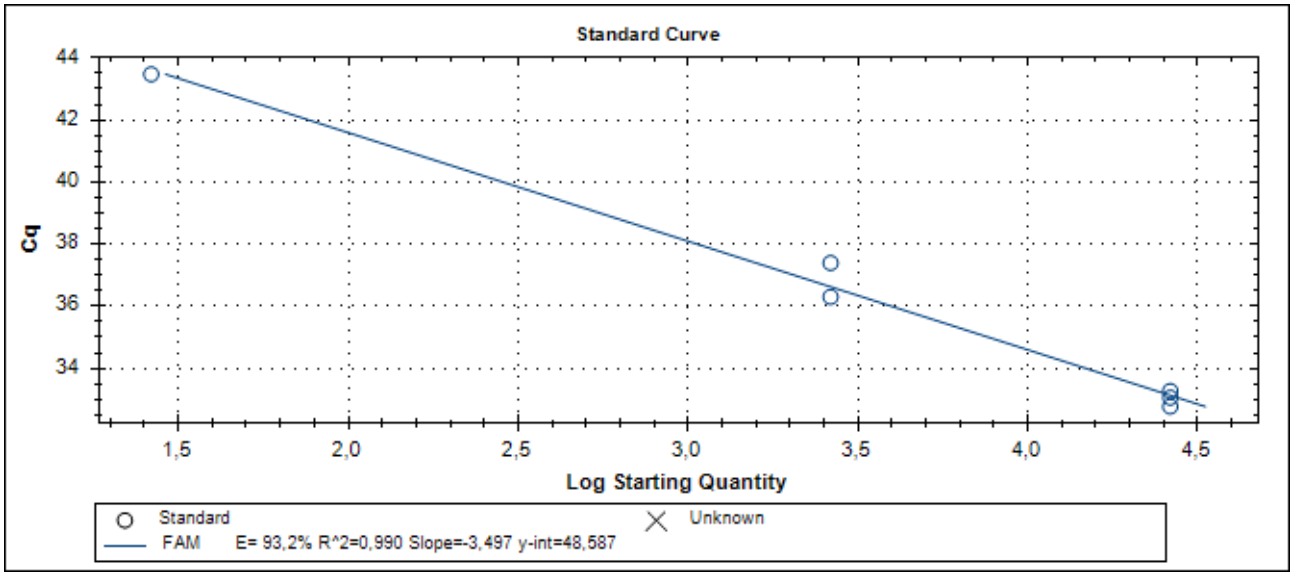
Addendum E :- Amplification chart of HAV standard LoD and Spike recovery





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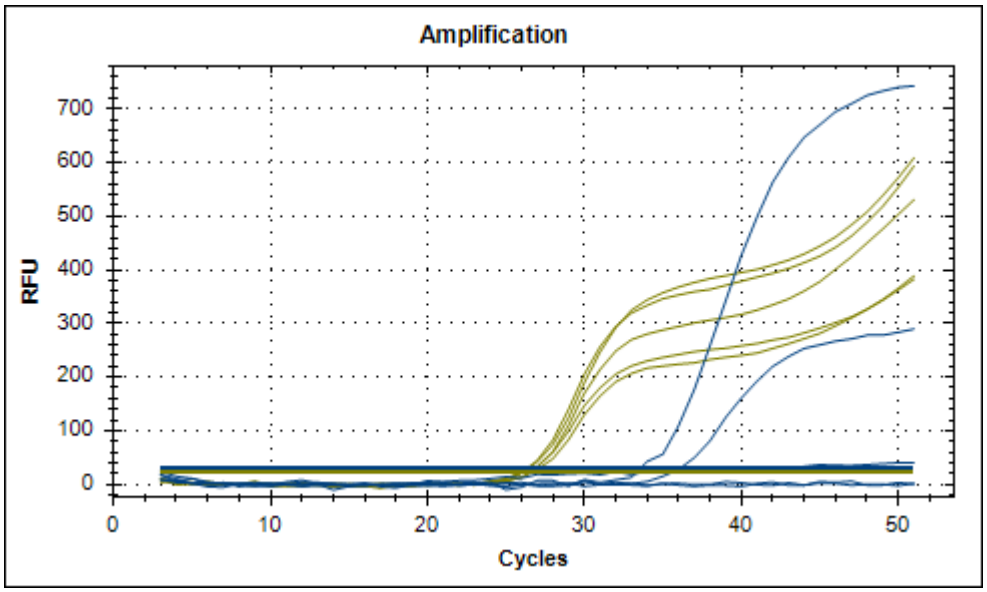
Recovery experiment



Well	Fluor	Target	Content	Sample	Cq	Cq Mean
A01	FAM		Unkn	MT clean		0.00
B01	FAM		Unkn	MT spike at 5ml	33.63	33.63
C01	FAM		Unkn	MT spiked 50ml	41.73	41.73
D01	FAM		Pos Ctrl		36.19	36.19
E01	FAM		NTC	NC		0.00
A01	VIC		Unkn	MT clean	26.71	26.71
B01	VIC		Unkn	MT spike at 5ml	26.07	26.07
C01	VIC		Unkn	MT spiked 50ml	26.07	26.07
D01	VIC		Pos Ctrl		26.53	26.53
E01	VIC		NTC	NC	26.11	26.11

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Addendum F:- Absorbance readings

Sample ID	Size (ng/µl)	A260/A280	A260/A230
TST1482	93.10	1.73	1.33
TST6773	61.10	2.08	0.32
ST4990	91.50	3.17	2.06
TX3898	60.10	0.10	0.00
TXN2490	26.50	2.20	0.57
TST2763	74.90	3.14	1.87
TST4007	55.90	2.83	2.22
TXM2269	24.60	2.69	0.89
WVII7042	35.60	2.87	1.13
WVJ7414	22.50	0.84	0.11
WVII7030	31.60	3.01	1.24
WVII7027	81.90	2.78	1.21
TXK5989	32.80	2.76	1.62
TXM0164	51.40	2.31	0.73
TST8549	46.20	2.45	1.29
GXJ4241	80.10	0.63	0.07
TST0503	41.80	2.73	1.09
GXD2093	61.00	0.76	0.11
WVII5270	79.10	0.68	0.10
WVII0848	25.90	0.67	0.15
WVII7023	24.40	1.41	0.11
WVII5281	21.10	0.87	0.15
TXK2595	42.60	2.61	0.78
TXF3470	148.40	1.63	0.37
TST0533	42.20	0.14	0.00
TST9201	40.20	2.68	1.47
GSA7647	35.60	2.83	1.23
TVH4233	76.30	0.89	0.15
WVII5283	25.50	2.15	0.48
WVII7003	61.80	1.88	0.23
WVII5263	89.20	2.32	0.45
WVII5297	30.10	2.71	1.21
TST9373	75.30	2.81	1.70
TST3590	40.00	1.96	0.24
GSG2517	97.00	2.28	1.80
TST1824	39.40	3.11	2.06

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Sample ID	Size (ng/µl)	A260/A280	A260/A230
GXJ6688	41.80	2.16	0.74
TST0168	40.30	2.55	0.99
WVII7047	89.20	2.32	0.45
WVII0873	125.70	2.38	0.67
WVII7040	38.40	3.25	1.14
WVII7014	122.20	3.22	1.09
TXN7714-	21.30	2.36	2.15
TST2897	32.30	2.35	0.34
GXD1976	21.30	2.87	2.52
TST1531	75.80	2.63	1.25
TST8714	75.30	2.44	0.90
GXD9493	98.10	2.35	0.87
GXD5835	45.30	2.83	0.65
TXN8921	48.70	1.93	0.09
GXD3612	100.00	0.62	0.22
TST2984	20.10	1.90	2.92
TST6706	24.00	3.14	2.85
GXK0249	127.50	2.34	0.75
GXC0903	32.20	2.90	1.42
WVII5277	71.00	2.81	1.81
WVII0855	50.10	2.45	1.11
WVII7018	62.30	2.21	1.13
WVII7017	41.50	2.35	1.14
WVII0850	32.60	2.25	1.10
WVJ7413	30.10	2.01	1.01
WVII7024	25.70	2.31	1.02
WVII0875	20.10	2.84	1.45
GXD9525	61.90	2.99	1.92
TXK3448	56.50	2.08	1.19
TXN2007	33.20	2.24	2.11
GSG2839	23.20	2.36	0.68
GST9314	59.40	3.26	3.61
TXG8247	206.80	2.77	1.66
GXD9007	20.30	2.44	0.77
TST8730	51.80	2.56	0.89
GXD3532	29.30	3.22	2.29
GSA0194	48.00	1.02	0.23
TPA8170	71.00	3.11	2.51

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Sample ID	Size (ng/µl)	A260/A280	A260/A230
GSG4032	63.90	2.69	1.79
WVII7035	63.90	2.96	1.76
WVII7421	63.90	2.66	1.52
WVII0871	63.90	2.69	1.79
WVII7001	63.90	2.99	1.92
WVII7025	88.70	2.08	1.19
WVII7015	90.50	2.83	0.65
WVII7039	43.10	1.93	0.09
WVII7029	45.00	2.36	1.03
GSA0077	35.60	2.69	0.89
GXD5547	24.60	2.88	0.93
TST6437	41.90	2.28	0.39
TXM1652	71.10	3.09	2.52
TXG2446	147.00	2.69	1.79
TXN5266	71.90	2.96	1.76
GXD9175	40.30	2.66	1.52
GXC0243	24.90	3.00	1.33
GXD8749	95.00	2.79	1.49
TST1229	101.60	2.60	0.24
GSA0388	28.20	3.21	2.59
TST2503	98.30	3.01	1.70
WVII0858	34.70	3.27	0.85
WVII7007	20.60	0.98	0.12
WVII7051	47.00	3.28	1.71
WVII7031	32.00	2.93	1.96
WVII0863	42.10	3.30	1.61
WVII7004	40.80	3.67	2.16
WVII7043	28.60	3.30	2.36
WVII5296	124.30	3.18	3.20
TST6897	26.60	2.92	1.96
TXG6038	67.60	3.05	1.39
TST0100	51.10	2.46	0.70
TST2724	23.50	3.16	1.61
TXN8390	69.10	2.97	1.57
GXD1026	47.30	2.04	0.85
GXD7260	22.50	3.42	4.22
TST3282	39.40	3.55	0.88
GXD3891	61.10	3.22	2.29

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Sample ID	Size (ng/µl)	A260/A280	A260/A230
TXL6745	48.00	3.40	1.05
GSA3022	60.50	2.86	1.73
TXF9423	50.50	3.05	0.36
WVII7009	39.90	1.92	0.13
WVII5299	41.00	4.18	0.28
WJ7416	41.00	2.95	0.37
WVII0854	41.00	1.78	3.37
WVII0877	41.00	2.76	1.97
WVII0879	41.00	3.17	2.27
WVJ0741	68.20	2.27	0.89
WVII5276	41.70	2.36	1.03
TST7545	35.60	2.61	0.74
GXC1917	41.80	2.62	1.03
TXN3545	58.40	2.49	1.04
TST7556 -ST0116	46.50	2.12	1.20
TST3249	51.20	0.70	0.30
TST5559	51.50	2.17	1.27
TXE2030 - XF0109	100.10	3.19	0.71
GXD7994	57.90	3.18	1.79
GXQ8801	26.60	2.84	1.61
TXF8715	57.70	3.29	3.97
TXM2288	56.70	2.87	0.71
TST0681	61.00	1.33	0.25
TXF1958	24.70	1.90	0.52
WVII5288	45.10	2.46	0.65
WVII7048	24.60	2.24	0.92
WVII7049	22.60	3.30	2.44
WVII5286	45.20	1.11	0.70
WVII5261	20.50	1.76	0.72
WVII7021	71.10	2.10	0.70
WVII7033	22.20	2.46	0.75
WVII7019	31.10	1.21	0.25
WVII0852	96.60	2.06	0.72
WVII7008	61.30	2.39	1.30
TST5985	41.00	2.30	1.32
TXG0653	26.30	2.65	0.45
GXQ4529	36.60	2.86	2.02
TQK1995	78.40	2.66	1.12

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Sample ID	Size (ng/µl)	A260/A280	A260/A230
TXF7357	60.60	1.19	0.25
GXQ5084	67.00	2.05	0.36
TPA8160	44.10	2.18	0.77
TST3751 - TST7232	94.10	3.19	4.09
TXE6201	67.30	1.95	0.52
TST1247	61.10	2.78	1.15
WVII5287	55.10	2.93	2.51
WVII0868	39.20	3.09	2.07
WVII5290	39.90	2.46	0.92
WVII5272	30.10	3.17	2.02
WVII5268	33.10	2.09	0.87
WVII7011	21.10	2.82	2.69
WVJ4731	61.90	2.93	1.46
WVII7050	43.50	3.16	2.31
WVII0867	35.10	3.03	1.73
WVII5266	53.10	2.65	1.97
TXK1271	20.30	2.68	1.47
TXF7706	74.10	3.16	1.61
TXN9891	69.10	2.80	1.23
TST2485	35.40	3.87	0.25
WVII5282	40.30	1.92	0.12
WVII7036	31.20	2.30	2.73
WVII7005	24.40	1.22	2.51
WVII5285	70.00	3.14	2.14
TXG9563	63.30	0.10	0.00
TXK6216	51.00	2.97	1.86
TXO4520	60.60	2.11	0.30
TXK2109	31.10	2.40	1.16
GXC9378	93.10	1.36	0.23
TXN4254	61.10	2.86	1.78

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Addendum G: - Real-time RT-PCR data

Episode Number	Experiment Number	Location of retrieval	Sex (M/F)	Age (years)	Clinical Sample Type	Sample Ct value results	IC Ct values	HAV RNA detection outcome
TST1482	32; 36	TBH	F	1	Stool	31.15	31.53	Positive
TST6773	26	TBH	F	2	Serum	32.76	33.02	Positive
ST4990	35	TBH	F	2	Serum	34.04	31.84	Positive
TX3898	32	TBH	M	2	Stool	29.97	35.54	Positive
TXN2490	33; 46	TBH	M	2	Serum	32.01	32.35	Positive
TST2763	29	TBH	M	3	Serum	27.61	0.00	Positive
TST4007	33; 36	TBH	M	3	Serum	28.60	31.49	Positive
TXM2269	50	TBH	F	3	Serum	36.39	31.37	Positive
WVII7042	22; 23; 25	WCBS	F	53	Plasma	0.00	31.80	Negative
WVJ7414	22; 23; 25	WCBS	F	36	Plasma	0.00	32.56	Negative
WVII7030	22; 23; 25	WCBS	F	36	Plasma	0.00	32.25	Negative
WVII7027	22; 23; 25	WCBS	M	51	Plasma	0.00	32.78	Negative
TXK5989	28	TBH	F	4	Serum	28.34	0.00	Positive
TXM0164	24; 25	TBH	F	18	Serum	0.00	32.06	Negative
TST8549	28	TBH	M	4	Serum	29.18	0.00	Positive
GXJ4241	24; 25; 31; 36	GSH	F	8	Serum	0.00	32.64	Negative
TST0503	22; 23; 25	TBH	F	4	Serum	31.97	0.00	Positive
GXD2093	24; 25	GSH	F	7	Serum	0.00	32.79	Negative
WVII5270	24; 25	WCBS	F	33	Plasma	0.00	32.56	Negative
WVII0848	24; 25	WCBS	M	39	Plasma	0.00	32.25	Negative
WVII7023	24; 25	WCBS	M	30	Plasma	0.00	30.43	Negative
WVII5281	24; 25	WCBS	M	57	Plasma	0.00	30.46	Negative
TXK2595	27	TBH	F	4	Serum	31.41	0.00	Positive
TXF3470	26	TBH	F	56	Serum	0.00	30.42	Negative
TST0533	32	TBH	M	5	Stool	29.22	35.79	Positive
TST9201	33; 36	TBH	M	5	Serum	28.24	33.78	Positive
GSA7647	29	GSH	F	5	Serum	25.61	0.00	Positive
TVH4233	27	TBH	F	5	Serum	33.86	38.59	Positive
WVII5283	26; 31	WCBS	F	60	Plasma	0.00	29.33	Negative
WVII7003	26; 31; 36	WCBS	F	43	Plasma	0.00	32.30	Negative
WVII5263	26; 31	WCBS	F	23	Plasma	0.00	0.00	Negative
WVII5297	26; 31; 36	WCBS	F	26	Plasma	0.00	30.76	Negative
TST9373	28	TBH	F	7	Serum	30.51	0.00	Positive
TST3590	32	TBH	F	7	Serum	29.36	32.45	Positive

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Episode Number	Experiment Number	Location of retrieval	Sex (M/F)	Age (years)	Clinical Sample Type	Sample Ct value results	IC Ct values	HAV RNA detection outcome
GSG2517	32	GSH	F	7	Serum	29.03	32.46	Positive
TST1824	29	TBH	F	7	Serum	28.27	0.00	Positive
GXJ6688	27; 31; 36	GSH	F	13	Serum	0.00	30.02	Negative
TST0168	29	TBH	M	7	Serum	28.63	0.00	Positive
WVII7047	27	WCBS	M	56	Plasma	0.00	29.87	Negative
WVII0873	27; 31	WCBS	M	26	Plasma	0.00	0.00	Negative
WVII7040	27; 31	WCBS	M	39	Plasma	0.00	0.00	Negative
WVII7014	27; 31; 36	WCBS	F	33	Plasma	0.00	31.29	Negative
TXN7714-TST2897	34	TBH	M	7	Serum	31.44	32.40	Positive
GXD1976	26; 31	GSH	F	8	Serum	27.04	30.90	Positive
TST1531	33; 36	TBH	M	8	Serum	31.71	32.23	Positive
TST8714	28	TBH	M	13	Serum	0.00	29.99	Negative
GXD9493	22; 23; 25	GSH	M	8	Serum	32.11	31.57	Positive
GXD5835	22; 23; 25	GSH	F	8	Serum	29.29	30.71	Positive
TXN8921	26	TBH	F	9	Serum	29.17	0.00	Positive
GXD3612	24; 25	GSH	F	9	Serum	28.91	30.65	Positive
TST2984	33; 46	TBH	F	9	Serum	27.36	27.78	Positive
TST6706	32	TBH	M	9	Serum	28.99	44.23	Positive
GXK0249	28	GSH	M	30	Serum	0.00	33.02	Negative
GXC0903	28	GSH	M	25	Serum	0.00	32.95	Negative
WVII5277	28; 31; 36	WCBS	F	21	Plasma	0.00	29.91	Negative
WVII0855	28	WCBS	F	47	Plasma	0.00	0.00	Negative
WVII7018	28	WCBS	M	37	Plasma	0.00	28.05	Negative
WVII7017	28	WCBS	M	34	Plasma	0.00	31.01	Negative
WVII0850	28; 36	WCBS	F	37	Plasma	0.00	30.11	Negative
WVJ7413	28	WCBS	M	70	Plasma	0.00	34.06	Negative
WVII7024	28	WCBS	F	47	Plasma	0.00	34.12	Negative
WVII0875	28	WCBS	F	50	Plasma	0.00	37.51	Negative
GXD9525	30; 31	GSH	M	9	Serum	3.17	31.97	Positive
TXK3448	35	TBH	F	10	Serum	32.64	31.40	Positive
TXN2007	32	TBH	M	10	Serum	28.69	0.00	Positive
GSG2839	32	GSH	M	12	Serum	29.97	0.00	Positive
GST9314	22; 23; 25	GSH	M	12	Serum	29.92	32.64	Positive
TXG8247	29; 31	TBH	F	10	Serum	0.00	29.61	Negative
GXD9007	29	GSH	F	13	Serum	29.59	32.37	Positive

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Episode Number	Experiment Number	Location of retrieval	Sex (M/F)	Age (years)	Clinical Sample Type	Sample Ct value results	IC Ct values	HAV RNA detection outcome
TST8730	22; 23; 25	TBH	M	13	Serum	30.74	32.06	Positive
GXD3532	29; 31; 36	GSH	M	13	Serum	0.00	30.13	Negative
GSA0194	29; 31; 36	GSH	M	14	Serum	30.06	29.91	Positive
TPA8170	15; 16	TBH	M	14	Stool	33.00	32.50	Positive
GSG4032	29	GSH	F	7	Serum	0.00	30.15	Negative
WVII7035	29; 31	WCBS	M	34	Plasma	0.00	29.61	Negative
WVII7421	29	WCBS	F	31	Plasma	0.00	33.09	Negative
WVII0871	29	WCBS	F	44	Plasma	0.00	36.71	Negative
WVII7001	29	WCBS	F	39	Plasma	0.00	0.00	Negative
WVII7025	29	WCBS	M	32	Plasma	0.00	0.00	Negative
WVII7015	29; 31	WCBS	F	46	Plasma	0.00	29.73	Negative
WVII7039	29	WCBS	M	50	Plasma	0.00	32.99	Negative
WVII7029	29; 31; 36	WCBS	F	25	Plasma	0.00	29.22	Negative
GSA0077	29; 31	GSH	M	14	Serum	5.73	30.12	Positive
GXD5547	32	GSH	M	15	Serum	30.14	35.21	Positive
TST6437	34	TBH	M	17	Serum	34.47	31.56	Positive
TXM1652	30	TBH	F	5	Serum	0.00	0.00	Negative
TXG2446	30	TBH	M	36	Serum	0.00	31.33	Negative
TXN5266	29	TBH	F	17	Serum	31.00	0.00	Positive
GXD9175	30	GSH	M	23	Serum	0.00	0.00	Negative
GXC0243	28	GSH	F	18	Serum	22.77	0.00	Positive
GXD8749	30; 31	GSH	M	36	Serum	0.00	31.79	Negative
TST1229	30; 31	TBH	F	18	Serum	29.03	29.65	Positive
GSA0388	27	GSH	F	18	Serum	32.96	0.00	Positive
TST2503	30	TBH	F	19	Serum	26.49	0.00	Positive
WVII0858	30	WCBS	M	49	Plasma	0.00	31.82	Negative
WVII7007	30	WCBS	F	48	Plasma	0.00	33.09	Negative
WVII7051	30	WCBS	F	37	Plasma	0.00	31.78	Negative
WVII7031	30	WCBS	M	33	Plasma	0.00	31.82	Negative
WVII0863	30; 31; 36	WCBS	F	23	Plasma	0.00	29.47	Negative
WVII7004	30	WCBS	M	57	Plasma	0.00	31.82	Negative
WVII7043	30	WCBS	M	19	Plasma	0.00	32.13	Negative
WVII5296	30	WCBS	F	42	Plasma	0.00	32.39	Negative
TST6897	34; 46	TBH	M	19	Serum	31.88	32.61	Positive
TXG6038	32; 36	TBH	F	27	Serum	0.00	33.75	Negative
TST0100	32; 36	TBH	M	6	Serum	0.00	39.62	Negative

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Episode Number	Experiment Number	Location of retrieval	Sex (M/F)	Age (years)	Clinical Sample Type	Sample Ct value results	IC Ct values	HAV RNA detection outcome
TST2724	32; 36	TBH	F	23	Serum	0.00	33.83	Negative
TXN8390	27	TBH	M	19	Serum	30.27	0.00	Positive
GXD1026	28; 31; 36	GSH	F	21	Serum	34.75	29.71	Positive
GXD7260	28; 31; 36	GSH	F	21	Serum	33.78	29.14	Positive
TST3282	32; 36	TBH	F	11	Serum	0.00	35.02	Negative
GXD3891	30; 31	GSH	M	21	Serum	25.21	31.43	Positive
TXL6745	50	TBH	F	22	Serum	31.38	31.17	Positive
GSA3022	30; 31; 36	GSH	F	24	Serum	25.57	30.05	Positive
TXF9423	45	TBH	F	25	Serum	27.75	30.58	Positive
WVII7009	32; 36	WCBS	M	75	Plasma	0.00	36.20	Negative
WVII5299	32; 36	WCBS	M	41	Plasma	0.00	31.24	Negative
WJ7416	32; 36	WCBS	F	16	Plasma	0.00	33.44	Negative
WVII0854	32; 36	WCBS	F	50	Plasma	0.00	32.29	Negative
WVII0877	32; 36	WCBS	F	34	Plasma	0.00	0.00	Negative
WVII0879	32; 36	WCBS	F	44	Plasma	0.00	35.55	Negative
WVJ0741	32; 36	WCBS	M	40	Plasma	0.00	33.73	Negative
WVII5276	32; 36	WCBS	M	52	Plasma	0.00	34.57	Negative
TST7545	50	TBH	F	25	Serum	40.40	32.27	Positive
GXC1917	26; 31; 36	GSH	F	26	Serum	27.11	30.02	Positive
TXN3545	28	TBH	F	27	Serum	31.44	0.00	Positive
TST7556 - ST0116	34; 46	TBH	M	28	Serum	33.97	31.89	Positive
TST3249	33; 36	TBH	F	16	Serum	0.00	31.34	Negative
TST5559	33; 45	TBH	M	3	Serum	0.00	30.81	Negative
TXE2030 - XF0109	34;46	TBH	M	28	Serum	32.39	31.70	Positive
GXD7994	32	GSH	M	29	Serum	32.02	34.25	Positive
GXQ8801	30; 31; 36	GSH	F	29	Serum	29.43	30.30	Positive
TXF8715	33; 36	TBH	M	35	Serum	0.00	32.48	Negative
TXM2288	33; 45	TBH	M	7	Serum	0.00	31.10	Negative
TST0681	22; 23; 25	TBH	F	30	Serum	32.19	32.78	Positive
TXF1958	33; 36	TBH	F	4	Serum	0.00	31.03	Negative
WVII5288	33; 36	WCBS	F	25	Plasma	0.00	30.00	Negative
WVII7048	33; 36	WCBS	M	37	Plasma	0.00	30.27	Negative
WVII7049	33; 36	WCBS	F	30	Plasma	0.00	30.15	Negative
WVII5286	33; 36	WCBS	F	57	Plasma	0.00	30.71	Negative

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Episode Number	Experiment Number	Location of retrieval	Sex (M/F)	Age (years)	Clinical Sample Type	Sample Ct value results	IC Ct values	HAV RNA detection outcome
WVII5261	33; 36	WCBS	M	45	Plasma	0.00	30.56	Negative
WVII7021	33; 36	WCBS	M	27	Plasma	0.00	30.58	Negative
WVII7033	33; 36	WCBS	F	40	Plasma	0.00	30.16	Negative
WVII7019	33; 36	WCBS	F	62	Plasma	0.00	30.44	Negative
WVII0852	33; 36	WCBS	M	33	Plasma	0.00	30.10	Negative
WVII7008	33; 36	WCBS	F	21	Plasma	0.00	31.87	Negative
TST5985	32	TBH	F	31	Serum	29.05	33.83	Positive
TXG0653	34	TBH	M	32	Serum	0.00	30.51	Negative
GXQ4529	26	GSH	F	31	Serum	30.68	32.53	Positive
TQK1995	30	TBH	F	31	Serum	24.83	0.00	Positive
TXF7357	30; 31	TBH	F	31	Serum	29.45	29.67	Positive
GXQ5084	27	GSH	M	35	Serum	31.61	0.00	Positive
TPA8160	15; 16	TBH	M	35	Stool	25.79	0.00	Positive
TST3751 - TST7232	34	TBH	F	37	Serum	0.00	32.08	Negative
TXE6201	34; 46	TBH	M	21	Serum	0.00	32.02	Negative
TST1247	34; 46	TBH	F	27	Serum	0.00	32.09	Negative
WVII5287	34	WCBS	F	37	Plasma	0.00	33.55	Negative
WVII0868	34	WCBS	M	30	Plasma	0.00	30.48	Negative
WVII5290	34	WCBS	M	51	Plasma	0.00	30.27	Negative
WVII5272	34	WCBS	M	51	Plasma	0.00	30.27	Negative
WVII5268	34	WCBS	F	59	Plasma	0.00	30.13	Negative
WVII7011	34	WCBS	M	55	Plasma	0.00	30.40	Negative
WVJ4731	34	WCBS	F	24	Plasma	0.00	30.40	Negative
WVII7050	34	WCBS	M	51	Plasma	0.00	31.80	Negative
WVII0867	34	WCBS	F	27	Plasma	0.00	32.08	Negative
WVII5266	34	WCBS	M	33	Plasma	0.00	32.33	Negative
TXK1271	34	TBH	M	36	Serum	27.79	30.51	Positive
TXF7706	35; 45	TBH	F	36	Serum	30.55	29.86	Positive
TXN9891	50	TBH	M	36	Serum	36.08	32.49	Positive
TST2485	28	TBH	F	37	Serum	28.36	0.00	Positive
WVII5282	35	WCBS	F	56	Plasma	0.00	29.62	Negative
WVII7036	35	WCBS	F	37	Plasma	0.00	30.02	Negative
WVII7005	35	WCBS	F	51	Plasma	0.00	29.91	Negative
WVII5285	35	WCBS	M	35	Plasma	0.00	30.07	Negative
TXG9563	35; 46	TBH	F	38	Serum	26.75	31.77	Positive

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Episode Number	Experiment Number	Location of retrieval	Sex (M/F)	Age (years)	Clinical Sample Type	Sample Ct value results	IC Ct values	HAV RNA detection outcome
TXK6216	50	TBH	F	7	Serum	0.00	31.25	Negative
TXO4520	29	TBH	M	41	Serum	28.41	0.00	Positive
TXK2109	24; 25	TBH	F	47	Serum	31.34	31.57	Positive
GXC9378	28	GSH	F	49	Serum	23.60	33.14	Positive
TXN4254	24; 25; 25	TBH	F	71	Serum	30.82	31.13	Positive

Experiment numbers as corresponding to laboratory book.

Environmental samples

Sample ID	Experiment number	Sample original location	Months of collection 2021	Sample CT Values	IC Ct Values	HAV RNA DETECTION
ATC120421	54	ATC	April	37.76	31.99	Positive
ZTV120421	54	ZTV	April	0.00	32.07	Negative
ATC190421	54	ATC	April	39.33	30.22	Positive
ZTV190421	54	ZTV	April	0.00	30.52	Negative
ATC080821	55	ATC	August	0.00	0.00	Negative
ATC180821	55	ATC	August	0.00	0.00	Negative
ZTV080821	55	ZTV	August	0.00	0.00	Negative
ZTV180821	55	ZTV	August	0.00	30.65	Negative
MET080821	55	MET	August	0.00	0.00	Negative
MH2080821	55	MH2	August	0.00	33.39	Negative
MH1080821	55	MH1	August	0.00	33.36	Negative
MET180821	55	MET	August	0.00	0.00	Negative
MH2180821	55	MH2	August	0.00	0.00	Negative
MH1180821	55	MH1	August	0.00	0.00	Negative
ATC030821	56	ATC	August	0.00	0.00	Negative
ATC150821	56	ATC	August	0.00	0.00	Negative
ZTV030821	56	ZTV	August	0.00	0.00	Negative
ZTV150821	56	ZTV	August	0.00	32.13	Negative
MET030821	56	MET	August	0.00	0.00	Negative
MH2150821	56	MH2	August	0.00	39.20	Negative
MH1150821	56	MH1	August	0.00	0.00	Negative
MET150821	56	MET	August	0.00	0.00	Negative
MH260821	56	MH2	August	0.00	0.00	Negative
MH1260821	56	MH1	August	0.00	0.00	Negative
ATC280821	57	ATC	August	0.00	21.68	Negative

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Sample ID	Experiment number	Sample original location	Months of collection 2021	Sample CT Values	IC Ct Values	HAV RNA DETECTION
MH2300821	57	MH2	August	0.00	21.04	Negative
ZTV280821	57	ZTV	August	0.00	21.35	Negative
HIPPO300621	57	HIPPO	June	0.00	20.25	Negative
MET300821	57	MET	August	0.00	20.82	Negative
MH1280821	57	MH1	August	0.00	20.37	Negative
MH2280821	57	MH2	August	0.00	20.63	Negative
MET280221	57	MET	August	0.00	21.47	Negative
ATC300821	57	ATC	August	0.00	20.65	Negative
ZTV300821	57	ZTV	August	0.00	20.50	Negative
MH1300821	57	MH1	August	0.00	20.28	Negative
ATC050721	58	ATC	July	0.00	17.87	Negative
MH1010921	58	MH1	September	0.00	21.09	Negative
ZTV050721	58	ZTV	July	35.49	20.54	Positive
MH2010921	58	MH2	September	35.49	18.67	Positive
ATC120721	58	ATC	July	36.85	22.39	Positive
MET010921	58	MET	September	0.00	22.97	Negative
ZTV120821	58	ZTV	August	39.56	21.90	Positive
ATC020821	58	ATC	August	30.23	22.21	Positive
ZTV020821	58	ZTV	August	0.00	22.18	Negative
ATC090821	58	ATC	August	0.00	21.92	Negative
ZTV090821	58	ZTV	September	31.68	22.01	Positive
ATC190721	59	ATC	July	37.52	23.83	Positive
MH1270721	59	MH1	July	0.00	0.00	Negative
ZTV190721	59	ZTV	July	0.00	24.15	Negative
MH2270721	59	MH2	July	0.00	0.00	Negative
ATC290721	59	ATC	July	0.00	21.30	Negative
MET270721	59	MET	July	0.00	0.00	Negative
ZTV290721	59	ZTV	July	37.38	22.85	Positive
MH2130721	59	MH2	July	0.00	22.77	Negative
MH2210728	59	MH2	July	0.00	22.78	Negative
MH2210721	59	MH2	July	0.00	22.40	Negative
MH1130721	59	MH1	July	0.00	22.79	Negative
ATC180121	60	ATC	January	36.07	26.40	Positive
MH1060621	60	MH1	June	38.01	26.98	Positive
ZTV180121	60	ZTV	January	38.02	26.51	Positive
MH2060621	60	MH2	June	35.94	26.63	Positive
ATC250121	60	ATC	January	0.00	25.25	Negative
MET060621	60	MET	June	26.58	26.39	Positive

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Sample ID	Experiment number	Sample original location	Months of collection 2021	Sample CT Values	IC Ct Values	HAV RNA DETECTION
ZTV250121	60	ZTV	January	38.50	25.94	Positive
ATC010221	60	ATC	February	0.00	26.34	Negative
ZTV010221	60	ZTV	February	0.00	26.64	Negative
ATC080221	60	ATC	February	0.00	26.54	Negative
ZTV080221	60	ZTV	February	0.00	26.59	Negative
ATC150221	61	ATC	February	0.00	26.59	Negative
ATC150321	61	ATC	March	35.67	26.36	Positive
MH1050521	61	MH1	May	34.92	26.46	Positive
ZTV150221	61	ZTV	February	0.00	26.35	Negative
ZTV150321	61	ZTV	March	36.32	26.83	Positive
ATC220221	61	ATC	February	0.00	26.13	Negative
ATC220321	61	ATC	March	34.83	26.61	Positive
ZTV220221	61	ZTV	February	0.00	26.36	Negative
ZTV220321	61	ZTV	March	36.09	26.71	Positive
ATC010321	61	ATC	March	0.00	25.85	Negative
ATC290321	61	ATC	March	35.43	26.64	Positive
ZTV010321	61	ZTV	March	0.00	26.43	Negative
ZTV290321	61	ZTV	March	31.46	26.66	Positive
ATC080321	61	ATC	March	0.00	26.14	Negative
MET040521	61	MET	May	34.99	26.02	Positive
ZTV080321	61	ZTV	March	0.00	25.98	Negative
3SUS080521	61	3SUS	May	0.00	25.95	Negative
ATC260721	62	ATC	July	0.00	27.50	Negative
ZTV260721	62	ZTV	July	37.12	26.92	Positive
ATC020821	62	ATC	August	36.21	26.97	Positive
ZTV020821	62	ZTV	August	40.29	27.49	Positive
ATC090821	62	ATC	August	0.00	27.01	Negative
ZTV090821	62	ZTV	August	39.23	27.15	Positive
ATC160821	62	ATC	August	38.01	27.25	Positive
ZTV160821	62	ZTV	August	0.00	27.50	Negative
ATC230821	62	ATC	August	0.00	0.00	Negative
ZTV230821	62	ZTV	August	0.00	26.66	Negative
ATC300821	62	ATC	August	0.00	28.08	Negative
ZTV300821	62	ZTV	August	0.00	27.15	Negative
ATC80921	62	ATC	September	0.00	27.00	Negative
ZTV080921	62	ZTV	September	0.00	26.90	Negative
HIPPO180821	62	HIPPO	August	0.00	26.81	Negative
MET240821	62	MET	August	0.00	26.93	Negative

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Sample ID	Experiment number	Sample original location	Months of collection 2021	Sample CT Values	IC Ct Values	HAV RNA DETECTION
3SUS250821	62	3SUS	August	35.01	27.68	Positive
MH1170821	62	MH1	August	34.47	27.39	Positive
ATC130921	63	ATC	September	35.83	27.66	Positive
MET110821	63	MET	August	38.96	27.79	Positive
ZTV130921	63	ZTV	September	35.50	26.48	Positive
HIPPO080921	63	HIPPO	September	37.25	27.09	Positive
ATC200921	63	ATC	September	35.46	27.25	Positive
ZTV200921	63	ZTV	September	35.40	27.33	Positive
ATC270921	63	ATC	September	0.00	27.26	Negative
ZTV270921	63	ZTV	September	0.00	27.16	Negative
ATC041021	63	ATC	October	0.00	27.20	Negative
3SUS110821	63	3SUS	August	0.00	26.97	Negative
ATC030521	64	ATC	May	37.63	26.32	Positive
ZTV310521	64	ZTV	May	34.37	26.52	Positive
MET070621	64	MET	June	0.00	26.18	Negative
ZTV030521	64	ZTV	May	0.00	26.64	Negative
MH2210721	64	MH2	July	0.00	26.01	Negative
MH1070721	64	MH1	July	37.15	26.32	Positive
ATC100521	64	ATC	May	0.00	26.40	Negative
MH2140721	64	MH2	July	0.00	26.34	Negative
MET070721	64	MET	July	0.00	26.30	Negative
ZTV100521	64	ZTV	May	34.67	26.04	Positive
MH1140721	64	MH1	July	0.00	36.87	Negative
ATC170521	64	ATC	May	0.00	0.00	Negative
MH1210721	64	MH1	July	39.19	25.93	Positive
ZTV170521	64	ZTV	May	35.92	26.62	Positive
MET161121	64	MET	November	35.69	26.01	Positive
ATC240521	64	ATC	May	35.06	26.41	Positive
MH1080921	64	MH1	September	0.00	25.68	Negative
ATC310521	64	ATC	May	35.42	26.33	Positive
MH2210721	64	MH2	July	0.00	25.84	Negative
MH1010621	65	MH1	June	0.00	28.47	Negative
MH2010621	65	MH2	June	0.00	28.51	Negative
MH1080621	65	MH1	June	0.00	28.18	Negative
MH2080621	65	MH2	June	0.00	27.23	Negative
MH1150621	65	MH1	June	0.00	27.14	Negative
MH2150621	65	MH2	June	0.00	27.70	Negative
MH1220621	65	MH1	June	0.00	20.11	Negative

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Sample ID	Experiment number	Sample original location	Months of collection 2021	Sample CT Values	IC Ct Values	HAV RNA DETECTION
MH2220621	65	MH2	June	0.00	27.33	Negative
MH1290621	65	MH1	June	0.00	26.97	Negative
MH1290621	65	MH2	June	0.00	2.13	Negative
MH1060721	65	MH1	July	0.00	27.62	Negative
MH2060721	65	MH1	July	0.00	27.09	Negative
MH1130721	65	MH2	July	0.00	27.99	Negative
MH2130721	65	MH1	July	0.00	28.49	Negative
MH1210721	65	MH1	July	0.00	28.34	Negative
MH2210721	65	MH1	July	0.00	27.04	Negative
MH1270721	65	MH2	July	0.00	26.61	Negative
MH2270721	65	MH1	July	0.00	27.03	Negative
MH1040821	65	MH2	August	0.00	27.03	Negative
MH2040821	65	MH1	August	0.00	28.37	Negative
MET120821	65	MH2	August	0.00	26.89	Negative
MET230821	65	MH1	August	28.38	27.43	Positive
MH1100821	66	MH2	August	0.00	27.41	Negative
MH2100821	66	MH2	August	0.00	27.47	Negative
MH1170821	66	MH2	August	0.00	27.68	Negative
MH2170821	66	MH1	August	0.00	27.47	Negative
MH1240821	66	MH1	August	0.00	27.37	Negative
MH2240821	66	MH1	August	0.00	27.23	Negative
MH1010921	66	MH2	August	35.98	27.58	Positive
MH2010921	66	MH1	September	36.32	27.16	Positive
MH1080921	66	MH2	September	37.21	27.02	Positive
MH2080921	66	MH1	September	35.77	26.97	Positive
MH1130921	66	MH1	September	36.30	26.87	Positive
MH2130921	66	MH2	September	36.24	26.60	Positive
MH1290921	66	MH1	September	35.23	26.67	Positive
MH2290921	66	MH2	September	36.44	26.83	Positive
MH1061021	66	MH1	October	0.00	27.14	Negative
MH2061021	66	MH2	October	37.58	26.47	Positive
MH1131021	66	MH1	October	0.00	27.62	Negative
MH2131021	66	MH2	October	0.00	0.00	Negative
MH1201021	66	MH1	October	0.00	0.00	Negative
MH2201021	66	MH1	October	34.68	26.85	Positive

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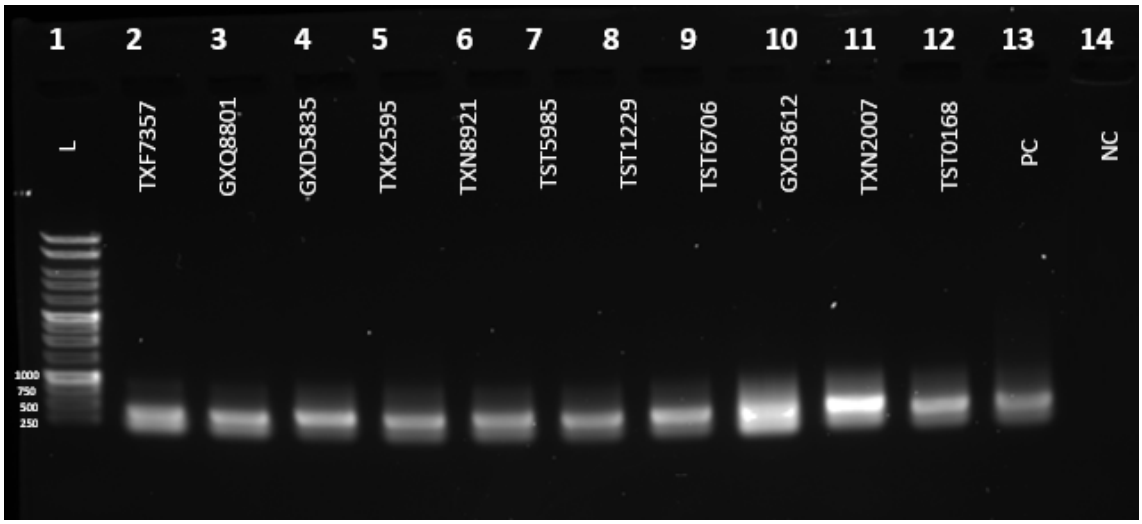
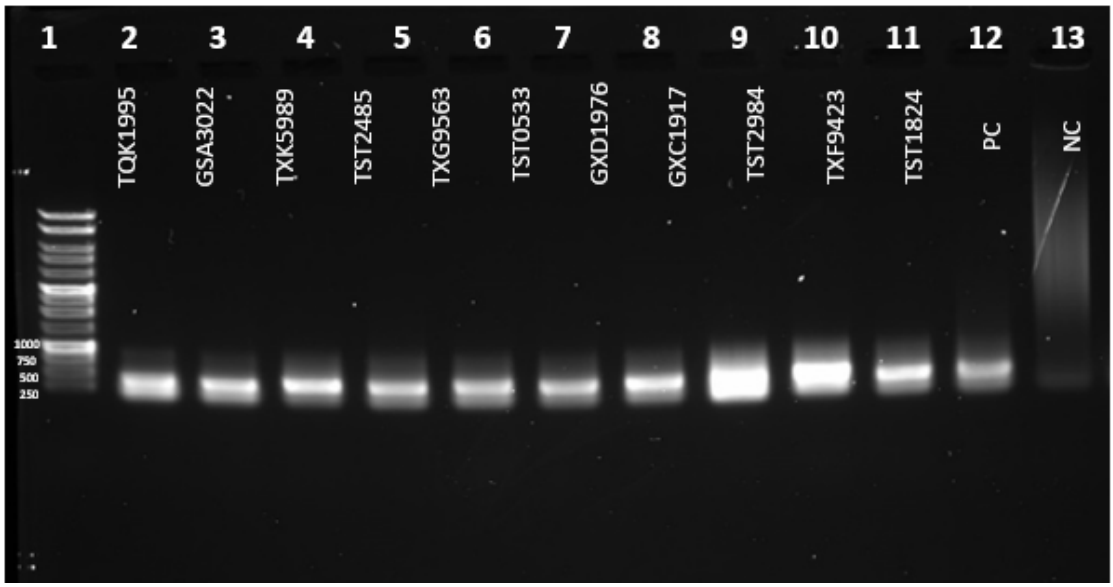
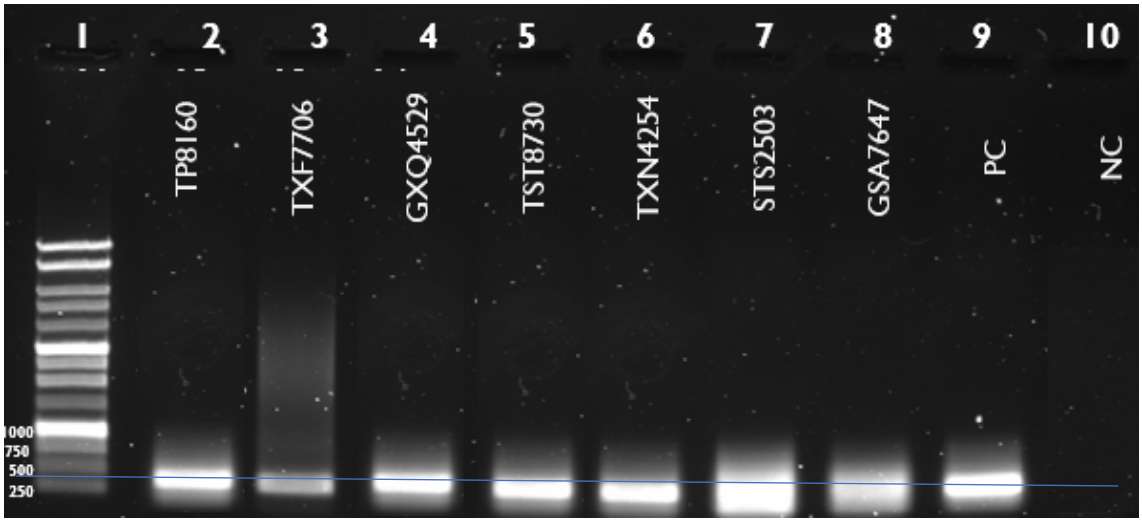
Addendum H: - Agarose gel images



Genotyping gel run 1



Genotyping gel run 2



Genotyping gel run 3

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Addendum I: - Genome Detective genotypic tool files

SEQUENCE ASSIGNMENT

Name TXG9563

Length 222

GENOGROUP ASSIGNMENT

Genogroup assignment Hepatovirus A

GENOTYPE RESULT

Genotype assignment HAV I.B

Supported with phylogenetic analysis and bootstrap 74.0 (>= 70.0)

Sub-clustering N/A

Sequence does not sufficiently overlap with region or subcluster is not available for the type

GENOME REGION

Sequence starts at position 2982 and ends at position 3203 relative to the NC_001489.1 reference sequence for Hepatovirus A (taxon:12092).

SEQUENCE ASSIGNMENT

Name TST0533

Length 391

GENOGROUP ASSIGNMENT

Genogroup assignment Hepatovirus A

GENOTYPE RESULT

Genotype assignment HAV I.B

Supported with phylogenetic analysis and bootstrap 72.0 (>= 70.0)

Sub-clustering N/A

Sequence does not sufficiently overlap with region or subcluster is not available for the type

GENOME REGION

Sequence starts at position 2898 and ends at position 3288 relative to the NC_001489.1 reference sequence for Hepatovirus A (taxon:12092).

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SEQUENCE ASSIGNMENT

Name TXN5226

Length 298

GENOGROUP ASSIGNMENT

Genogroup assignment Hepatovirus A

GENOTYPE RESULT

Genotype assignment HAV I.B

Supported with phylogenetic analysis and bootstrap 73.0 (>= 70.0)

Sub-clustering N/A

Sequence does not sufficiently overlap with region or subcluster is not available for the type

GENOME REGION

Sequence starts at position 2946 and ends at position 3243 relative to the NC_001489.1 reference sequence for Hepatovirus A (taxon:12092).

SEQUENCE ASSIGNMENT

Name TST9201

Length 367

GENOGROUP ASSIGNMENT

Genogroup assignment Hepatovirus A

GENOTYPE RESULT

Genotype assignment HAV I.B

Supported with phylogenetic analysis and bootstrap 83.0 (>= 70.0)

Sub-clustering N/A

Sequence does not sufficiently overlap with region or subcluster is not available for the type

GENOME REGION

Sequence starts at position 2925 and ends at position 3291 relative to the NC_001489.1 reference sequence for Hepatovirus A (taxon:12092).

SEQUENCE ASSIGNMENT

Name GSG2839

Length 303

GENOGROUP ASSIGNMENT

Genogroup assignment Hepatovirus A

GENOTYPE RESULT

Genotype assignment HAV I.B

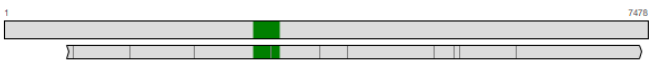
Supported with phylogenetic analysis and bootstrap 78.0 (>= 70.0)

Sub-clustering N/A

Sequence does not sufficiently overlap with region or subcluster is not available for the type

GENOME REGION

Sequence starts at position 2898 and ends at position 3200 relative to the NC_001489.1 reference sequence for Hepatovirus A (taxon:12092).



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SEQUENCE ASSIGNMENT

Name	GXD5547
Length	314

GENOGROUP ASSIGNMENT

Genogroup assignment	Hepatovirus A

GENOTYPE RESULT

Genotype assignment	HAV I.B
	Supported with phylogenetic analysis and bootstrap 83.0 (>= 70.0)
Sub-clustering	N/A
	Sequence does not sufficiently overlap with region or subcluster is not available for the type

GENOME REGION

Sequence starts at position 2925 and ends at position 3238 relative to the NC_001489.1 reference sequence for Hepatovirus A (taxon:12092).

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Addendum J: - Sanger sequencing clean-up and genotyping file

Macherey Nagel NucleoSpin® Gel and PCR Clean-up

1. **Preparation of the sample**
   a) DNA extraction from agarose gels:
      - Add 200 µL Buffer NT1 for each 100 mg of agarose gel < 2%
      - Incubate sample for 5-10 min at 50°C. Vortex the sample briefly every 2-3 min until the gel slice is completely dissolved.

   b) PCR clean-up:
      - Mix 1 volume of sample with 2 volumes of buffer NT1 (e.g., mix 100 µL PCR reaction and 200 µL Buffer NT1)

2. **Bind DNA**
   Place a NucleoSpin® Gel and PCR Clean/up column into a collection tube (2 mL) and load up to 700 µL sample. Centrifuge for 30 s at 11,000 x g. Discard flow/through and place the column back into the collection tube.

   Load remaining sample if necessary and repeat the centrifugation step.

3. **Wash silica membrane**
   Add 700µL Buffer NT3 to the NucleoSpin® Gel and PCR Clean/up column. Centrifuge for 30 s at 11,000 x g. Discard flow/through and place the column back into the collection tube.

   Repeat this washing step to minimiye chaotropic salt carrz/over and improve A_{260}/A_{230} values.

4. **Dry silica membrane**
   Centrifuge for 1 min at 11,000 x g to remove Buffer NT3 completely. Make sure the spin column does not come in contact with the flow-through while removing it from the centrifuge and the collection tube.

5. **Elute DNA**
   Place the NucleoSpin® Gel and PCR Clean/up column into a new 1.5 mL microcentrifuge tube (not provided). Add 15-30 µL Buffer NE and incubate at room temperature (18-25°C) for 1 min. Centrifuge for 1 min at 11,000 x g.