Genetic assessment of infectious hepatitis A virus strains detected in selected water sources in Gauteng, South Africa

by

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DECLARATION

I, Saïd Rachida, declare that this work was not copied or repeated from any other studies either from national or international publications. Procedures were carried out in accordance with the ethical rules as prescribed by the Faculty of Health Science Research Ethics Committee, University of Pretoria.

Signature:.....

Date: 17th DECEMBER 2019

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In the Name of Allah, Most Gracious, Most Merciful

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SUPERVISOR: PROF MB TAYLOR
DEPARTMENT: MEDICAL VIROLOGY
DEGREE: DOCTOR OF PHILOSOPHY (MEDICAL VIROLOGY)

SUMMARY

Hepatitis A is a vaccine preventable liver inflammation caused by the hepatitis A virus (HAV). Hepatitis A virus is the most common cause of acute viral hepatitis worldwide that is transmitted via the faecal-oral route with waterborne transmission recognised as a major public health concern. Hepatitis A virus is classified in the genus *Hepatovirus* of the family *Picornaviridae*. The stability of HAV regarding pH, temperature and different treatment systems contributes to the virus' persistence in the environment. The virion of HAV has a 7.5 kb positive-sense single-stranded RNA genome. Nucleotide sequence analysis of the VP1 region has identified six geographically distinct genotypes infecting humans (genotypes I, II and III) and non-human primates (genotypes IV, V and VI). In South Africa (SA), unique HAV IB strains have been detected in surface and wastewater samples, as well as on fresh produce at the point of retail. However, as the viruses were detected by molecular-based assays it is unknown whether the detected strains were still infectious. Although hepatitis A is a notifiable disease in SA there is gross underreporting, no routine surveillance system and a paucity of epidemiological data on HAV strains in circulation. Recently, the pretreatment of water and food samples with intercalating dyes prior to nucleic acid extraction was successfully applied for the quantification of potentially infectious HAV using molecular assays. Given

that analysing sewage, wastewater and surface waters would provide a more accurate estimation of the HAV strains circulating in the country, the present study aimed to detect and characterise infectious HAV strains from selected South African water sources.

From April 2015 to March 2016, 118 samples consisting of sewage, treated wastewater discharge and a downstream dam water were collected monthly from five wastewater treatment plants (WWTP 1, 2, 3, 4, 5). High titres of HAV were detected in the sewage (1.34×10^5 and 3.70×10^{10} genome copies [gc]/litre [L]) and treated discharge (4.74×10^3 to 3.39×10^7 gc/L) samples. None of the dam water samples tested positive for HAV. Genetic characterisation of the detected strains by Sanger sequencing revealed the circulation of HAV IB strains that carried the R298K amino acid change over the VP1 region or the R63K and R71S change over the VP1/P2B junction or the C70S and M104I change over the VP1/P2B junction. The quasispecies dynamic of HAV has been detected in sewage samples. Hepatitis A virus strains carrying amino acid mutation at the immunodominant and neutralisation epitopes were characterised in both the sewage and treated discharge samples. The virus concentrates of HAV-positive sewage and treated discharge samples were treated with a combination of PMA-water (50 µM) and Tween®20 (0.5%) and the quantification of HAV from the samples was repeated. Potentially infectious HAV was quantified from the wastewater samples, with titres ranging up to 10^6 and 10^4 gc/L of sewage and treated discharge samples, respectively. Characterisation of these potentially infectious strains by Sanger sequencing confirmed the circulation of HAV strains carrying the R298K amino acid change over the VP1 region or the R63K and R71S change over the VP1/P2B junction or the C70S and M104I change over the VP1/P2B junction. The complete coding sequence, obtained from sewage and treated discharge samples by NGS, confirmed the circulation of HAV strains carrying the R63K and R71S changes but not the C70S and M104I changes over the VP1/P2B junction. The present study provides a methodology for the quantification and genetic characterisation of potentially infectious HAV from wastewaters.

PRESENTATIONS

National Presentations

Rachida S, Taylor MB. Hepatitis A virus in wastewater discharge: the great escape [Presentation]. Faculty Day, Faculty of Health Sciences, University of Pretoria 21-22 August 2018: Tswelopele Building, Prinshof Campus, Pretoria, South Africa.

International Presentations

Rachida S, Taylor MB. Hepatitis A virus in wastewater discharge: a potential public health concern? [Presentation]. 2nd International Conference for Food Safety and Security 15-17 October 2018: Saint George's Hotel and Conference Centre, Pretoria, South Africa.

Rachida S, Taylor MB. Viability PCR for the detection of potentially infectious hepatitis A virus in wastewater sources, Gauteng, South Africa [Presentation]. Virology Africa 10-14 February 2020: Radisson Blu Hotel, Cape Town, South Africa.

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ABBREVIATIONS ACRONYMS AND SYMBOLS

µg	Microgram
µL	Microlitre
µM	Micromolar
AMPI	Age at midpoint of population immunity
BLAST	Basic local alignment search tool
bp	Base pairs
cDNA	Complementary DNA
CDS	Coding sequence
Cf	HAV titres after viability treatment
Ci	HAV titres before viability treatment
CPE	Cytopathic effect
cre	Cis-acting replication element
C_s	Concentration of HAV in sewage
CV	Coxsackievirus
C_{wd}	Concentration of HAV in treated wastewater discharge
DMSO	Dimethyl sulfoxide
ds-cDNA	Double-stranded cDNA
eHAV	Quasi-enveloped hepatitis A virus
EMA	Ethidium monoazide
EMA-DMSO	EMA dissolved in dimethyl sulfoxide
E-MEM	Eagle's Minimal Essential Medium
FCS	Foetal calf serum
g	Gram
GBD	Global Burden of Disease
gc	Genome copies
G	Genogroup
h	Hour

HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDV	Hepatitis D virus
HEV	Hepatitis E virus
HIV	Human immunodeficiency virus
IC	Internal control
ICC-PCR	Integrated cell culture PCR
IDUs	Injection drug users
Ig	Immunoglobulins
IMS-PCR	Immunomagnetic separation PCR
IRES	Internal ribosome entry site
kb	Kilobases
kDa	Kilodalton
L	Litres
Log	Base 10 logarithm
LTR-PCR	Long target region PCR
MAR	Monoclonal antibody resistant
min	Minutes
mL	Millilitre
mM	Millimolar
MSM	Men who have sex with men
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
NGS	Next generation sequencing
NICD	National Institute for Communicable Diseases
NoV	Norovirus
nt	Nucleotide
ORF	Open reading frame
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PMA	Propidium monoazide
PMA-	PMA dissolved in dimethyl sulfoxide

DMSO	
PMA-water	PMA dissolved in nuclease-free water
qPCR	Quantitative PCR
QS5	QuantStudio™ 5 Real-Time PCR System, 96 well
RT-PCR	Reverse-transcription polymerase chain reaction
RT-qPCR	Reverse-transcription quantitative PCR
RT-qvPCR	Reverse transcription quantitative viability PCR
SA	South Africa
SAn	South African
tagmented	Tagged and fragmented
$TCID_{50}$	50% tissue culture infectious dose
U	Units
USA	United States of America
USSR	Union of Soviet Socialist Republics
UTR	Untranslated region
vdye	Viability dye
VIRADEL	Virus adsorption-elution
VirusTAP	Viral genome-targeted assembly pipeline
VP	Viral Protein
vPCR	Viability PCR
WHO	World Health Organization
WWTP	Wastewater treatment plant

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CHAPTER 1

GENERAL INTRODUCTION

Viral hepatitis caused 1.34 million deaths in 2015, a mortality rate similar to that of tuberculosis.[1] As mortality due to human immunodeficiency virus (HIV) infection, tuberculosis and malaria is decreasing, viral hepatitis mortality is increasing.[1] In 2016, the World Health Assembly has adopted a Global Health Sector Strategy on viral hepatitis with a goal to eliminate hepatitis by 2030.[1] Hepatitis A, B, C, D and E viruses (HAV, HBV, HCV, HDV and HEV) are responsible for most viral hepatitis cases. Efforts to eliminate viral hepatitis are directed more towards HBV and HCV infections, which are responsible for 96% of all mortality due to viral hepatitis[1] and it is likely that infections due to HBV and HCV will decrease by 2030. Hepatitis A virus is a human food and waterborne pathogen that is the leading cause of acute hepatitis.[2] Currently, 2.1 billion people lack access to safe drinking water at home and for 4.5 billion people safe sanitation is non-existent.[3] The 2030 targets for the United Nations Sustainable Development Goal 6 include universal access to safe affordable drinking water, universal access to adequate sanitation and hygiene and improved water quality and wastewater treatment as well as the safe reuse of water.[4] Given that the incidence of HAV is linked to access to safe drinking water and sanitation, improvements in water and sanitation could increase economic burden from hepatitis A in a community.[5-6] This increase is likely to reverse once universal access to safe drinking water and sanitation has been completely achieved.

Hepatitis A is a vaccine preventable liver inflammation.[7-8] In 2015, HAV was responsible for approximately 11000 deaths worldwide.[1] Hepatitis A virus infections are usually self-limiting, but complications including fulminant hepatitis A may occur, especially in old age or in the presence of underlying liver conditions such as hepatitis B or C.[7-8]

Hepatitis A virus is mainly transmitted via the faecal-oral route with food and waterborne transmission recognised as a major public health concern.[9-10] The HAV particle is shed in faeces of infected individuals and can remain infectious in wastewaters (treated and untreated) for days to months.[11] Consequently, rivers receiving contaminated wastewater discharge could in turn become polluted, exposing users to potential infection with HAV.[9] Epidemiological data on the type of HAV strains circulating within a population is best sought through the detection and characterisation of the virus in wastewaters[12], as they contain viral strains shed by asymptomatically and symptomatically infected individuals. Molecular-based assays are the method of choice for the detection of HAV in wastewater, but cannot distinguish between infectious and non-infectious viruses.[13] Once detected, HAV can be classified into six geographically distinct genotypes[14] This information could assist source tracking the origin of an outbreak or potential source(s) of contamination. In addition, genetic characterisation of HAV can help identify the circulation of potential vaccine escape mutants.[15]

In South Africa (SA), HAV has been detected in environmental samples, namely sewage, treated wastewater discharge, various water sources and on fresh produce at the point of retail.[16-19] The South African (SAn) strains predominantly belong to subgenotype IB but carry unique mutations which distinguish them from IB strains detected in the rest of the world.[20-21] A novel HAV strain as well as a potential vaccine escape mutant have also been detected in separate single irrigation water samples from two different provinces in SA.[21-22] However, as the viruses were detected by molecular-based assays it is not known whether the strains were potentially infectious or not. Recently, molecular-based assays that can distinguish potentially infectious from non-infectious viruses have been developed. Pretreatment of samples with intercalating dyes prior to nucleic acid extraction was successful at detecting potentially infectious HAV particles in environmental samples.[13]

Although hepatitis A is a notifiable disease in SA, there is gross underreporting and until recently there was no routine surveillance system. Early seroprevalence studies showed that HAV is hyperendemic in SA and two well-defined

epidemiological patterns were recorded: the lower-socioeconomic population was infected by the age of six, while the higher-socioeconomic population was only exposed to the virus at an older age.[23-24] Recent serological studies showed that HAV is still prevalent in SA but the endemicity of the virus could be shifting from high to intermediate levels.[25-26] These studies were limited by the use of public health laboratory results, which underestimate the true prevalence of HAV in SA. These results not only exclude asymptomatic infections, but also the proportion of the population that consult in the private sector. There is also a paucity of molecular epidemiological data on HAV strains circulating in SA. Analysing sewage, wastewater and surface waters would provide a more accurate estimation of the HAV strains circulating in the country, since all infected individuals, be they symptomatic or not, shed high titres of the virus in faeces.[27] This passive surveillance method also provides valuable information on the efficacy of wastewater treatment and an early warning system of potential waterborne outbreaks. Additionally, coupling the detection with viral quantification and viability studies will provide valuable data on potentially infectious HAVs circulating in the community and environment and for future risk assessment studies.

1.1 AIM

The study aims to detect and characterise infectious HAV strains from selected SAn water sources.

(*It should be noted that the outcome of the present study is limited to the sampling area and may not necessarily apply to the rest of the Gauteng region or SA*)

1.2 HYPOTHESES

For this study, two hypotheses were formulated: (1) wastewater treatment is ineffective and does not completely remove and/or inactivate HAV from raw sewage; (2) recombinant and novel HAV strains circulate in SA.

1.3 OBJECTIVES

In order to investigate the hypotheses, five specific objectives were set:

1. To detect and quantify HAV strains from selected SA water sources
2. To determine the infectivity of detected HAV strains using molecular-based assays
3. To characterise the HAV strains detected by nucleotide sequence and phylogenetic analysis
4. To characterise novel and/or emerging strains using full genome analysis
5. To determine the clinical relevance of the potentially infectious environmental HAV strains by comparison to those detected in clinical specimens

1.4 ETHICAL APPROVAL

The research protocol of the present study was reviewed and approved by the Faculty of Health Sciences Research Ethics Committee (Ethics Reference No.: 327/2016), University of Pretoria (Appendix B). This committee complies with the International Conference on Harmonisation-Good Clinical Practice guidelines and has US Federal wide Assurance. Clinical specimens were not collected for the purpose of this study. However, nucleotide sequence data of clinical specimens from a previous study (Ethics Reference No.: 211/2012) were used for comparison at a molecular level.

The results of the study are presented in the following format. In addition to the introductory chapter (chapter 1), six chapters will address the following:

1. Chapter 2 will present the background of the study by reviewing the literature;
2. Chapter 3 will present and discuss the results obtain after quantifying HAV in wastewaters using real-time reverse-transcription quantitative polymerase chain reaction (RT-qPCR);
3. Chapter 4 will present how a viability PCR assay was optimised and used to determine the infectivity of HAV in wastewaters;

4. Chapter 5 will present the genetic diversity of HAV strains detected in wastewater, as determined by Sanger and next-generation sequencing; the genetic diversity of HAV in wastewater will be compared to nucleotide sequence data from clinical specimens;
5. Chapter 6 will summarise and discuss the main findings of the study, to prove or disprove the hypotheses formulated at the beginning of the study;
6. Chapter 7 will list the references used for the study.

CHAPTER 2

LITERATURE REVIEW

2.1 HISTORY

Hepatitis A virus is an ancient virus that causes jaundice.[8,28-29] Reports of catarrhal jaundice can be found in ancient Greek, Roman and Chinese literature.[28-29] Epidemic jaundice was commonly reported among troops on campaign, especially in America during the civil war.[28] In SA epidemic jaundice was reported during the Boer war and for expeditions in the Mediterranean region during World War I.[30] The disease was mild and mortality rate was very low, but the troops were incapacitated for duty.[30] The HAV particle was first detected in 1973[31], shortly followed by propagation in cell culture[32] and the development of a vaccine occurred in the early 1990s.[33-34] The implementation of HAV vaccine saw a dramatic decrease in the incidence of hepatitis A in geographical regions such as the United States of America (USA)[8,35], Catalonia (Spain)[36-37], Puglia (Italy)[36], Greece[38] and Argentina[39]. Despite a safe and effective vaccine being available, sporadic and outbreak cases of hepatitis A are still being reported. In 2010, HAV was classified as one of the major causes of foodborne disease with 14 million foodborne illnesses and 27,731 foodborne deaths worldwide.[8,40] The current multistate outbreak of hepatitis A taking place in the USA and involving 27,634 people with a hospitalisation rate of 60%[41], further highlights the importance of HAV from a public health perspective.

2.2 HEPATITIS A VIRUS

2.2.1 Virion morphology

2.2.1.1 Capsid structure

The naked HAV particle was first observed, by electron microscopy, in the stool of infected individuals in 1973.[31] The viral particle is a 27 nm icosahedron that has a smooth negatively charged external surface with no canyon.[42] There are four capsid proteins 1A, 1B, 1C and 1D, also known as viral protein (VP) 4 (VP4), VP2, VP3 and VP1, respectively. A biological protomer consists of one copy each of the surface protein VP0 (uncleaved or partially cleaved VP4VP2), VP3 and VP1. A total of 60 biological protomers organised into 12 pentamers give HAV the shape of a facetted triakis icosahedron (Figure 2.1).[42]

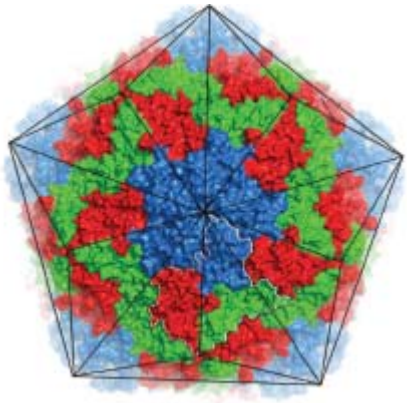


Figure 2.1: The structure of the HAV capsid. The surface proteins VP1, VP2 and VP3 are coloured in blue, green and red, respectively.[42]

The high resolution structure of the HAV capsid revealed that it is tightly packed and there is a 'domain swap' at the N terminus of the VP2 that connects two adjacent pentamers. This implies that the protomers of a pentamer are joined together by the VP2 of an adjacent protomer. This structural arrangement is absent in other picornaviruses and only present in insect picorna-like viruses such as cricket paralysis virus.[42]

2.2.1.2 Capsid stability

The stability of many picornaviruses is determined by the way in which the 12 pentamers that make up the capsid are connected.[43-44] Hepatitis A virus differs from other picornaviruses by its ability to withstand high temperatures and acidic pH. It has been hypothesised, but not yet proven, that the VP2 'domain swap'

observed for the HAV capsid could explain how the virus can withstand temperatures as high as 80°C and a pH as low as 2.0.[8,42]

2.2.1.3 Morphology

Hepatitis A virus can exist as either a naked or as a quasi-enveloped viral particle referred to as eHAV (Figure 2.2).[8,45] The eHAV particle is about 50 – 110 nm in size and consists of one to four naked HAV particles enclosed in a host membrane. Structurally, the eHAV capsid contains the VP1pX precursor protein that is cleaved in naked viral particles.[45] The naked HAV particles are shed in faeces while eHAV is found in the blood of an infected individual and the supernatant of infected cell cultures. In contrast to conventional enveloped viruses, eHAV particles do not express viral glycoproteins on the envelope surface.[8,45]

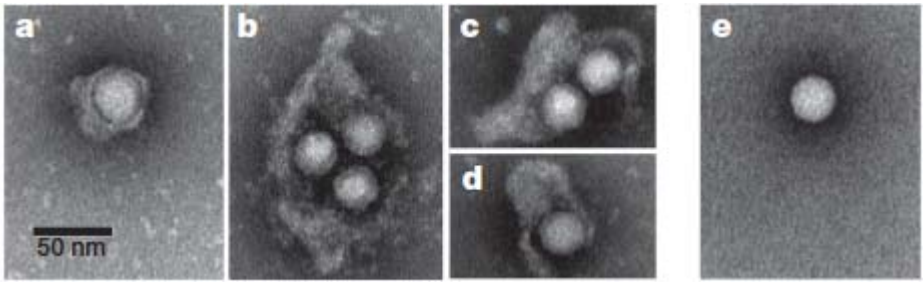


Figure 2.2: Electron micrograph of the quasi-enveloped (a to d) and naked (e) HAV particles.[45]

2.2.2 Genome organisation

The genome of HAV is a positive-sense single stranded non-segmented RNA molecule of approximately 7500 nucleotides (nt).[8,46] The genome of HAV consists of two untranslated regions (UTR) that enclose a single open reading frame (ORF) as depicted in Figure 2.3.

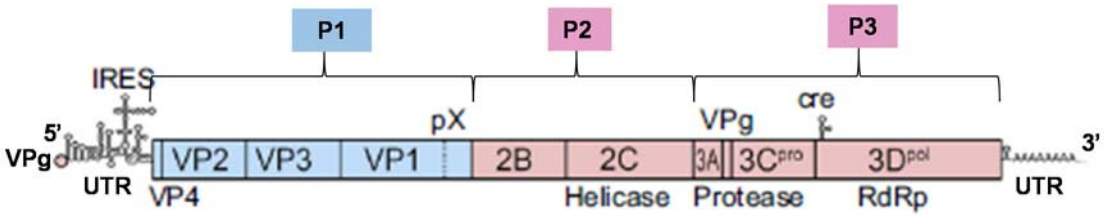


Figure 2.3: Genome map of HAV. The 5' and 3' untranslated regions enclose one open reading frame (highlighted in blue for structural protein and pink for non-structural proteins.[8]

2.2.2.1 Genomic untranslated regions

The 5'UTR of the HAV genome is approximately 734 nt long. From the 5' terminus the genomic region contains the following structures: the VPg (also known as 3B) protein covalently attached to the region, a 5' terminal hairpin structure, two putative RNA pseudoknots, a short (approximately 40 nt) conserved pyrimidine-rich [poly(U/UC)] tract and a type III internal ribosome entry site (IRES) (Figure 2.3).[8,46] The IRES controls translation of the ORF, while the uridylated VPg may initiate RNA synthesis.[47] Since it is highly conserved among HAV strains, the 5'UTR is used in real-time reverse-transcription PCR (RT-PCR) to detect the virus in clinical specimens and environmental samples.[48] The 3'UTR is shorter than the 5'UTR. It is about 53-59 nt long with a poly(A) tail at the end.[8,46]

2.2.2.2 Genomic translated region

The ORF of the HAV genome is broadly divided into three main regions: P1, P2 and P3 (Figure 2.3). A distinguishing feature of the HAV ORF is the cis-acting replication element (cre) (Figure 2.3), which is located within the portion of the P3 region that encodes for the polymerase.[49] Translation of the ORF produces one long polyprotein that is cleaved into 10 mature viral proteins. The structural proteins VP4, VP2, VP3 and VP1pX are cleaved from the P1 region (Figure 2.3). The non-structural proteins 2B, 2C are cleaved from the P2 region, and, 3A, 3B, $3C^{pro}$ and $3D^{pol}$ are cleaved from the P3 region. The $3D^{pol}$ protein is a RNA-dependent RNA polymerase and the $3C^{pro}$ is a cysteine protease. Cleavages of

the polyprotein are mediated by the $3C^{pro}$ protein except for the VP4-VP2 and VP1pX cleavages that may be performed by cellular proteases.[8,45-46]

2.2.2.3 Antigenicity

Hepatitis A virus has two biotypes that can be differentiated by monoclonal antibodies.[46] The first biotype infects humans, chimpanzees, owl monkeys and marmosets, while the second biotype infects green monkeys and cynomolgus monkeys.[46] Hepatitis A virus is an antigenically stable RNA virus with a single serotype.[46,50] This stability is attributed to a number of factors that ensure a proper capsid folding. An example of such factor is the presence of rare codons on the surface of the capsid that are located in the epitope region and whose substitutions are negatively selected, even under severe immune suppression.[50]

The antigenic structure of HAV has yet to be completely characterised, but findings suggest that it is defined by four epitopes represented by escape mutants to four monoclonal antibodies, namely K34C8, H7C27, 4E7 and R10.[50-53] Studies of monoclonal antibody resistant (MAR) mutants to K34C8 revealed that the immunodominant antigenic site involves the amino acid residues 102, 171 and 176 of VP1, and, 70, 71 and 74 of VP3.[50-52] The second antigenic site, known as the glycophorin A binding site, involves K221 of VP1 and was identified by studying the MARs to H7C27.[50,52] The third epitope represented by MARs to 4E7 have not yet been identified.[50] The fourth epitope, identified by studying MARs to R10 involves the amino acid residues 64 – 71 of VP2, and, 68 – 78, 143 – 150, 209 and 246 of VP3.[53] Replacement of a single amino acid residue at the epitopes could be enough to confer resistance to antibody neutralisation.[50,52,54]

In vitro studies of MARs to K34C8 and H7C27 allowed for the identification of additional amino acid residues around the epitopes that could interfere with antibody binding.[54] Furthermore, it has been shown that MARs to K34C8 have a lower fitness than wild-type HAV, while escape mutants to H7C27 have a similar fitness to wild-type HAV in terms of replication.[54] However, antigenic variants of HAV carrying amino acid replacement(s) at the immunodominant antigenic site have been isolated from patients during a hepatitis A outbreak among men who

have sex with men (MSM).[15] It has been suggested that in immunocompromised persons or persons who have not completed the vaccination schedule of two doses, HAV could replicate in the presence of antibodies leading to the emergence of antigenic escape variants.[15] This has been further proven by quasispecies analyses which demonstrated a higher diversity of antigenic variants of HAV in vaccinated patients in contrast to unvaccinated patients.[55]

An *in vitro* study of MAR C6 to K34C8 which carry a W170C change on the VP1 protein showed that the mutant is resistant to neutralisation by the Havrix™ and AVAXIM™ vaccines against hepatitis A.[15] On the other hand, MAR D23 to H7C27 which carry a G217D change on the VP1 protein can be neutralised by Havrix™ but maybe partially resistant to AVAXIM™.[15] The evidence presented here suggest that vaccine escape mutants of HAV could possibly emerge in partially vaccinated persons and spread to the rest of the population. It is therefore important to establish a surveillance system that can monitor the circulation of HAV strains in different communities to detect emerging and/or mutant strains early and prevent subsequent transmission.

2.2.3 Classification

2.2.3.1 Family Picornaviridae

Classified within the *Picornavirales* order, the *Picornaviridae* family groups small, non-enveloped, icosahedral positive-sense RNA viruses. Generally, the origin of the word *Picorna* is known to have been derived from the Italian word “piccolo” which means “small” and “rna”. However, the name *Picorna* is also an acronym derived from poliovirus, insensitivity to ether, coxsackievirus, orphan virus, rhinovirus, ribonucleic acid.[46] Picornaviruses cause symptomatic and asymptomatic infections in vertebrates. They can cause severe diseases of the heart, liver or the central nervous system. Four distinctive features characterise picornaviruses:

- all members have three capsid proteins with b-barrel folding;
- the size of the RNA genome ranges from 6.7 - 10.1 kilobases (kb);
- the polyprotein is processed by virus-encoded cysteine proteinase(s);

- an RNA-dependent RNA polymerase with YGDD sequence-motif catalyses replication.

The family *Picornaviridae* includes 80 species classified within 35 genera. These genera are differentiated by the following three criteria:

- genome maps are distinctive to each genus;
- amino acid divergence for members of the same genus is <67% over the P1 polyprotein and <64% over the $2C^{hel}$, $3C^{pro}$ and $3D^{pol}$ proteins;
- absence of homology of proteins L, 2B, 3A and 3B is observed.

Protein L is a leader protein that precedes the P1 region and is found in some, but not all, picornaviruses like aphtoviruses and erboviruses. It is a protease that releases itself from the polyprotein.[46]

2.2.3.2 Genus Hepatovirus

The genus *Hepatovirus*, with HAV as the type species, is the second most speciose genus of the *Picornaviridae* family.[46] Species demarcation within the genus is based on genome organisation and sequence divergence. Hepatoviruses, which have been described so far, share a common genome organisation. Nucleotide divergence within the genus ranges from 18%-40% and 19%-49% over the P1 and 3CD genomic regions, respectively.[46] There are 14 types of hepatoviruses that are classified into nine species (Table 2.1), all of which infect mammals.[46] Recently, a novel marsupial HAV has been described,[56] but it is still waiting classification by the International Committee on Taxonomy of Viruses.

Hepatoviruses are characterised by YPX3L “late domain” motifs, in the VP2 protein, that are involved in the synthesis of eHAV. Hepatoviruses have a unique type III IRES element but may lack protein L. The VP4 protein is small [16 to 26 amino acids long] and lacks an N-terminal myristoylation signal. The antigenic structure of non-primate hepatoviruses remain to be elucidated, however, bat hepatoviruses have a highly conserved antigenic structure that resembles that of HAV.[57] A unique feature of hepatoviruses is the highly variable pX-2B genomic

region that may be involved in host adaptation through insertion of exogenous sequence elements. The pattern of infection is also conserved within the genus and all novel HAV-like viruses are hepatotropic.[57] The discovery of novel HAV-like viruses in bats and small mammals that share unique properties different to other mammalian viruses, like the VP2 'domain swap', together with phylogenetic and ancestral recognition analyses suggest that HAV is an ancient virus that might have originated in a small insectivorous mammal that infected small mammals before forming the primate hepatovirus lineage.[8,57]

Table 2.1: List of species of the genus *Hepatovirus* and their host.[46]

Species	Name and abbreviation of representative virus	Type(s)	Mammalian host
Hepatovirus A	hepatitis A virus (HAV)	A	Primates
Hepatovirus B	phopivirus (PhV)	B	Seals
Hepatovirus C	hepatovirus C (HepV-C)	C	Bats
Hepatovirus D	hepatovirus D (HepV-D)	D1, D2	Rodents
Hepatovirus E	hepatovirus E (HepV-E)	E	Rodents
Hepatovirus F	hepatovirus F (HepV-F)	F1, F2	Rodents
Hepatovirus G	hepatovirus G (HepV-G)	G1, G2	Bats
Hepatovirus H	hepatovirus H (HepV-H)	H1	Hedgehog
		H2	Bats
		H3	Shrew
Hepatovirus I	hepatovirus I (HepV-I)	I	Shrew

2.2.3.3 Species Hepatovirus A

Classification of genotypes: At the species level, HAV strains are characterised by nucleotide sequence analysis of the full genome or subgenomic regions. Three subgenomic regions are commonly used to differentiate HAV strains: the VP1, VP1/P2A and VP3/VP1 regions.[14,58-59] There are six genotypes of HAV differentiated by nucleotide sequence analysis of the VP1 (900 nt) genomic region. The first three genotypes (I, II and III) infect humans, while the remaining three (IV, V and VI) infect non-human primates.[14] The simian HAV strains have a unique VP3/VP1 junction that distinguishes them from the human HAV strains.[9,60] Hepatitis A virus strains, classified in the same genotype, have VP1 sequences that are between 76.5% and 89.4% similar at the nucleotide, and, between 89.5% and 99.3% similar at the amino acid level.[14] The human HAV strains can be further subdivided into subgenotypes; namely IA, IB, IC, IIA, IIB, IIIA and IIIB.[14,37,58] Based on the VP1 genomic sequence, the nucleotide sequence of HAV strains classified in the same subgenotype should be >88.9% and >94.4% similar at the nucleotide and amino acid level, respectively.[14]

The first classification of HAV strains, based on the VP1/P2A genomic region, classified strains that are 15% and 7.5% similar, at the nucleotide level, in the same genotype and subgenotype, respectively.[58] Seven genotypes were thus defined. Genotype II and VII were represented by strains detected in France (CF-53) and Sierra Leone (SLF88), respectively.[58] However, the current classification of HAV strains based on the VP1 region showed that CF-53 and SLF88 strains are 89.4% and 97.2% similar at the nucleotide and amino acid levels, respectively. Hence, CF-53 and SLF88 strains have been reclassified as type strains of subgenotype IIA and IIB respectively.[14]

Geographical distribution of genotypes: Hepatitis A virus genotypes have a distinct geographical distribution.[9,58-59] In 1992, HAV genotype I was found to make up to 79% (82 out of 104 clinical specimens analysed) of HAV types, with subgenotype IA (67%) reported more than IB (13%).[58] Subgenotype IA was recorded as the predominant HAV type in North and South America, Japan, China, the former Union of Soviet Socialist Republics (USSR) and Thailand.[58] Genotype III was found to be the second most abundant HAV type (19%).

Subgenotype IIIA (13%) was isolated from Panamanian owl monkeys and was identified in humans from India, Sri Lanka, Nepal, Malaysia, Sweden and the USA.[58] Hepatitis A virus type IIIB was also identified in humans but from Japan and Denmark. The simian HAV strains classified within genotypes IV, V and VI, were recovered from Old World monkey species in the Soviet Union (genotypes V and VI) and in Atlanta, GA (genotype IV).[58]

A recent report considered nucleotide sequences of HAV submitted to the GenBank database of the National Center for Biotechnology Information (NCBI) and to the HAVNet network database.[59] The HAVNet network database is an online password-protected database platform where virologists from universities and public health institutes submit HAV nucleotide sequences and corresponding epidemiological data. The geographical signal of HAV sequences submitted to GenBank and the HAVNet network database up to 2017 was investigated.[59]

The analysis was based on a 100 nt fragment of the VP1/P2A genomic region. Based on the available epidemiological data, the different human HAV genotypes analysed were distributed over the endemic Global Burden of Disease (GBD) regions. The report only considered regions with intermediate to high endemicity of HAV. The GBD regions reported were: Latin America, Central Europe, Eastern Europe, Central Asia, South Asia, East and South-east Asia, Oceania, North Africa and Middle East, as well as Sub-Saharan Africa. The analysis revealed that the distribution of human HAV genotype resembled the findings reported in 1992 by Robertson and colleagues.[58-59] To date, genotype I remains greater than 80% of all human HAV genotypes detected, with IA more prevalent than IB. However, it should be noted that the gap between IA and IB is closing. Currently, 32.6% of human HAV types are IB as opposed to 13% reported by Robertson et al (1992). This could be due to a paucity of data from Sub-Saharan Africa in 1992.[58] Subgenotype IA was reported as predominant in all GBD regions considered, except for Sub-Saharan Africa and South Asia where HAV types IB and IIIA predominate, respectively.[59]

Hepatitis A virus type IIIA was also recorded in all GBD regions. Subgenotype IIA was reported in Sub-Saharan Africa. Subgenotypes IIB and IIIB have not been reported since 1992. The reported subgenotypes IA, IB, IIA and IIIA each

form different cluster(s) that belong to a single country or multiple countries.[59] The simian HAV types IV, V and VI were excluded from the Kroneman report.[59] As far as it is known there have been no reports of genotypes IV and VI after 1992, but new strains of HAV genotype V have been identified by partial or full genome analysis in SA, Uganda, Tanzania and Russia.[22,61-62]

2.3 CLINICAL ASPECTS

2.3.1 Pathobiology

Hepatitis A virus is mainly transmitted via the faecal-oral route.[8-9] Once ingested, HAV travels to the small intestine from where it uses the hepatic portal circulation to the liver.[8] Hepatitis A virus mainly replicates within hepatocytes.[8-9] The virus interacts with an unidentified receptor at the basolateral membrane of hepatocytes to enter and replicate.[8] An early study by Kaplan and colleagues suggested HAVCR1 to be the receptor for HAV.[63] However, recent studies in cell culture and mice reveal that HAVCR1 is not essential for HAV and eHAV entry into hepatocytes.[8,64]

The mechanisms underlying the replication cycle of HAV have yet to be completely understood. Based on the current knowledge, HAV (and eHAV at a later stage) may enter hepatocytes via an endocytic pathway.[8,45] Due to its ability to withstand low pH, HAV survives degradation by lysosomal enzymes. The attachment, entry and uncoating of HAV is faster than that for eHAV.[8,45] Because of its quasi-envelope, eHAV is first subjected to post-endocytic neutralisation.[8,45] Interaction with a specific receptor within the endosome allows HAV and eHAV to release their genome into the cytoplasm.[8] Within the cytoplasm, the genome of HAV is translated into a single polyprotein that is cleaved into the various proteins. Positive-strand RNA is transcribed into a negative-strand RNA which is then used as a template to produce more positive-strand RNA molecules. Once synthesised, positive-strand RNA molecules are encapsidated and released.[8] Hepatitis A virus is secreted from the hepatocytes as eHAV.[8,45] Through interactions between VP2 and cellular components, HAV capsids are recruited as cargo and exported via the multivesicular body pathway.[8,45] The eHAV particle

is released through both the apical and basolateral membranes.[8,65] When HAV is released through the apical membrane, eHAV loses its membrane due to the detergent action of the bile salts and is shed as naked particles in faeces. When released through the basolateral membrane, eHAV enters the bloodstream, gains access to the liver and the replication cycle is repeated.[8,45,65]

Hepatitis A virus actively replicates, undetected by the immune system, for two to four weeks.[8] Hepatitis A virus has been detected in the faeces and blood of infected persons.[7-9] Faecal shedding and viraemia peak just before the onset of symptoms, at which point an infected individual is highly infectious, and decreases dramatically once symptoms appear (Figure 2.4).[8,50] However, virus shedding in faeces and viraemia can persist for weeks to months after onset of symptoms, especially in premature children, immunocompromised patients, or patients co-infected with HBV or HCV.[7-8] It must be noted, however, that infectivity studies in humans and tamarins showed that virions shed in faeces more than 30 days after the onset of symptoms may not necessarily be infectious.[66] Hepatitis A virus has also been detected in the saliva and urine of patients.[67-68]

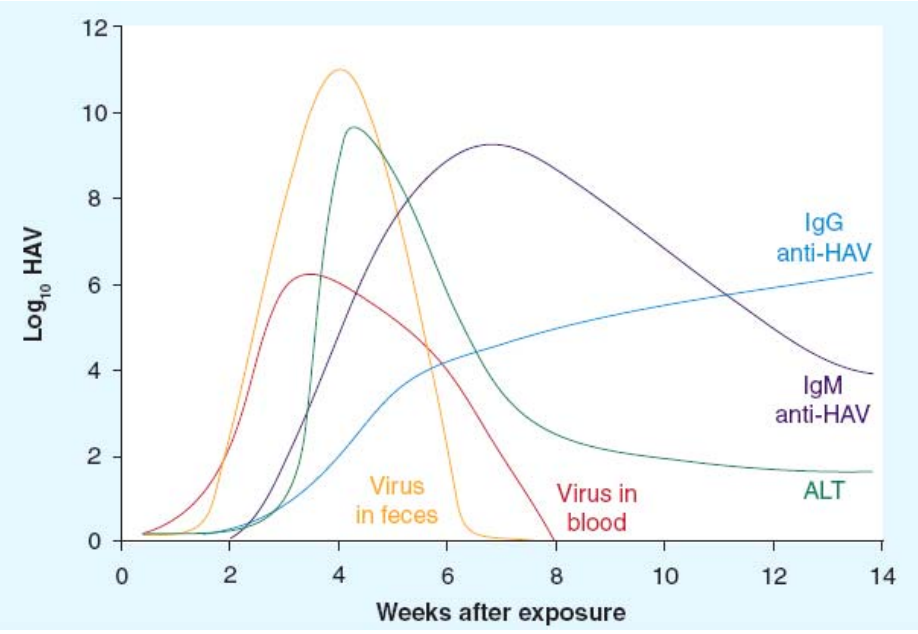


Figure 2.4: Evolution of virologic and biochemical markers of HAV infection.[50]

2.3.2 Clinical outcome

Hepatitis A virus infection causes a wide range of clinical manifestation that range from asymptomatic to acute liver failure.[7-9] Age is the main determinant in the outcome of HAV infection. [7-9] Children under six years infected with HAV seldom develop symptoms. In older children and adults, hepatitis A usually presents with symptoms, which resolve within two months. Recurrent, protracted and fulminant forms of the disease can occur and are more frequent at older ages.[7-8] Fulminant hepatitis A can also occur in the paediatric population.[69] This is a concern for children under six years, as the infection might not be clinically detected until it reaches the end stages.

The severity of hepatitis A may also depend on the genetic susceptibility of infected individuals as specific genes of the histocompatibility loci were detected in patients with severe hepatitis A mono-infection.[7,9] The presence of viral aetiologies such as HBV, HCV, HEV, HIV and dengue virus could also lead to severe or fatal outcome of hepatitis A.[7-8,70-71] In particular patients with chronic hepatitis C are at a higher risk of fulminant hepatitis and death, when superinfected with HAV.[71]

In addition to host factors, it has been suggested that viral loads at the onset of symptoms or specific nucleotide sequence variations within the 5'UTR, the P2 and P3 genomic regions could potentially lead to severe outcome of hepatitis A.[29,70,72] Additionally, it has been hypothesised, but not yet proven, that people infected with HAV subgenotype IIIA had a higher risk of developing fulminant hepatitis A.[73] More evidence is needed to prove or refute the hypothesis, because HAV subgenotype IB has also been detected in patients with acute liver failure.[74]

2.3.3 Immune response

During HAV infection, three classes of antibodies are produced in different proportions just before or at the onset of symptoms: immunoglobulins (Ig) M, G and A (Figure 2.4).[7-8,50] Anti-HAV IgM antibodies appear first and serve as diagnostic marker of acute hepatitis A. These antibodies can be detected in both

asymptomatic and symptomatic individuals, but their titres start declining after the onset of symptoms.[8] In contrast, the levels or titres of anti-HAV IgG antibodies steadily increase after symptoms appear. The anti-HAV IgG antibodies provide life-long protection against HAV infection.[8] Recent studies have shown that asymptomatic reinfection at an older age is possible for people that have been exposed to HAV early in life.[8]

2.4 EPIDEMIOLOGY

Faecal-oral and parenteral are routes of HAV transmission. The parenteral route of transmission of HAV includes transmission through transfusion of infected blood or blood products and the sharing of needles by users of injection drugs.[7,9,75] The main route for transmission of HAV is faecal-oral and it includes person-to-person contact, ingestion of contaminated water and/or food as well as contact with faecally contaminated environment. Transmission of HAV through contact with an infected individual can be seen in closed settings like daycares or schools and in households or shelters where congregate living conditions exist, resulting in poor sanitation.[9,29,75-76] Hepatitis A virus transmission can also occur as a result of oro-anal and oro-genital contact as seen within the MSM population.[9] In line with the faecal-oral route of transmission, living in a faecally contaminated environment could also facilitate the transmission of the virus. Such a situation could happen within an area inhabited by the homeless population or users of injection and non-injection drugs that lack proper sanitation facilities, thus cannot practice good hygiene. This is exemplified by the current ongoing outbreak of hepatitis A in the USA that primarily affects drug users and homeless people.[75,77]

Waterborne transmission of HAV has been well documented.[9,27,29,78-79] Hepatitis A virus can be transmitted through contaminated drinking,[78,80-81] or recreational waters.[9,82] Surface waters and groundwater can become contaminated by the discharge of inadequately treated or untreated wastewaters, but also by run-off from contaminated manure or human defecation.[9,79] Hepatitis A virus is very stable in the environment, especially when associated with organic matter.[83] The

physicochemical properties of the capsid could potentially allow it to escape wastewater treatment.[79,84]

The foodborne transmission of HAV is also well recognised. Contamination of fresh produce can occur at various points from farm-to-fork. An example is the use of contaminated water for irrigation, for washing purposes, or for the mixing of agrichemicals.[85-87] Food produce can also become contaminated through the use of polluted sludge as manure[79] or by infected food handlers.[85-87] Particularly at risk are produce that are minimally processed before consumption, such as tomatoes,[88] green onions,[89] frozen berries,[90] strawberries[91] and pomegranate.[92-93] In addition to fresh produce, shellfish can be a vehicle for the transmission of HAV. When grown in polluted water, these filter-feeders can concentrate the virus from water and transmit it.[79] The largest foodborne outbreak of hepatitis A occurred in Shanghai, China, where approximately 300 000 people became infected after consuming clams that were harvested from sewage-contaminated harbour waters.[94] Recently, raw scallops have been implicated in an outbreak of hepatitis A in the state of Hawaii.[95] In SA, HAV has been detected on fresh tomatoes and lettuce.[17,96]

Hepatitis A virus can survive for days to months on the surface of food products and cause multistate outbreaks as exemplified by the recent outbreak of hepatitis A linked to contaminated strawberries, imported from Egypt, which affected nine states in the USA.[97] In Europe, contaminated strawberries have also been implicated in a multistate foodborne hepatitis A outbreak with cases recorded in Austria and six counties of Sweden.[91]

Worldwide, a minimum of 10 million individuals become infected with HAV annually.[6] Three main factors influence the incidence rate of HAV: socioeconomic development, human demographics and the age of a population.[6] The epidemiology of HAV is directly proportional to markers of socioeconomic development like water, sanitation, hygiene and the human development index.[6] The endemicity of HAV decreases within communities that are provided with safe and clean drinking water sources, suitable sanitation and hygiene facilities.[6,98] In addition, factors like income, levels of education, family and home sizes greatly influence the circulation of HAV.[6,99-100] Human demographics that influence the

epidemiology of HAV consist of people migrating or travelling from high or intermediate endemic regions to low endemic regions.[6] Lastly, the age of a population can impact HAV endemicity. Hepatitis A virus is rarely endemic in countries that have an old population (>40 years). On the other hand, HAV endemicity can be maintained by child-to-child transmission, especially in communities that have a large proportion (between 3 and 10%) of children <5 years.[6]

Traditionally, the World Health Organization (WHO) uses seroprevalence data, namely age-specific anti-HAV IgG, to define the endemicity of a specific region.[101] In highly endemic regions anti-HAV IgG is ≥90% by age 10 years, while in intermediate regions anti-HAV IgG must be ≥50% by age 15 years, but <90% by age 10 years. The seroprevalence in low endemic regions should be ≥50% by age 30 years, but <50% by age 15, and in very low endemic regions it should be <50% by age 30 years.[101]

Recently, the age at midpoint of population immunity (AMPI) in conjunction with the level of income has been proposed as an alternative definition of the epidemiology of HAV in a specific region. The AMPI is the age at which half of the population has been exposed to HAV.[6] In low-income countries, the AMPI is <5 years (Figure 2.5), suggesting that HAV is highly endemic and virtually all the older children and adult population are immune to the virus.[6] In high-income countries the AMPI is >40 years, while in middle-income countries the AMPI varies between 5 years and 40 years (Figure 2.5).[6]

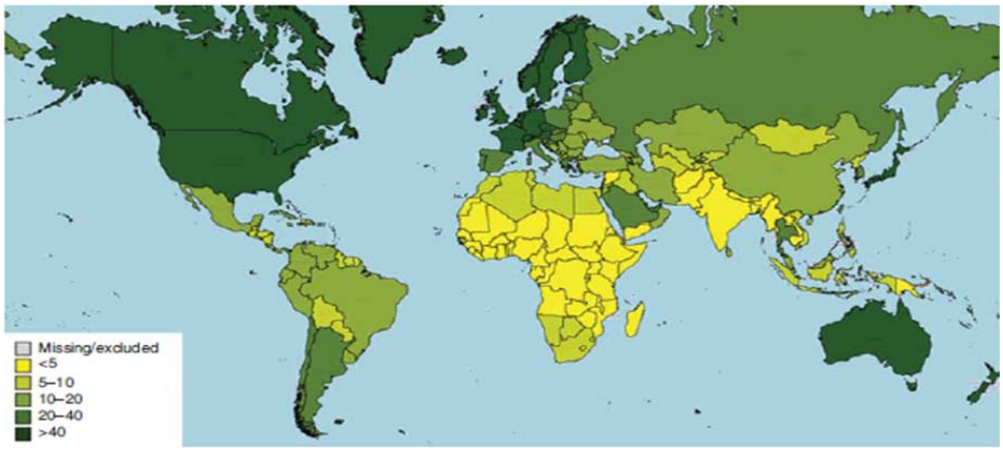


Figure 2.5: Map of AMPI to hepatitis A per country in 2015. Data was estimated using the age (in years) at midpoint of population immunity.[6]

Low-income countries are mostly located in Sub-Saharan Africa (24 out of 31), while the largest proportion of high-income countries are found in Europe and Central Asia (37 out of 80).[102] The middle-income countries can be further divided into lower-middle and upper-middle. The lower-middle countries are found in Sub-Saharan Africa and East Asia and Pacific (30 out of 47) while the upper-middle income countries are mostly found in Latin America and the Caribbean (20 out of 60).[102]

Middle-income countries exemplify the "paradox of HAV epidemiology".[6] The urbanisation taking place in those regions is accompanied by rising incomes and improved access to safe drinking water sources and sanitation. As a result, the incidence of HAV decreases, but the AMPI increases causing a spike in the number of severe forms of hepatitis A.[6] The incidence of HAV decreases unevenly, leaving pockets of the population susceptible to the virus.

One phenomenon influencing HAV endemicity in middle-income countries is rural-to-urban migration.[103] Water and sanitation in rural areas tend to be poorer than urban areas in middle-income countries. As a result, the endemicity of HAV is higher in rural areas than in urban areas. An infectious rural resident that visits an urban area could infect susceptible residents with HAV. Higher-income countries that receive migrants or travellers from high or intermediate endemic regions could face the same situation.[104-105]

South Africa is one of six Sub-Saharan African countries classified among the upper-middle income countries.[102] Recent studies indicate that SA is transitioning from a high to an intermediate HAV endemicity.[25-26] As the overall incidence of HAV is decreasing in SA, it is expected that the rate of symptomatic disease might increase for several decades as has been shown in other upper-middle income countries like Brazil, Mexico and Thailand.[6,103]

Similarly to SA, the incidence of HAV infection is decreasing worldwide.[6] Efforts to map the risk of infection is slowed by the lack of epidemiological data, especially in regions experiencing transition to a lower endemicity.[6] In the absence of epidemiological data, adequate measures cannot be taken to reduce the incidence of HAV. In addition to improvements in water and sanitation, the HAV vaccine may be recommended to reduce transmission of the virus. In

regions with intermediate endemicity, universal childhood vaccination may be recommended,[101] while in low endemic regions, vaccinating high risk populations would be more appropriate.[8,101] High risk group of people usually targeted for HAV vaccination include, but it is not limited to, travellers to higher endemic regions, MSM, injection drug users (IDUs) and food handlers.[8,101] Recently, homeless persons have been added to the list of high risk populations in response to the current multistate outbreak of hepatitis A in the USA.[106]

Another limitation in mapping the risk of HAV infection is that seroprevalence studies provide information on symptomatic infections only, which is an underestimation since HAV infections can be asymptomatic. Wastewater-based surveillance studies include both symptomatic and asymptomatic hepatitis A cases and could complement seroprevalence studies. Successful wastewater-based surveillance studies require the use of appropriate methods and techniques to recover, detect and characterise HAV from wastewater samples.

2.5 MOLECULAR DETECTION

Enteric viruses such as HAV, are shed in high titres (up to 10^{11} genome copies [gc]/gram [g] of faeces[107]] by infected individuals, but are present in contaminated water and food sources at titres too low for direct detection.[27,108-109] The exception is sewage samples where high titres of viruses are anticipated.[108,110] For this reason, the analysis of water matrices for enteric viruses is a multi-stage process which usually starts with a recovery and concentration step to increase the titre of targeted virus to detectable levels, followed by appropriate methods and techniques for detection and characterisation.[27,108,110-111]

2.5.1 Virus concentration

One of the seven criteria listed for an ideal concentration method is high efficiency.[108] The efficiency of virus concentration methods depends on factors like water quality and sample volume, which could impact downstream detection processes.[110] In addition, environmental inhibitors and chemicals used to

concentrate viruses from water samples can inhibit downstream molecular-based detection.[110] In general, the recovery of viruses from water samples occur in two stages, namely primary and secondary concentration. Additional stages may be required to remove inhibitors for downstream detection processes, namely viral isolation and PCR-based methods. The choice of method for primary concentration, also referred to as viral recovery, is influenced by the physicochemical properties of the targeted virus, like particle size, density and sedimentation coefficient, and, ionic charge.[108]

2.5.1.1 Ultrafiltration

Ultrafiltration is an entrapment method used to recover viruses from water samples based on their size.[108,111] The filter matrices used for entrapment include capillaries, membranes and hollow fibres.[108] These filters allow water and low molecular mass solutes to flow through but trap viruses and macromolecules.[108] Since preconditioning of water samples is not required, ultrafiltration has the advantage of concentrating a wide range of viruses simultaneously.[108-109] Generally, virus recovery rate is good, but the processing time of turbid surface water samples may be lengthy. Additional drawbacks in the use of ultrafiltration are the co-concentration of inhibitors and the expensive system required.[108]

2.5.1.2 Ultracentrifugation

Ultracentrifugation can concentrate a wide range of viruses simultaneously and separate them based on their particle size and density.[108-109] The method has been successfully used to concentrate HAV and HEV from sewage samples,[112] however it has also been shown to co-concentrate inhibitors.[111] The use of ultracentrifugation for viral analysis of water samples is limited because it necessitates expensive and highly specialised equipment.[109,111] Since the method only accommodates small volumes of pure water samples, it is typically used for secondary concentration.[108-109]

2.5.1.3 Coagulation, flocculation and immunocapture

Primary concentration of viruses from water sample can also be done by hydroextraction with hygroscopic solids, iron oxide flocculation, two-phase separation, freeze-drying or immunocapture.[108] The immunocapture method uses antibody-coated affinity columns or magnetic beads to recover viruses from water samples. The main advantage of immunocapture is that it removes RT-PCR inhibitors.[108]

2.5.1.4 Adsorption-elution

The virus adsorption-elution (VIRADEL) method is used to recover viruses from large volumes of water samples based on the ionic charge of the viral particle.[108-109] The VIRADEL method is one of the earliest methods developed in environmental virology[113] and it is still widely used. Under specific pH and ionic strength, viruses are recovered from water samples by adsorption to a solid matrix, then released using an appropriate elution buffer containing beef extract, skimmed milk or glycine.[108-109] The efficiency of the VIRADEL method is variable and depends on the water matrix and target virus.[110] These two factors also determine the choice of the adsorbent matrix which could be negatively charged membranes and filters, positively charged membranes and filters, glass wool or glass powder.[108] Except for glass powder and negatively charged cartridges, these adsorbents can be used to process up to 1000 litres (L) of water samples.[108] The main disadvantage of using negatively charged membranes and filters is the requirement for preconditioning water samples, while complex apparatus is needed with glass powder.[108] Positively charged membranes and filters do not require preconditioning of water samples,[108-109] but the running cost may be high.[108] The glass wool adsorption-elution is a cost-effective method for primary concentration, or recovery, of viruses from water samples.[27,108] The volume of the eluate obtained after applying the VIRADEL method is often too large for direct analysis and requires secondary concentration.[108-109]

Concentration of viruses from the eluate, of the VIRADEL method, can occur using ultrafiltration, ultracentrifugation, organic flocculation or polyethylene glycol

(PEG) precipitation.[108-109,111] Using PEG and sodium chloride (NaCl) or dextran T40, viruses and proteins are precipitated from the sample eluate.[108,111] The resulting pellet may be further purified prior to nucleic acid extraction.[111] The PEG/NaCl precipitation has been successfully applied as secondary concentration, following a glass wool adsorption-elution, to concentrate noroviruses (NoV),[114] HAV,[16,19,22] and enteroviruses[115] from surface water samples in SA. The method has also been used as primary concentration to recover NoV, sapoviruses and HAV from sewage samples in previous studies in SA.[19,116] Precipitation of viruses using PEG is simple, inexpensive and can be performed at neutral pH.[108,111] However, sample processing time is lengthy. To reduce the waiting period, liquid PEG_{8000} can be used.[117]

2.5.2 Virus detection

Currently, the detection of viruses in water sources is best achieved using molecular techniques like PCR, nucleic acid sequence-based amplification, microarray or metagenomics.[109] The PCR-based methods are the 'gold standard' for the detection of viruses in water samples[109,111] Despite their high sensitivity and specificity, PCR-based methods are susceptible to inhibitors in the water samples and cannot differentiate between naked nucleic acid, infectious and non-infectious viruses.[13,109,118] Therefore, the impact of HAV-contaminated water samples on public health, using this approach, is difficult to interpret.[13,119]

The infectivity of viruses in water samples can be assessed via production of cytopathic effects (CPE) in infected cell cultures.[29,109] The isolation of viruses in cell culture is the 'gold standard' for the detection and quantification of infectious viral particles from water samples,[109,111] but it is an expensive and laborious technique.[109] Wild-type HAV grows poorly in cell culture, has low viral yield and seldom produces CPE.[11,29] Furthermore, wild-type HAV goes through an adaptation period before successful propagation in cell culture. Thereafter, isolation in cell culture can only be confirmed using immunological assays, like fluorescent focus assay or *in situ* radioimmunoassays[120] or PCR-based methods.[29,121-122] The integrated cell culture PCR (ICC-PCR) method combines the benefits of cell culture and PCR to detect infectious viruses from cell culture

suspensions.[109,121] The method is less prone to PCR inhibitors[109] and has been successfully applied to differentiate between infectious and formalin-inactivated HAV particles in water samples.[122] However, HAV strains detected by ICC-PCR could be genetically different than the wild-type strain, since wild-type HAV may mutate to facilitate adaptation to grow *in vitro*.[11,29]

The infectivity of viruses in water samples can also be assessed by genome integrity.[109] It has been hypothesised that amplifying long target regions (~1000 base pairs [bp]) could indicate the presence of an infectious virus.[109,121,123] The method is called long target region PCR (LTR-PCR) and was used to test the inactivation of HAV by chlorine treatment.[123] It was found that amplification of 1000 bp fragments at the beginning (5'UTR) or at the end (3'UTR) of the HAV genome correlated with infectivity after chlorine treatment.[123] However, the application of LTR-PCR for viral detection in water sample is limited due to decrease sensitivity compared to short target region PCR.[109] In addition, the amplification of a 1000 bp fragment alone is insufficient to assess HAV infectivity.[109] Determining the capsid integrity of viruses present in water samples offers better correlations between PCR-based detection and infectivity.

A number of PCR-based methods, developed to detect infectious viruses in water samples, assess for capsid integrity.[109] Intact viral particles could be recovered from the water samples using antibody-coated paramagnetic beads as in immunomagnetic separation PCR (IMS-PCR). The IMS-PCR method is an antigen-detection based PCR.[109] The real-time format of IMS-PCR has been applied to detect HAV in groundwater samples.[124] The IMS-PCR method is advantaged by the fact that HAV has a single serotype and co-concentration of PCR inhibitors is reduced.[109] However, the method is expensive and can detect empty or damaged particles, since the antibody-coated paramagnetic beads bind to a specific portion of the capsid.[109,121] In addition, the water matrices may interfere with antibody-antigen binding.[109-110,121]

Enzymatic and intercalating dye pretreatments have also been used to enable PCR detection of infectious viral particles based on capsid integrity.[109,121,125] Enzymatic treatment of recovered viruses prior to PCR involves the use of proteases and/or nucleases before nucleic acid extraction.[109,126] Such a

pretreatment would remove naked nucleic acid and nucleic acid associated with damaged viral particles. Enzymatic pretreatment processes combining RNase and proteinase K have been previously used to differentiate intact HAV, poliovirus and feline calicivirus from viruses inactivated by ultraviolet light, sodium hypochlorite and high temperature (72°C).[126] Intercalating dye pretreatment of water samples is the most recent and promising strategy developed to assess viral infectivity.[109,118,121,125,127] The pretreatment is carried out before nucleic acid extraction. Once added to the samples, the intercalating dyes would bind to naked nucleic acid, or nucleic acid from damaged viral particles, upon photoactivation, thus preventing PCR amplification (Figure 2.6). Therefore PCR signals detected from samples pretreated with intercalating dyes come from potentially infectious viruses (Figure 2.6).[118,125] Viability PCR (vPCR) is the term used to refer to a combination of intercalating dye (also known as viability dye [vdye]) pretreatment and PCR.[118,125] The vdye pretreatment has also been used in combination with quantitative PCR (qPCR), RT-qPCR[13] and next generation sequencing (NGS).[128]

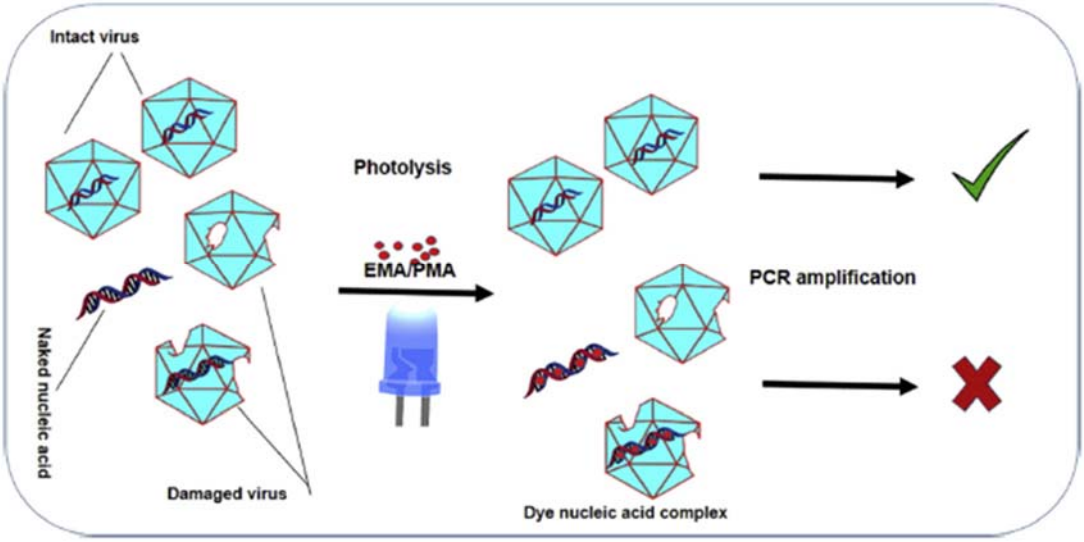


Figure 2.6: Theory of intercalating dyes pretreatment.[109]

The first intercalating dye, used to develop a vPCR was ethidium monoazide (EMA),[129] followed by propidium monoazide (PMA).[130] The EMA and PMA are membrane impermeant dyes that selectively enter viruses with damaged capsid, while an intact capsid would prevent entry of the dyes (Figure 2.6). The mechanism of how both dyes bind to nucleic acid upon photoactivation has yet

to be completely understood. The EMA and PMA possess azide groups that induce covalent crosslinkage between the dyes and the nucleic acid after photoactivation.[125] It has been hypothesised that the nucleic acid complex, thus formed, becomes insoluble and is washed away with cell debris during nucleic acid extraction.[125] Another hypothesis suggests that bound dyes prevent PCR amplification by causing nucleic acid fragmentation.[131] It is worth noting that the efficiency with which the EMA or PMA dyes bind to nucleic acid and how much they prevent PCR amplification are still unclear, but it is greatly influenced by the targeted virus.[125,132]

Pretreatment with PMA followed by RT-PCR could successfully differentiate between infectious and inactivated coxsackievirus (CV), poliovirus, echovirus and NoV in water, after heat and hypochlorite treatments.[133] In another study, enteric virus positive drinking water samples were retested, by real-time PCR, after treatment with EMA. The results showed that none of the enteric viruses (adenovirus, aichi virus, cosavirus, enterovirus, NoV genogroup [G] I and GII) detected were infectious.[134] In practice, it was found that pretreatment of lettuce wash water with EMA and PMA reduced PCR amplification from heat-inactivated HAV particles.[13]

The reduction of PCR signal was further improved by combining PMA and the surfactant Triton® X100.[13] Pretreatment with PMA and Triton® prior to RT-qPCR was also successfully applied for the detection of infectious HAV particles after heat treatment of river water and raw sewage samples.[135] However, it was found that PMA treatment alone without added surfactants was better at reducing PCR signal from chlorine-inactivated HAV in water samples.[135] A modified PMA known as PMAxx was also found to successfully detect infectious HAV in sewage samples.[136] Another vdye known as reagent D was also found to differentiate between infectious and heat inactivated HAV particles suspended in phosphate buffered saline (PBS).[137]

Studies on the application of vPCR to detect infectious HAV in raw sewage and associated treated wastewater or effluent are scarce. One study used PMAxx/Triton-RT-qPCR to detect potentially infectious HAV in influent and associated effluent water samples.[138] However, the study was unsuccessful

because the initial quantification required to test the efficacy of PMAxx/Triton pretreatment failed or the concentration of HAV was below the detection limit of the RT-qPCR assay.

On the African continent in general, and SA in particular, HAV was detected by RT-PCR and RT-qPCR in samples of treated wastewater discharge samples.[19,139] However, the infectivity of the detected strains was unknown. Pretreatment of wastewaters samples with an appropriate vdye combined or not with a surfactant could provide information on the infectivity of detected HAV strains.

Intercalating dye pretreatment overcomes a major limitation with traditional PCRs i.e. the inability to discriminate between infectious and non-infectious viruses. However, the approach does have a few drawbacks. The vPCR has been extensively used to differentiate between live and dead bacterial cells.[125] Results from those studies indicate that efficiency of the pretreatment depends on several factors like type of vdye used and incubation conditions (namely concentration of vdye, time, temperature, photoactivation), targeted microbial species, ratio of live/dead cells and matrices of the sample analysed.[125] Given that studies on the pretreatment of wastewater samples are limited for the detection of potentially infectious viruses, the impact of these factors is mostly unknown. Another main disadvantage of vdye pretreatment is that it relies on capsid integrity to assess infectivity of viruses.[109,125] However, loss of infectivity can occur without damage being done to the capsid. As a result, the vdye may not enter the viral particle, leading to an overestimation of infectious viruses. Despites the limitations of vdyes pretreatment, vPCR is currently the best approach to detect potentially infectious viruses in water samples using PCR.[109,125]

2.5.3 Virus characterisation

Detected viruses can be characterised by serotyping or genotyping. [111] Given that HAV has a single serotype, nucleotide sequence analysis is the “gold standard” for characterising HAV strains.[111] Nucleotide sequence and phylogenetic analysis of HAV strains is an important tool for microbial source

tracing, because it can help trace back the origin of an outbreak by identifying the source of contamination (be it a specific food, water source or individual).[80,91] Genotyping can also help differentiate sporadic from outbreak cases and link geographically separate sporadic cases which have the same risk factors.[59,77,140-142] Genotyping HAV strains detected in sewage and effluent samples would also give an overview of the HAV diversity circulating in the population surrounding the sewage works.[12,139,143] Knowledge of the genetic identity of HAV strains circulating within a population coupled to clinical studies could help differentiate locally acquired from imported hepatitis A cases.[12,144] In addition, this information could help formulate suitable vaccination policies should the need arise. If vaccine escape variants of HAV strains are circulating in a specific group of people, the currently available vaccines may not protect against the new strains.

The genotyping of HAV strains from wastewaters is usually performed separately from the detection step and include PCR, nucleotide sequencing and phylogenetic analyses.[19,139,143-145] Cloning of detected HAV strains may be required as multiple strains may be present in a sample.[19] Recent studies have designed subtype-specific RT-PCR assays that couple the detection and genotyping of HAV strains.[146-147] A number of these assays can identify co-infection in clinical specimens (serum and faecal), but they have not yet been validated for the analysis of food and water samples.[146] Sanger sequencing of subgenomic regions of the HAV genome is commonly used to identify the genetic identity of HAV strains detected in wastewaters. [19,139,143-145] However, minor variants of HAV could be missed by Sanger sequencing.[143]

Next generation sequencing can be applied to detect both major and minor variants of HAV in wastewaters. In a study, RT-qPCR assay coupled to high-throughput sequencing was used to analyse effluent samples over a period of one year. The study identified a diverse viral population of astrovirus and NoV circulating in the population inhabiting an urban area of Paris, France.[148] In addition, the viral diversity found in effluent samples was higher than that identified by clinical studies.[148]

Given that HAV has a low degree of genetic variability and the emergence of vaccine escape mutants is possible, it is important to detect major and minor

variants of the virus that are circulating. However, sequencing of subgenomic regions instead of full genomes could potentially mislead molecular epidemiological investigations. Hepatitis A virus strains implicated in four major foodborne outbreaks in USA were characterised using the VP1/P2B genomic region and the full genome.[149] The outbreak strains were compared to non-outbreak strains with identical VP1/P2B sequences. The study found that genotyping based on the VP1/P2B genomic region could only identify transmission among epidemiological linked cases, however accurate source tracking of the implicated food products was only achieved using full genome. This was due to the fact that the food products were contaminated with multiple HAV strains.[149] Detection and characterisation of HAV in wastewaters is an important tool that could complement clinical data in molecular epidemiological studies. However, the choice of methods and genomic targets must be appropriate in order to obtain accurate information that could be used from a public health perspective.

CHAPTER 3

QUANTIFICATION OF HEPATITIS A VIRUS IN WASTEWATER

3.1 INTRODUCTION

Hepatitis A virus is shed as non-enveloped viral particles in high amounts in human faeces from both asymptomatic and symptomatic infected individuals, with most of the virus ending up in sewage.[79,150-151] Inadequately treated sewage discharged into the environment can spread HAV by contaminating downstream water sources used for domestic, agricultural or recreational purposes.[9,79] Given that less than 100 infectious particles of HAV can cause illness,[151-152] ensuring the successful removal or inactivation of HAV from sewage could potentially decrease faecal-oral transmission in a given region.[110]

As wild-type HAV grows poorly, if at all in routine cell culture, viral isolation from water samples is unrealistic.[11,29] Currently, RT-PCR is the "gold standard" for the detection of HAV in food and water sources[153] and enables both the quantitative and qualitative detection of HAV. The RT-qPCR method is now widely used for the detection of HAV because it is sensitive, specific and rapid.[154] Several studies have reported the use of RT-qPCR to detect HAV in wastewater.[139,155-157] The quantification of HAV not only facilitates quantitative risk assessment,[154] but can be applied to evaluate the efficacy of wastewater treatment plants (WWTP) for inactivating and/or removing HAV from sewage[158] and can also help identify an outbreak during surveillance studies [144]

In SA, HAV has previously been detected in the surface water samples in Gauteng[16,19,82] and the Eastern Cape[159] and the potential health risk has been quantified.[82,160] From April 2015 to March 2016 a comprehensive water sampling programme was initiated to establish the contribution of wastewater discharge from WWTPs to contamination in the surface catchment and source water, i.e. dam, for a large water treatment utility. Five WWTPs, which release treated wastewater discharge directly into the dam or rivers feeding the dam,

were selected for investigation. Water from the dam is used for irrigation purposes, for domestic purposes by the lower socio-economic community, for recreational purposes by the higher socio-economic population and as source water for a water purification plant. Sewage inflow and treated wastewater from the WWTPs, dam water and distribution network water samples from the water utility were collected for analysis for selected enteric viruses, namely enteroviruses,[161] NoV,[162] HAV, and rotaviruses[163] clinically relevant bacteria[164] and endocrine disrupting chemicals[165-166]

The aim of this study was to detect and quantify HAV in the influent sewage, treated wastewater discharge from the five WWTPs and downstream dam water by RT-qPCR.

3.2 MATERIALS AND METHODS

3.2.1 Recovery of viruses from wastewater and dam water samples

Sewage (1 L), treated wastewater discharge (10 L) and dam water (10 L) were collected monthly from the five WWTPs (Figure 3.1, Table 3.1) and transported on ice to the laboratory within 24 hours (h) of collection.

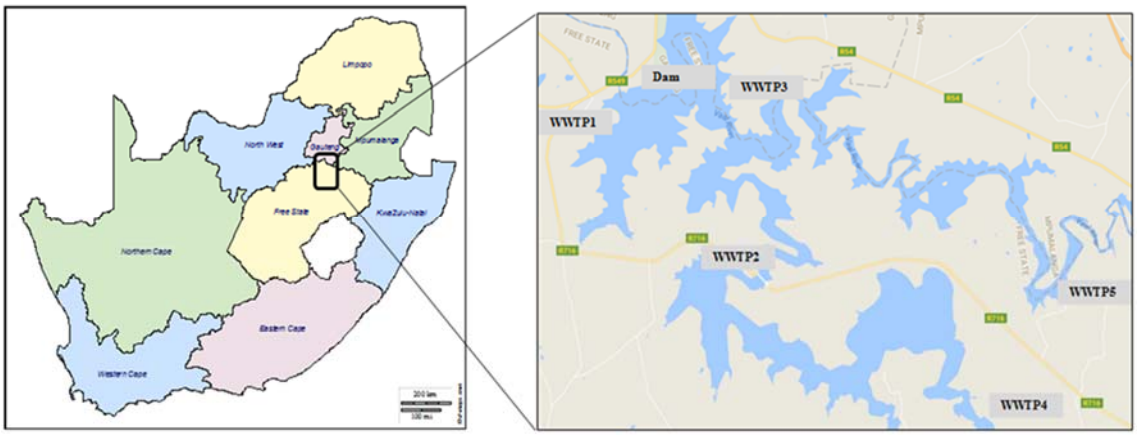


Figure 3.1: Map showing the location of the five WWTPs and dam from which the water samples were collected.[167]

Table 3.1: Operational information and performance of the five WWTPs.[164,168-169]

Wastewater treatment plant	Type of treatment process	Population served		[a]Risk profile
		Size	Age groups	
WWTP1	Biofilter and sludge drying beds	19479	0-14: 14%	High risk (79.4%)
			15-64: 70.4%	
			65+: 15.6%	
WWTP2	Biofilter and sludge drying beds	5166	0-14: 31.9%	High risk (79.4%)
			15-64: 62.4%	
			65+: 5.7%	
WWTP3	Activated sludge, diffused air	701	0-14: 9%	High risk (79.1%)
			15-64: 72.3%	
			65+: 18.7%	
WWTP4	Aerated and facultative ponds	26144	0-14: 25.7%	Critical risk (97.7%)
			15-64: 63.4%	
			65+: 10.9%	
WWTP5	Biofilter and oxidation ponds	17315	0-14: 20.2%	Critical risk (97.7%)
			15-64: 64.6%	
			65+: 15.2%	

a: calculated by the Green Drop Certification program and proportional to effluent quality non-compliance trends

Viruses were recovered and concentrated from the treated discharge (10 L) and dam water (10 L) samples to a final volume of 10 (millilitre [mL]) in PBS (pH 7.2, [Sigma-Aldrich Co., St Louis, MO]). The glass wool adsorption-elution method, as previously described by Mans et al[114], Saïd et al[19] and Kiulia et al[170], was used to recover the viruses which were then further concentrated by PEG_{8000} (Amresco, Solon, OH)/NaCl (Merck KGaA, Darmstadt, Germany) precipitation.[117] The PEG_{8000}/NaCl precipitation method alone was used to concentrate viruses from the 1 L sewage samples to a final volume of 10 mL in PBS (Sigma-Aldrich Co.) Aliquots of virus concentrates were stored at -20°C until further investigation.

3.2.2 Mengovirus

Mengovirus, strain MC_0 (kindly provided by Professor Albert Bosch, Department of Microbiology, Facultat de Biologia, University of Barcelona, Barcelona, Spain) was used to monitor the efficacy of total nucleic acid extraction. The virus had been further propagated and titrated in a Vero African Green Monkey Kidney cell line. An aliquot (10 microlitre [µL]) containing 5 x 10^4 gc of mengovirus was added to the virus concentrates before extraction.

3.2.3 Extraction of total nucleic acid

Total nucleic acid was extracted from the virus concentrate (1 mL) seeded with mengovirus. The extraction was performed on the semi-automated NucliSENS® EasyMAG® platform (BioMérieux, Marcy l'Etoile, France), using associated reagents according to manufacturer's instructions. The nucleic acid was eluted into 100 µL and 20-50 µL aliquots were stored at -70°C until further analyses.

3.2.4 Molecular detection and quantification of viruses

3.2.4.1 RT-qPCR for the detection of mengovirus

A previously prepared RNA standard was used for the quantification of mengovirus.[167] Briefly, the standard was prepared using the Mengo Extraction Control (Ceeram s.a.s, La Chappelle-Sur-Erdre, France) provided at a concentration of 4.87 x 10^3 gc/µL. Serial ten-fold dilutions of the extraction control were prepared in nuclease-free water (Promega Corp. Madison, WI). For each dilution, quantification was performed in duplicate using the mengo@ceeramTools™ (Ceeram s.a.s) on the LightCycler® v2.0 (Roche Diagnostics GmbH, Mannheim, Germany). The software version LCS4 4.1.1.2.1 on the LightCycler® v2.0 (Roche Diagnostics) prepared the mengovirus standard curve using the concentration for each dilution and stored it as an external standard curve for downstream RT-qPCR assays.

The mengo@ceeramTools™Kit (Ceeram s.a.s), uses proprietary primers and probe and 5 µL nucleic acid in a 25 µL reaction. The reverse transcription step was performed at 45°C for 10 minutes (min) followed by initial denaturation at 95°C for 10 min. Thereafter, amplification was performed with 45 cycles of 95°C for 15 s and 60°C for 45 s in the LightCycler® v2.0 (Roche Diagnostics). A positive control (mengovirus RNA) and a negative control (nuclease-free water [Promega Corp.]) were added to every mengovirus RT-qPCR assay. If the mengovirus assay failed, a new aliquot (1 mL) of the virus concentrate was re-seeded with mengovirus. Total nucleic acid was re-extracted and re-tested.

The extraction efficiency of mengovirus was calculated using the following formula:

(mengovirus titre after RT-qPCR) / (titre of seeded mengovirus)

3.2.4.2 RT-qPCR for the detection of HAV

The DNA standard used for the quantification of HAV was prepared using the HAV quantification standard for RT-PCR assays from CeeramTools® (Ceeram s.a.s). The standard was supplied as plasmid at a concentration of 2 x 10^6 gc/µL. Serial ten-fold dilutions of the plasmids were prepared in nuclease-free water (Promega Corp.). For each dilution, quantification was performed in triplicate using the hepatitisA@ceeramTools™ Kit (Ceeram s.a.s) on the LightCycler® v2.0 (Roche Diagnostics). The software version LCS4 4.1.1.2.1 on the LightCycler® v2.0 (Roche Diagnostics) prepared the HAV standard curve using the concentration for each dilution and stored it as an external standard curve for downstream RT-qPCR assays.

The hepatitisA@ceeramTools™ Kit (Ceeram s.a.s), which uses proprietary primers and probe, and 5 µL nucleic acid in a 25 µL reaction was used. The RT step was performed at 45°C for 10 min followed by initial denaturation at 95°C for 10 min. Thereafter, amplification was performed with 45 cycles of 95°C for 15 s and 60°C for 45 s. The hepatitisA@ceeramTools™ Kit includes an internal control (IC) that monitors amplification progress. In addition, a positive control, provided with the kit, and a negative control (nuclease-free water [Promega Corp.]) was included in every HAV RT-qPCR assay.

3.2.4.3 Calculation of HAV concentrations

The concentration of HAV in the WWTP samples, expressed as gc/L, was calculated from the quantified HAV expressed as gc per 5 µL of reaction (Figure 3.2). It was assumed that the nucleic acid extraction efficiency of mengovirus was equal to that of HAV extraction.

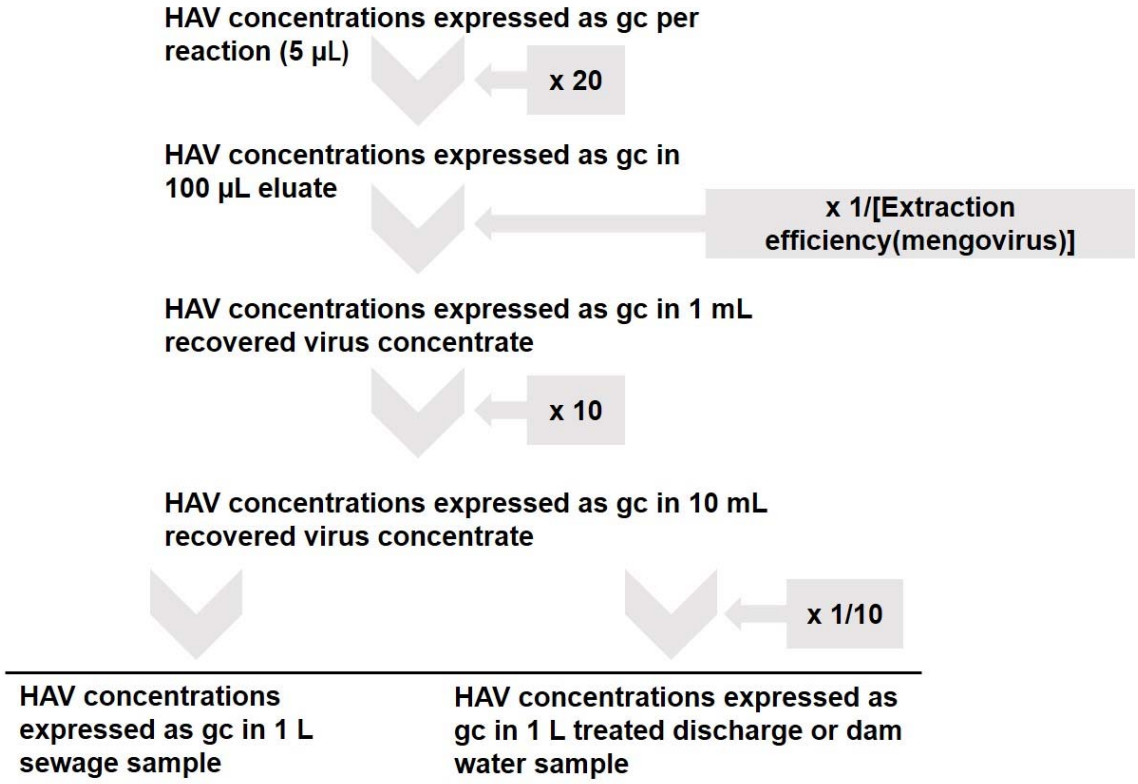


Figure 3.2: Schematic diagram of the calculation of HAV concentration in 1 L sewage, treated wastewater discharge or dam water sample.

A 100% recovery efficiency was assumed for the glass wool adsorption-elution and the PEG_{8000}/NaCl precipitation methods. The efficacy of wastewater treatment for each WWTP was determined by calculating the Log reduction of the viral load between sewage and treated wastewater discharge using the formula:

$$C_s - C_{wd}$$

Where C_s and C_{wd} represent the concentration of HAV in sewage and treated wastewater discharge, respectively.

The results of the RT-qPCR assays were used to determine the rate of detection, the seasonal profile of HAV and the efficacy of WWTPs studied.

3.3 RESULTS

3.3.1 Construction of HAV standard curve

The efficiency of the HAV standard curve was 2.062 with an error of 0.009 (Figure 3.3) as determined on the LightCycler® v2.0 (Roche Diagnostics). The

detection range of the standard curve was 10^6-10^2 copies/reaction. The detection limit of the assay was between 5 and 50 gc/reaction (User Manual supplied with kit, Ceeram s.a.s).

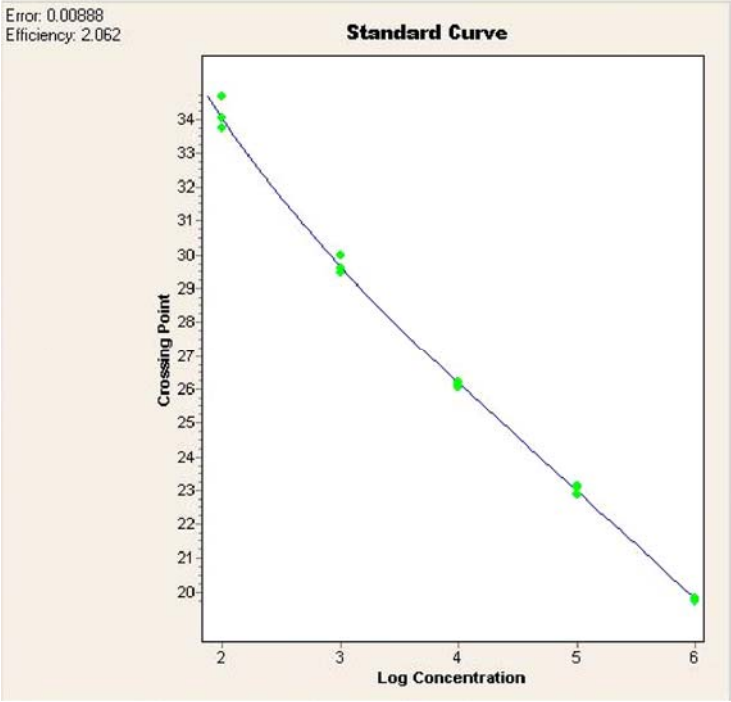


Figure 3.3: Hepatitis A DNA standard curve for the quantification of HAV on the LightCycler® v2.0. The standard curve was prepared from a HAV DNA plasmid standard and the hepatitisA@ceeramTools™ Kit.

3.3.2 Validity of RT-qPCR assays

Recovered virus concentrates from a total of 118/120 WWTP samples, which included sewage (*n=54*), treated wastewater discharge (*n=52)* and dam water (*n=12*), were tested for mengovirus and HAV by RT-qPCR. The RT-qPCR assays for both mengovirus and HAV were considered to be valid as the negative controls were negative and the positive controls were positive. For the HAV RT-qPCR using the hepatitisA@ceeramTools™ Kit (Ceeram s.a.s), the IC was amplified in all the assays. Hepatitis A virus was not detected in 11 sewage, nine treated wastewater discharge and 12 dam water samples. The mengovirus (extraction control) was amplified in the 20 HAV negative WWTP samples and in 8/12 dam water samples. The virus concentrates of the remaining 4/12 dam water samples tested negative for mengovirus, even after

re-seeding, re-extracting and re-testing. The results of the RT-qPCR performed on these virus concentrates were considered as false negative and the samples were excluded from further analysis and discussion. The dam water samples excluded were those collected in April, June, October and February.

3.3.3 Detection of HAV

Hepatitis A virus was detected in 79.6% (43/54) sewage and 82.7% (43/52) treated wastewater discharge samples (Table 3.2).

For most of WWTPs, the rate of HAV detection was higher in treated wastewater discharge than in sewage (Table 3.2). The same rates of detection were recorded for sewage (90.9%) and treated wastewater discharge (100%) samples collected from WWTP1 and WWTP4. For WWTP2 and WWTP5, the detection rate was higher in the discharge (100% rate for WWTPs 2 and 5) than in the sewage (91.7% and 83.3%, respectively). In comparison to the previously mentioned WWTPs, the rate of HAV detection in the sewage and treated discharge samples collected from WWTP3 was low, 41.7% and 27.3%, respectively (Table 3.2).

The rate of HAV detection according to season is highlighted in Table 3.2. The highest rates (from 75% to 100%) of HAV detection were recorded in winter with a peak in June for both sewage and treated wastewater discharge. A second peak was recorded for treated wastewater discharge in July.

In summer, the rate of detection was low (from 50% to 80%) with a peak in January for both types of WWTP samples (Table 3.2). The same detection rate range was recorded for autumn/fall and spring (50% to 100%) (Table 3.2). In autumn/fall, the detection rate of HAV peaked in April for both sewage and treated wastewater discharge samples (Table 3.2). However, a second peak was recorded in March for treated wastewater discharge (Table 3.2). In spring, the highest detection rate was recorded in September for sewage and in November for treated wastewater discharge (Table 3.2).

Table 3.2: Detection rate of HAV in sewage and treated wastewater discharge samples collected from five WWTPs during a 12-month collection period. The ratio indicates number of positive sample / total number of samples; the colours represent South African seasons: autumn/fall (yellow), winter (white), spring (green) and summer (blue).

Month	Sample type	WWTP1	WWTP2	WWTP3	WWTP4	WWTP5	Prevalence (detection/ samples screened)
April	Sewage	1/1	1/1	1/1	1/1	1/1	100 (5/5)
	discharge	1/1	1/1	0/1	1/1	1/1	80 (4/5)
May	Sewage	1/1	1/1	0/1	[a]ND	1/1	75 (3/4)
	discharge	0/1	1/1	ND	ND	ND	50 (1/2)
June	Sewage	1/1	1/1	1/1	1/1	1/1	100 (5/5)
	discharge	1/1	1/1	1/1	1/1	1/1	100 (5/5)
July	Sewage	1/1	1/1	0/1	ND	1/1	75 (3/4)
	discharge	1/1	1/1	1/1	ND	1/1	100 (4/4)
August	Sewage	1/1	1/1	0/1	1/1	1/1	80 (4/5)
	discharge	1/1	1/1	0/1	1/1	1/1	80 (4/5)
September	Sewage	1/1	1/1	1/1	1/1	1/1	100 (5/5)
	discharge	1/1	1/1	0/1	1/1	1/1	80 (4/5)
October	Sewage	1/1	1/1	0/1	ND	1/1	75 (3/4)
	discharge	1/1	1/1	0/1	ND	1/1	75 (3/4)
November	Sewage	0/1	1/1	0/1	ND	1/1	50 (2/4)
	discharge	1/1	1/1	1/1	ND	1/1	100 (4/4)
December	Sewage	ND	0/1	0/1	1/1	1/1	50 (2/4)
	discharge	ND	1/1	0/1	1/1	1/1	75 (3/4)
January	Sewage	1/1	1/1	0/1	1/1	1/1	80 (4/5)
	discharge	1/1	1/1	0/1	1/1	1/1	80 (4/5)
February	Sewage	1/1	1/1	1/1	ND	0/1	75 (3/4)
	discharge	1/1	1/1	0/1	ND	1/1	75 (3/4)
March	Sewage	1/1	1/1	1/1	1/1	0/1	80 (4/5)
	discharge	1/1	1/1	0/1	1/1	1/1	80 (4/5)
Total sewage		90.9 (10/11)	91.7 (11/12)	41.7 (5/12)	100 (7/7)	83.3 (10/12)	79.6 (43/54)
Total wastewater discharge		90.9 (10/11)	100 (12/12)	27.3 (3/11)	100 (7/7)	100 (11/11)	82.7 (43/52)

a: ND = not done/tested.

3.3.4 Quantification of HAV

The concentration of HAV ranged from 1.34 x 10^5 to 3.70 x 10^{10} gc/L of sewage and 4.74 x 10^3 to 3.39 x 10^7 gc/L of treated wastewater discharge. The titres of HAV detected in sewage and treated wastewater discharge are presented for all five WWTPs in Figure 3.4. Except for few months, HAV was detected in both sewage and treated wastewater discharge throughout the sampling period (Figure 3.4). The highest concentration of HAV in sewage (3.70x10^{10} gc/L) was recorded for WWTP4 in March (Figure 3.4) and the highest concentration in treated wastewater discharge (3.39 x 10^7 gc/L) for WWTP2 in May (Figure 3.4).

The concentration ranges of HAV in samples collected for each WWTP are given in Table 3.3. The concentration range for treated wastewater discharge is lower than that of sewage for all five WWTPs (Table 3.3). This is also reflected in a decrease in the recorded mean concentration (Table 3.3).

Figure 3.5 illustrates the fluctuations in the concentrations of HAV recorded for each WWTP. An increase in the concentration of HAV in sewage is recorded from April, with a peak in August for WWTP2 (Figure 3.5B) and WWTP5 (Figure 3.5E), or a peak in September for WWTP3 (Figure 3.5C) and WWTP4 (Figure 3.5D). Despite the trend, WWTPs 2 (Figure 3.5B), 3 (Figure 3.5C) and 4 (Figure 3.5D) recorded the highest concentrations of HAV in sewage in March. Amplification of HAV in March failed for WWTP5 (Figure 3.5E). The trend is not observed for WWTP1 which records high concentration of HAV in sewage during July (Figure 3.5A). The treated wastewater discharge samples do not display the same trend observed in sewage samples. Hepatitis A virus concentrations in treated wastewater discharge peaked in January for WWTP1 (Figure 3.5A), WWTP4 (Figure 3.5D) and WWTP5 (Figure 3.5E). For WWTPs 2 and 3, the highest titres were recorded in May and November, respectively.

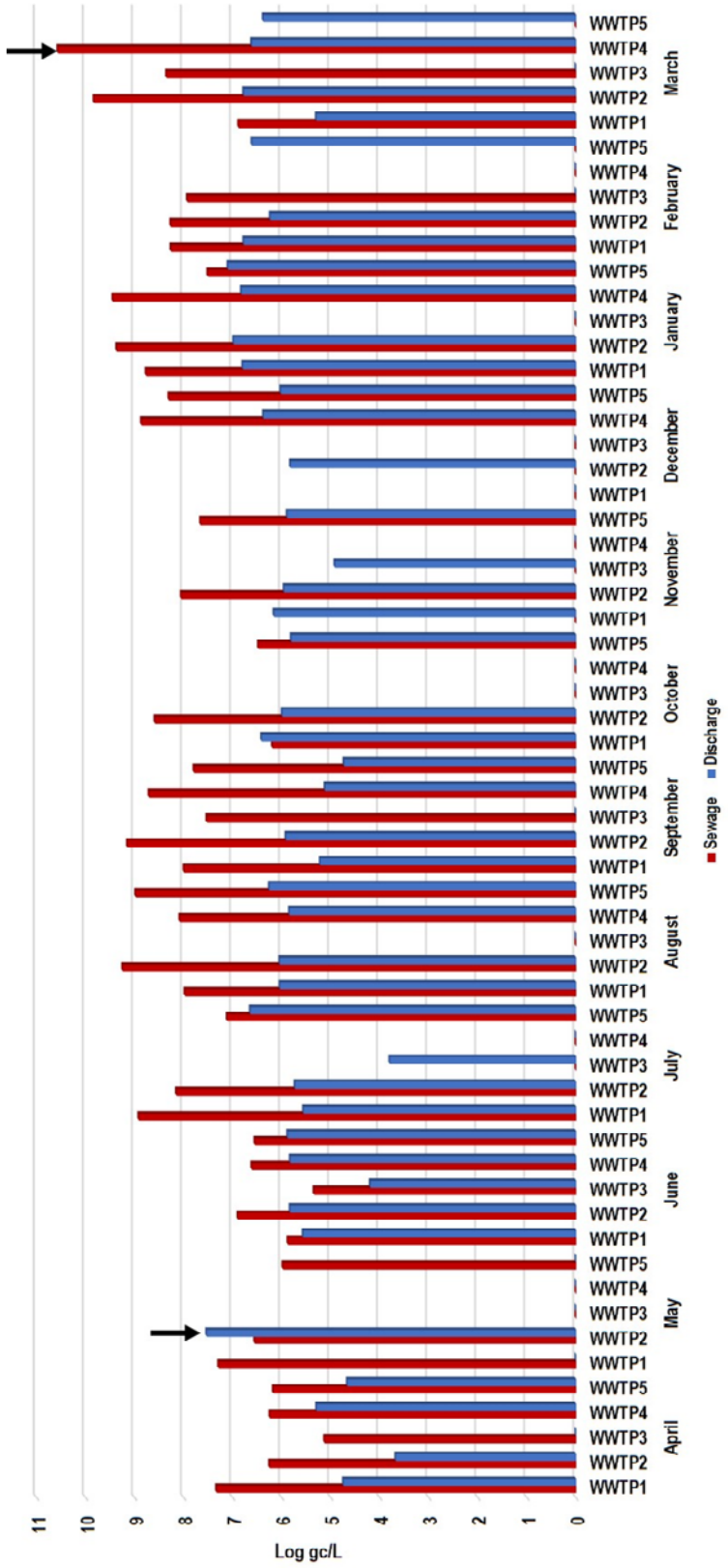


Figure 3.4: Quantification of HAV from five WWTPs, from April 2015 to March 2016. The arrows indicate highest concentrations in sewage and treated wastewater discharge.

Table 3.3: Quantification of HAV, expressed as gc/L, in sewage and wastewater discharge for each WWTP sampled between April 2015 and March 2016. The range of concentration and means for each WWTP are also given. Samples not tested are marked with ND.

	WWTP1		WWTP2		WWTP3		WWTP4		WWTP5	
	Sewage	Wastewater discharge	Sewage	Wastewater discharge	Sewage	Wastewater discharge	Sewage	Wastewater discharge	Sewage	Wastewater discharge
April	2.14E+07	5.54E+04	1.78E+06	4.74E+03	1.34E+05	0.00E+00	1.74E+06	1.95E+05	1.50E+06	4.62E+04
May	1.94E+07	0.00E+00	3.57E+06	3.39E+07	0.00E+00	ND	ND	ND	9.48E+05	ND
June	7.50E+05	3.68E+05	7.80E+06	6.78E+05	2.20E+05	1.57E+04	4.08E+06	6.74E+05	3.54E+06	7.60E+05
July	8.24E+08	3.62E+05	1.41E+08	5.38E+05	0.00E+00	6.28E+03	ND	ND	1.30E+07	4.39E+06
August	9.42E+07	1.09E+06	1.76E+09	1.11E+06	0.00E+00	0.00E+00	1.20E+08	7.00E+05	9.52E+08	1.80E+06
September	9.86E+07	1.65E+05	1.41E+09	8.23E+05	3.37E+07	0.00E+00	5.10E+08	1.32E+05	6.17E+07	5.37E+04
October	1.56E+06	2.58E+06	3.87E+08	9.82E+05	0.00E+00	0.00E+00	ND	ND	3.00E+06	6.43E+05
November	0.00E+00	1.44E+06	1.12E+08	9.00E+05	0.00E+00	8.30E+04	ND	ND	4.54E+07	7.76E+05
December	ND	ND	0.00E+00	6.67E+05	0.00E+00	0.00E+00	7.28E+08	2.36E+06	2.01E+08	1.05E+06
January	5.85E+08	6.24E+06	2.33E+09	9.60E+06	0.00E+00	0.00E+00	2.78E+09	6.68E+06	3.22E+07	1.25E+07
February	1.81E+08	5.99E+06	1.84E+08	1.73E+06	8.42E+07	0.00E+00	ND	ND	0.00E+00	4.07E+06
March	7.59E+06	1.98E+05	6.76E+09	6.00E+06	2.24E+08	0.00E+00	3.70E+10	4.13E+06	0.00E+00	2.39E+06
Range	0 - 8.24E+08	0 - 6.24E+06	0 - 6.76E+09	4.74E+03 - 3.39E+07	0 - 2.24E+08	0 - 8.24E+04	1.74E+05 -3.70E+09	1.32E+05 - 6.68E+06	0 - 9.52E+08	4.62E+04 - 1.25E+07
Mean	1.67E+08	1.68E+06	1.09E+09	4.75E+06	2.85E+07	9.54E+03	5.88E+09	2.12E+06	1.10E+08	2.59E+06

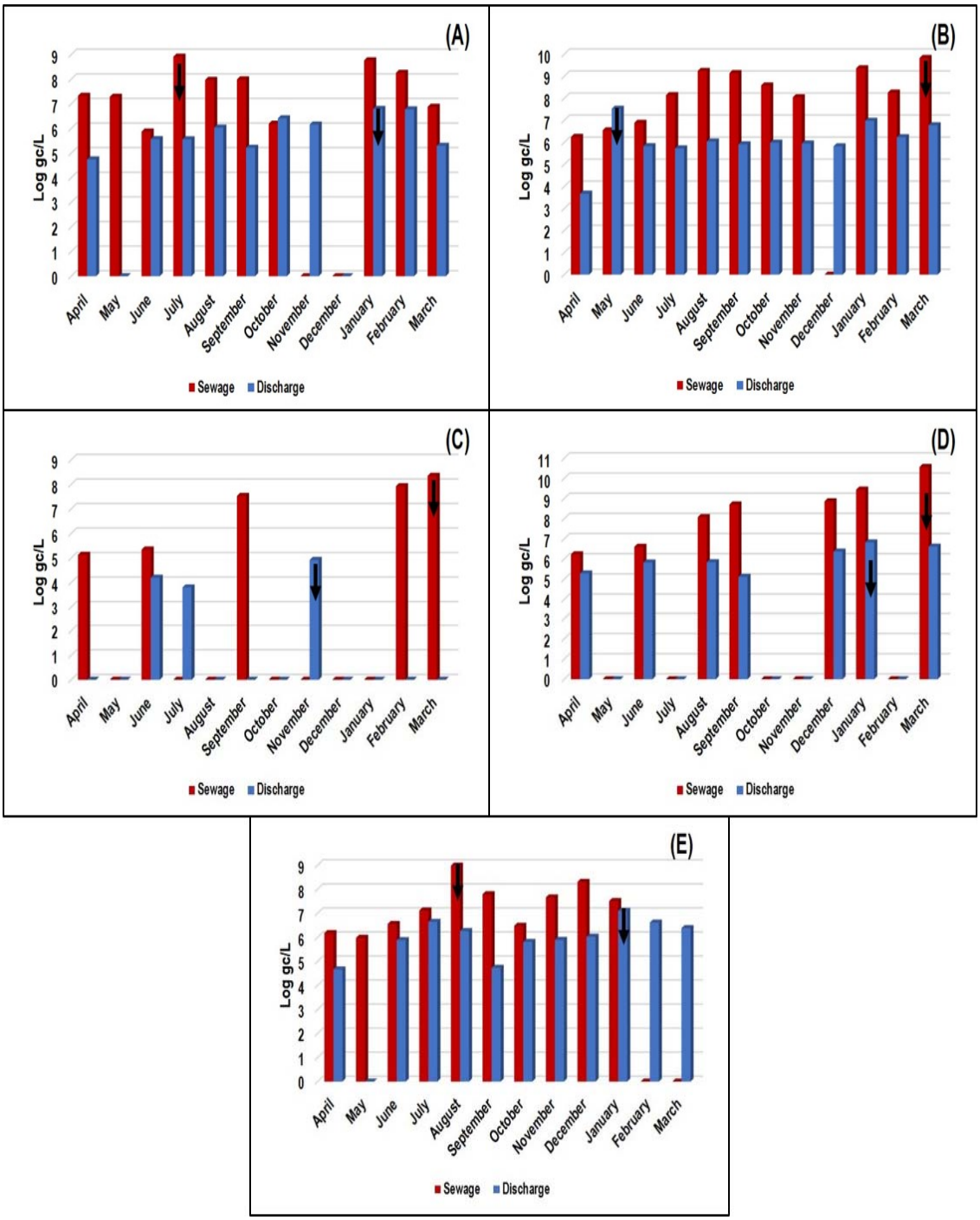


Figure 3.5: Quantification of HAV in samples collected from WWTP1 (A), WWTP2 (B), WWTP3 (C), WWTP4 (D) and WWTP5 (E), from April 2015 to March 2016. The arrows indicate highest concentrations in sewage ■ or in wastewater discharge ■.

3.3.5 Efficacy of treatments by WWTPs

The reduction in Log values of the HAV concentration between sewage and treated wastewater discharge for each WWTP is presented in Table 3.4. In addition, the table highlights the type of treatment used by each WWTP. All five WWTPs recorded a high percentage (>88%) positive Log reduction values ($C_s > C_{wd}$; Table 3.4) which reflects the decrease in the mean viral loads obtained (Table 3.3). The lowest percentages were recorded for WWTP1 (88.9%) and WWTP2 (90.9%). Negative Log reduction values ($C_s < C_{wd}$) were recorded for WWTP1 (11.1%) and WWTP2 (9.1%) which use the same wastewater treatment process (Table 3.4).

Table 3.4: Reduction in Log values of the concentration of HAV between sewage (C_s) and treated wastewater discharge (C_{wd}) for each WWTP. The type of treatment used by each WWTP is given. The colours highlight positive Log reduction (green), negative Log reduction (orange) or no reduction (purple).

	Biofilter and sludge drying beds		Activated sludge, diffused air	Aerated and facultative ponds	Biofilter and oxidation ponds
	WWTP1	WWTP2	WWTP3	WWTP4	WWTP5
April	2.59	2.58	ND	0.95	1.51
May	ND	-0.98	ND	ND	ND
June	0.31	1.06	1.15	0.78	0.67
July	3.36	2.42	ND	ND	0.47
August	1.94	3.20	ND	2.23	2.72
September	2.78	3.23	ND	3.59	3.06
October	-0.22	2.60	ND	ND	0.67
November	ND	2.09	ND	ND	1.77
December	ND	ND	ND	2.49	2.28
January	1.97	2.38	ND	2.62	0.41
February	1.48	2.03	ND	ND	ND
March	1.58	3.05	ND	3.95	ND
$C_s > C_{wd}$	88.9% (8/9)	90.9% (10/11)	100% (1/1)	100% (7/7)	100% (9/9)
$C_s < C_{wd}$	11.1% (1/9)	9.1% (1/11)	-	-	-
$C_s = C_{wd}$	-	-	-	-	-

a: ND = not done; sample was not collected or data on sewage and/or wastewater discharge was not available

3.4 DISCUSSION

Surveillance studies performed on sewage or treated wastewater provide information on the circulation of HAV within a given geographical area. This information is more accurate than clinical studies because HAV shed by symptomatic and asymptomatic infected individuals end up in the sewage.[79,150-151] In addition, the information can help assess the efficacy of the WWTP at removing and/or inactivating HAV.[139,158] In this study, HAV was detected and quantified by RT-qPCR.

Hepatitis A virus was previously considered highly endemic in SA. However, recent studies reported on a potential epidemiological shift to intermediate endemicity.[25-26] Hepatitis A virus was detected in 79.6% sewage samples. The percentage of HAV-positive sewage samples is similar to that obtained in studies from highly endemic areas like Egypt (71%)[143] and Tunisia (66-67%).[139,155] Previous studies have suggested a possible relationship between the detection of HAV in sewage and the level of HAV endemicity within a geographical area. In low endemic areas, like Spain[171] and Italy[172], less than 34% sewage samples tested positive for HAV. However, there is no clear definition between the detection of HAV in sewage and the epidemiology of the virus, since France which is considered a low endemic area, recorded 0% HAV-positive sewage samples in Paris but 60% HAV-positive sewage samples in Central France.[12] The results of the present study suggests that HAV is highly endemic in the sampling region.

Hepatitis A virus was detected in 82.7% treated wastewater discharge samples. This detection rate is higher than that for sewage samples. This observation could not be due to inhibitors in the sewage samples, as the amplification and extraction controls were both detected. The HAV detection assay used in this study was not inhibited as shown by the amplification of the IC in all the WWTP samples analysed. Hepatitis A virus is highly stable in the environment, especially when associated to organic matter.[83] The increase in the detection rate from sewage to treated wastewater discharge could have been due to the wastewater treatment which might have dissociated the virus from organic matter. The observations made on the overall detection rate is not reflected for

three out of the five WWTPs studied. The detection rate of HAV was lower in the treated wastewater discharge than in the sewage for WWTP3, but maintained for WWTPs 1 and 4. Thus, wastewater treatment used by WWTP3 may remove HAV from the sewage, while the treatments used by WWTPs 1 and 4 may not remove the virus. These observations are different to another highly endemic region, namely Tunisia, where the overall detection rate of HAV is higher in sewage than in treated wastewater discharge (67% and 41%, respectively).[139] In Tunisia, sewage and treated wastewater discharge samples were collected and analysed from five WWTPs. The overall detection rate showed a decrease between sewage and treated discharge which was observed by four out of the five WWTPs studied.[139] The methodology used in the current study may have been more efficient to quantify HAV. Alternatively, the wastewater treatment in Tunisia could be more effective than the ones used by the WWTPs investigated in this study. In addition, the differences between the present study and that performed in Tunisia could also be due to the fact that the composition of sewage and the type of wastewater treatment used differed which may affect the detection of HAV in wastewaters. Even though the detection rate was generally higher in the treated discharge, sewage samples analysed, in this study, recorded higher titres than treated discharge.

In SA, quantifying HAV in sewage is unprecedented. These concentrations are high (1.34×10^5 to 3.70×10^{10} gc/L), but similar to another highly endemic region, Tunisia, where HAV titres range between 10^1 and 10^7 gc/mL of sewage (10^4 and 10^{10} gc/L).[139] The quantification of HAV in wastewater effluent has previously been performed in SA. In 2015, in the Eastern Cape, a study tested the final effluent of five WWTPs and found no HAV.[156] A year later, a similar study, carried out on the effluent of two WWTPs in the same province, found HAV in 6.25% samples, but at concentrations below 1 gc/L.[173] In the present study, the concentration of HAV in treated wastewater discharge was much higher (4.74×10^3 to 3.39×10^7 gc/L) than the titres recorded in the Eastern Cape. The difference could be attributed to the correction factor applied for the mengovirus recovery or the methodology used to quantify HAV from the wastewaters, because effluents from the same five WWTPs in the Eastern Cape tested positive for adenovirus.[156] The observations made on the overall

range of HAV concentrations is also reflected in the range and mean titres obtained for all five WWTPs analysed in this study. This indicates that each of the five WWTPs released high titres of HAV into the dam or rivers feeding the dam.

The virus concentrates of the dam water samples analysed in this study tested negative for HAV. Water samples, previously collected and analysed from the same dam, tested positive for HAV. However, in earlier studies a qualitative detection, using conventional RT-PCR, was performed.[16,82] Titres of HAV ranging from 10^1 to 10^5 gc/L have been reported from three dams in the Eastern Cape, SA.[18] One of the three dams received discharge from an inefficient wastewater treatment and was located downstream densely populated area.[18] Given that high titres of HAV have been discharged into the dam analysed in this study, dilution factors or inhibitors of the RT-qPCR assays could account for the negative results. In comparison to the dams analysed in the Eastern Cape, the water samples analysed in this study were collected from a much larger dam. Therefore the HAV discharged might have been diluted and was below the detection limit of the RT-qPCR assay. The detection of viruses in water samples using RT-qPCR is sensitive, accurate and fast. But the accuracy of the process may be negatively impacted by the presence of inhibitory substances in the sample. That is why a number of controls are required to monitor the efficiency of virus detection in water and food sources.[27,110]

A RT-qPCR method was used to detect HAV in sewage, treated wastewater discharge and dam water. The extraction control and the RT-qPCR controls are important because they monitor the nucleic acid extraction efficiency and the amplification assay, respectively.[27,110] At least one control is required during analysis of water samples to monitor the effect of inhibitors.[27,110] For this study, two types of controls were included in the analysis of the sewage, treated wastewater and dam water samples. Mengovirus was added as an extraction control. The RT-qPCR controls consisted of the IC of the hepatitisA@ceeramTools™ Kit (Ceeram s.a.s) that served as an amplification control, a positive control provided by the kit and nuclease-free water as negative control. The successful amplification of the positive control and the

absence of amplification in the negative control indicated that the reagents used for the RT-qPCR assay were not contaminated and could amplify HAV RNA. The IC was successfully amplified in all WWTP and dam water samples that were tested for HAV. This indicates that there were no inhibitors to the HAV RT-qPCR assay within the WWTP and dam water samples. The failure to detect mengovirus in four dam water samples suggests that total nucleic acid extraction might have failed for these samples or inhibitors to the mengovirus RT-qPCR assay were present. The 1:10 diluted nucleic acid was not tested, as the IC for the mengovirus RT-qPCR assay, emerged at the correct cycle threshold value. This could have ruled out the presence of inhibitors in the four mengovirus-negative dam water samples. Nevertheless, the HAV RT-qPCR assay used in this study was sensitive and provided accurate detection of HAV in all WWTP and 8/12 dam water samples. Despite the accuracy of the HAV assay used, statistical significance of the results could not be inferred as a single sample was collected from each WWTP per month. Therefore, final opinions on the implications of the study with regard to HAV epidemiology in the sampling region, seasonal patterns and efficacy of WWTPs analysed cannot be made.

The WWTPs 2, 4 and 5 recorded the highest rate of HAV detection in sewage. Each of these three plants service a young population, with people 0 to 14 years of age making 20 to 32% of the total population (Table 3.1).[168] Establishing HAV circulation within such a young population would suggest that HAV has an intermediate to high endemicity in the area surrounding the WWTPs.[101] Depending on the duration of virus circulation, the adult and elderly population could be immune to HAV infection. In contrast, HAV was detected at lower levels in the sewage of WWTP3, which services a much older population (9% of the population is between 0 and 14 years of age[168]) (Table 3.1). Thus, the population serviced by WWTP3 could be less exposed to HAV at a young age and the adult and elderly population may be susceptible to HAV infection with an increased possibility of developing severe hepatitis A.[8,101] Given that a single sample was collected per month from the WWTP and no clinical data was obtained from the sampling region, it is not known if HAV detected in sewage came from sporadic cases, outbreak cases or is widely circulating in

the serviced population. The source or origin of HAV, detected in the wastewater samples analysed in the present study, could not be established. However, the highest HAV titres in sewage were recorded in March while highest HAV titres in treated wastewater discharges were mostly recorded in January.

Detection of viruses in water environments by RT-qPCR can enable the identification of seasonal patterns based on fluctuations of viral concentrations in samples. Human enteric viruses like NoV may exhibit a clear seasonal trend[174] or not,[162] according to the region where they prevail. Other viruses like Aichi virus may have the same concentration in sewage throughout the year.[175] The five WWTPs analysed in this study, are located within the same seasonal region, but the highest concentrations of HAV recorded for each plant were different. In March, high titres (10^8-10^{10} gc/L) of HAV in sewage were recorded for WWTPs 2, 3 and 4, while WWTPs 1 and 5 recorded high concentrations (10^8 gc/L) of the virus in July and August, respectively. Several factors like geographical location and service population can influence the quantification of HAV in sewage.[110,176] With regard to the serviced population, the size and structure of the population and the incidence of infection can affect the concentration of viruses in sewage. The observed variations in the concentration of HAV in sewage might be due to a difference in the prevalence of HAV in the population serviced by the plants. In the treated wastewater discharge samples, the concentration of HAV peaks in January (WWTPs 1, 4 and 5), May (WWTP2) and in November (WWTP3). The amount of HAV detected in treated wastewater discharge depends on the performance of the plant and may not reflect true occurrence of the virus in the serviced population. In SA, there is not enough data on the quantification of HAV in sewage and treated wastewater discharge, but other studies have shown that HAV was frequently detected in August and September.[16,18] In contrast, the rate of HAV detection was high in winter, for the five WWTPs analysed, especially in June, where the rate in sewage and treated wastewater discharge was 100%. A systematic review on the global seasonality of viral hepatitis could not identify consistent seasonal pattern for HAV. The various recorded incidences of infection were attributed to multiple sources of transmission. The absence of

proper sanitation and water supplies were identify as the most contributing factors.[177] However, it should be noted that a longitudinal study on the occurrence of HAV in sewage might help identify a potential seasonal variation shared by all five WWTPs. Such a study, combined with clinical data could help detect potential outbreaks of hepatitis A at their early stages.[144] The quantification of HAV in WWTPs samples could not help identify a pattern in the detected titres, but it indicated poor efficacy of wastewater treatment by the plants.

The concentration of HAV was higher in sewage than in treated wastewater discharge, for each WWTP, with the mean concentration in sewage and treated wastewater discharge differing by a factor between 100 and 10000. The reduction in the amount of HAV could be attributed to the efficiency of each WWTP. Wastewater treatment plants 1 and 2 recorded lowest percentages in positive Log reduction. This indicates that using a biofilter and sludge drying beds treatment of sewage is less effective at reducing the titre of HAV in sewage compared to the other three wastewater treatment processes. Therefore, WWTPs 3, 4 and 5 outperformed WWTPs 1 and 2. In contrast, the 2014 Green drop progress report classified WWTPs 4 and 5 as 'critical risk' WWTPs (Table 3.1).[169] The Green Drop Certification Programme is an incentive-based regulation, unique to SA that was developed to improve municipal wastewater management.[169] The Programme calculates the Cumulative Risk Rating for WWTPs and classifies each plant as medium, high or critical risk. The best performing WWTPs are given the medium risk rating while the worst performing ones are given the critical risk rating. One criterion used to calculate the Cumulative Risk Rating is the non-compliance trends in terms of effluent quality. According to the Green Drop Certification Programme WWTPs 1, 2 and 3 were classified as 'high risk' while WWTPs 4 and 5 were classified as 'critical risk'. This report was published a year before the sampling was done.[169] Thus, a year after the report was published, all five WWTPs analysed discharged HAV in the environment. The discrepancy between the results of this study and the Green Drop Certification Programme could be attributed to the use of sludge drying bed in the treatment process and the higher number of HAV-positive sewage and associated treated discharge

samples. Among the five WWTPs, WWTP2 had the highest number of HAV-positive sewage and associated treated discharge samples. The WWTPs 1 and 5 had the same number of HAV-positive sewage and associated treated discharge samples, but WWTP5, which uses oxidation ponds instead of sludge drying beds, outperformed WWTP1.

In summary, this study reports the first quantitative data on the occurrence of HAV in sewage and treated wastewater discharge in SA. The results presented here form a baseline for future risk assessment studies that will be carried out in the sampling region. All five WWTPs discharged HAV into the dam water or rivers feeding the dam. The virus was detected by RT-qPCR which amplify nucleic acid associated with both infectious and inactivated viruses. In terms of public health significance, further infectivity studies are required to determine if a risk of exposure to HAV exists by using water from the dam for irrigation, domestic or recreational purposes.

CHAPTER 4

OPTIMISATION AND APPLICATION OF A VIABILITY PCR ASSAY FOR THE QUANTIFICATION OF POTENTIALLY INFECTIOUS HEPATITIS A VIRUS IN WASTEWATER

4.1 INTRODUCTION

Water is one of the most critical routes of transmission of HAV.[79] Hepatitis A virus does not replicate in the environment,[110] but it is highly stable and can remain infectious in water bodies for at least 6 weeks.[79] The PCR-based detection methods cannot differentiate between infectious and non-infectious viruses.[13,109,118] Cell culture techniques are traditionally used to isolate infectious viruses, but the propagation of wild-type HAV in conventional cell cultures is a long and tedious process.[109] The application of ICC-PCR combines the benefit of PCR and pre-amplification in cell culture. However, a number of amino acid changes occur as HAV adapts to replicating in cell culture.[11,29] Thus, the infectious viruses detected by ICC-PCR could be genetically different to the wild type. Treating virus concentrates of water samples with a vdye prior to nucleic acid extraction and detection by real-time RT-qPCR offers the possibility of detecting genetically intact potentially infectious viruses from environmental samples.

The aim of this part of the study was to optimise a vPCR assay for the quantification of potentially infectious HAV in selected SA wastewater samples. As proof of concept, the initial objective was to develop and optimise the vPCR assay using a laboratory strain of CV-B6. Thereafter the developed assay would be optimised for HAV to establish which vdye, with or without surfactant, could be applied for the detection of potentially infectious HAV. The optimised vPCR assay would then be applied to selected sewage and treated wastewater

discharge samples to determine whether or not potentially infectious HAV was present in these water sources.

4.2 MATERIALS AND METHODS

4.2.1 Viruses and cell lines

4.2.1.1 CV-B6

A stock of CV-B6 (kindly provided by the National Institute for Virology [now National Institute for Communicable Diseases], Sandringham) was propagated in the Vero African Green Monkey cell line (European Collection of Cell Cultures [ECACC] 84113001) using Eagle's Minimal Essential Medium with Earle's Salts and L-Glutamine (E-MEM) (gibco®, Life Technologies™, Paisley, UK) supplemented with foetal calf serum (FCS) (FBS [gamma irradiated and heat-inactivated] gibco®) and with added penicillin (100 Units (U)/mL) and streptomycin (100 microgram (µg)/mL) (Lonza, Rockland, ME). Different concentrations of FCS were used for cell culture propagation (5% FCS), cell culture maintenance (2% FCS) and infection (1% FCS). The infected cell cultures were harvested at 100% CPE and the CV-B6 was partially purified by three freeze-thaw cycles followed by centrifugation (660 x g for 30 min) to precipitate the cell debris. The supernatant was chloroform-treated and retained. The virus titre, as 50% tissue culture infectious dose ($TCID_{50}$), was determined using the Kärber formula[178] after virus titration in a 96-well microtitre tray (Thermo Fisher Scientific, Nunc A/S, Roskilde, Denmark) using six wells per 10-fold dilution and 100 µL inoculum per well. The titrated virus was aliquoted and stored at -80°C until use. Heat-inactivated and control viruses for the vPCR assay were titrated and the $TCID_{50}$ determined using the same procedure and cell line as described above.

4.2.1.2 HAV

A stock of the cytopathogenic cell culture-adapted strain of HAV HM-175 43c (pHM-175) (previously provided by Professor Albert Bosch, Department of

Microbiology, Facultat de Biologia, University of Barcelona, Barcelona, Spain and maintained in the laboratory) was propagated on the rapidly growing variant (FRhK-4R) of the foetal rhesus monkey kidney (FRhK-4) cell line.[179] The FRhK-4R cells were kindly provided by Professor Albert Bosch and were imported into SA under the SA Department of Environmental Affairs CITES permit 208111 and the SA Department Agriculture, Forestry and Fisheries Veterinary Import Permit 13/1/1/30/0-201902002401. The FRhK-4R cell line was propagated using E-MEM (gibco®) supplemented with FCS (gibco®) and with added penicillin (100 U/mL) and streptomycin (100 µg/mL) (Lonza). Different concentrations of FCS were used for cell culture propagation (15% FCS), cell culture maintenance (5% FCS) and infection (1% FCS). A partially purified stock of HAV was obtained from infected FRhK-4R cells which showed 100% CPE 8 to 11 days post-infection. The harvested cell cultures were subjected to three freeze-thaw cycles, followed by centrifugation at 660 x g for 30 min to pellet the cell debris. The supernatant was chloroform-treated and retained. The concentration of the infectious virus stock, expressed as $TCID_{50}$, was determined using the Kärber formula[178] after virus titration in a 96-well microtiter tray (Nunc A/S) using six wells per 10-fold dilution and 100 µL inoculum per well. The titrated virus was aliquoted and stored at -80°C until further use. Heat-inactivated and control viruses for the vPCR assays were titrated and the $TCID_{50}$ determined using the same procedure and cell line as described above. Real-time RT-qPCR (section 4.2.3.3) was also used to quantify the viral stock with the concentration expressed as gc/mL.

4.2.2 Wastewater samples

4.2.2.1 Sewage

Sewage samples (*n=37*) from the five WWTPs that tested positive for HAV by initial RT-qPCR (Chapter 3) and where sufficient recovered virus concentrates (≥1000 µL) was available, were included for retesting using the optimised vPCR assay.

4.2.2.2 Wastewater effluent/treated wastewater discharge

Treated wastewater discharge samples (*n=42*) from the five WWTPs that tested positive for HAV by initial RT-qPCR (Chapter 3), where sufficient recovered virus concentrates (≥1000 µL) was available, were included for retesting using the optimised vPCR assay.

4.2.3 Molecular detection and quantification of viruses

4.2.3.1 Nucleic acid extraction

Total nucleic acid was extracted from 1 mL of the following samples: i) vdye treated and untreated virus suspensions, ii) vdye treated and untreated simulated water samples and iii) vdye treated virus concentrates of sewage and treated wastewater discharge samples from the five WWTPs. The extraction was performed using the semi-automated NucliSENS® EasyMAG® platform (BioMérieux) and associated reagents according to manufacturer's instructions. The extracted nucleic acid was eluted in 100 µL and virus concentration, expressed as gc/mL, was immediately determined or the nucleic acid was stored at -70°C with quantification within 24 h of the extraction.

4.2.3.2 Real-time RT-qPCR for the detection of CV-B6

A DNA standard used for quantification of CV-B6 was prepared using a commercial enterovirus quantification standard for RT-PCR assays from CeeramTools® (Ceeram s.a.s). The standard was supplied as a plasmid at a concentration of 2 x 10^6 gc/µL. Serial ten-fold dilutions of the plasmid were prepared in nuclease-free water (Promega Corp.). For each dilution, quantification was performed in triplicate using the enterovirus@ceeramTools® Kit (Ceeram s.a.s) on the LightCycler® v2.0 (Roche Diagnostics). The software version LCS4 4.1.1.2.1 on the LightCycler® v2.0 (Roche Diagnostics) prepared the CV-B6 standard curve using the concentration for each dilution and stored it as an external standard curve for downstream real-time RT-qPCR assays.

For further experiments, the CV-B6 suspensions were tested by RT-qPCR using the enterovirus@ceeramTools® Kit (Ceeram s.a.s), which uses proprietary primers and probe and 5 µL nucleic acid in a 25 µL reaction on the LightCycler® v2.0 (Roche Diagnostics). The RT step was performed at 45°C for 10 min followed by initial denaturation at 95°C for 10 min. Thereafter, amplification was performed with 45 cycles of 95°C for 15 s and 60°C for 45 s. For the vPCR assays the CV-B6 suspensions were tested in triplicate.

4.2.3.3 Real-time RT-qPCR for the detection of HAV

The DNA standard used for quantification was prepared using the HAV quantification standard for RT-PCR assays from CeeramTools® (Ceeram s.a.s). The standard was supplied as plasmid at a concentration of 2 x 10^6 gc/µL. Serial ten-fold dilutions of the plasmids were prepared in nuclease-free water (Promega Corp.). The dilutions were tested in triplicate using two different RT-qPCR assays, namely the QuantiFast® Pathogen RT-PCR + IC Kit (Qiagen, Hilden, Germany) (referred to as QuantiFast® Kit in the rest of the thesis) and the hepatitisA@ceeramTools™ Kit (Ceeram s.a.s). The two RT-qPCR assays were run on the QuantStudio™ 5 Real-Time PCR System, 96 well (QS5) (Applied Biosystems™, Foster City, CA). The software on the QS5 platform (Applied Biosystems™) prepared the HAV standard curves using the concentration for each dilution and stored it as external standard curves for downstream real-time RT-qPCR assays.

Using the QuantiFast® Kit (Qiagen), RT-qPCR was performed in a 25 µL reaction volume, with 5 µL RNA and 10 µM each of the primers HAV 240, HAV 68 and probe HAV 150, which target the 5' non coding region of HAV.[48] The cycling parameters were 20 min at 50°C, followed by 5 min at 95°C, 50 cycles of 15 s at 95°C, 30 s at 60°C and 30 s at 65°C, followed by a final stage of 30 s at 40°C. The QuantiFast® Kit (Qiagen) includes an IC to monitor amplification. In addition, a positive control obtained from the hepatitisA@ceeramTools™ Kit (Ceeram s.a.s) and a negative control (nuclease-free water [Promega Corp.]) were added to every RT-qPCR assay peformed using the QuantiFast® Kit (Qiagen).

The hepatitisA@ceeramTools™ Kit (Ceeram s.a.s) uses proprietary primers and probe and 5 µL nucleic acid in a 25 µL reaction. The RT step was performed at 45°C for 10 min followed by initial denaturation at 95°C for 10 min. Thereafter, amplification was performed with 45 cycles of 95°C for 15 s and 60°C for 45 s. The hepatitisA@ceeramTools™ Kit (Ceeram s.a.s) comes with an IC to monitor for PCR inhibitors. Additionally, a positive control provided with the kit, and a negative control (nuclease-free water [Promega Corp.]) were added to every RT-qPCR assay done using the hepatitisA@ceeramTools™ Kit (Ceeram s.a.s).

During the development of the vPCR assay, the QuantiFast® Kit (Qiagen) was used to test the HAV cell culture suspensions and simulated water samples. For the application of the vPCR assay, virus concentrates of HAV-positive WWTP samples were tested by RT-qvPCR using both the QuantiFast® Kit (Qiagen) and the hepatitisA@ceeramTools™ Kit (Ceeram s.a.s).

4.2.3.4 Quantification of HAV virus stock

The HAV viral stock was quantified in triplicate ($Qstock_1$, $Qstock_2$, and $Qstock_3$). Two assumptions were made: the nucleic acid extraction efficiency was 100% and that '1 in 200 quantified particles were infectious'.[180] If $Qstock_1$ is the first quantification of the stock, the following equation was used to express the concentration of infectious HAV in the suspension as gc/mL:

$$gHAVstock_1 = (Qstock_1 \times 20) / 200$$

where $gHAVstock_1$ is the concentration of infectious HAV in 1 mL cell culture suspension and expressed as gc/mL

The final titre of HAV stock was calculated using the following equation:

$$gHAVstock = (gHAVstock_1 + gHAVstock_2 + gHAVstock_3) / 3$$

4.2.3.5 Calculation of CV-B6 and HAV concentrations after RT-qPCR

CV-B6: Each treatment combination was quantified in triplicate (Q_{a1}, Q_{b1} and Q_{c1}). The quantification was performed on 5 µL nucleic acid. Given that a 100% nucleic acid extraction efficiency was assumed, the values obtained for Q_{a1}, Q_{b1} and Q_{c1} were multiplied by 20 to obtain the concentration of CV-B6 in 100 µL eluate, which corresponds to a final concentration of gCV-B6$_{a1}$, gCV-B6$_{b1}$ and gCV-B6$_{c1}$ in 1 mL cell culture suspension, respectively. The following equations were used:

$$gCV\text{-}B6_{a1} = Q_a \times 20;\ gCV\text{-}B6_{b1} = Q_{b1} \times 20;\ gCV\text{-}B6_{c1} = Q_{c1} \times 20$$

Q_{a1}, Q_{b1} and Q_{c1} are detected gc in 5 µL RNA
gCV-B6$_{a1}$, gCV-B6$_{b1}$ and gCV-B6$_{c1}$ are the concentration of CV-B6 in 1 mL cell culture suspension and expressed as gc/mL

For the purpose of analysis, the concentration values obtained were transformed using the base 10 logarithm (refer to as Log in the rest of the thesis). The average of Log transformed data was calculated using the following equation:
[Log gCV-B6$_{a1}$ + *Log* gCV-B6$_{b1}$ + *Log* gCV-B6$_{c1}$*] / 3*

HAV: For each vPCR assay, nucleic acid extracted after each treatment combination, was quantified in triplicate (Q_{a1}, Q_{a2} and Q_{a3}). A treatment combination was performed in triplicate (gHAV$_a$, gHAV$_b$ and gHAV$_c$). Two assumptions were made: the nucleic acid extraction efficiency was 100% and '1 in 200 quantified particles were infectious'. To calculate the concentration of HAV in 100 µL eluate, which corresponds to a final concentration of gHAV$_a$, gHAV$_b$ and gHAV$_c$ in 1 mL viral suspension, the following equations were used:

$$gHAV_a = \{[(Q_{a1}+Q_{a2}+Q_{a3})/3] \times 20\} / 200;\ gHAV_b = \{[(Q_{b1}+Q_{b2}+Q_{b3})/3] \times 20\} / 200;$$
$$gHAV_c = \{[(Q_{c1}+Q_{c2}+Q_{c3})/3] \times 20\} / 200$$

Q_{a1}, Q_{a2} and Q_{a3} are detected gc in 5 µL RNA

$gHAV_a$, $gHAV_b$ and $gHAV_c$ are the concentration of HAV in 1 mL viral suspension and expressed as gc/mL

For the purpose of analysis, the concentration values obtained were transformed using Log. The average of Log transformed data was calculated using the following equation:

$$[Log\ gHAV_a + Log\ gHAV_b + Log\ gHAV_c] / 3$$

The average of Log transformed data after running the vPCR assay in triplicate was also determined.

4.2.4 Development and optimisation of the viability PCR

4.2.4.1 Heat-inactivation

Heat was used to inactivate viruses for the development and optimisation of the vPCR assays.[13] Aliquots (1.0 – 1.2 mL) of the partially purified CV-B6 (3.16 x 10^4 $TCID_{50}$/mL) and HAV (2.67 x 10^4 gc/mL) cell culture virus suspensions were heat inactivated at 99°C for 5 min on a dry heating block (QBT2 Dry Block Heating System, Grant Instrument, Cambridge, UK). Each aliquot was divided into two equal portions before inactivation. After heat treatment, the halves were cooled down for 2 min on ice, followed by a brief centrifugation (5 s), then combined. An aliquot of 1 mL from each pool was used for the vPCR experiments. After heat-inactivation, the viral suspensions were assessed for viable viruses by performing the $TCID_{50}$ assay using the appropriate cell line.

4.2.4.2 Viability dyes and surfactants

The vdyes, PMA™ (Biotium, Fremont, CA) and EMA (Biotium) were used to develop the vPCR assay. The 20 millimolar (mM) working solution of PMA was made by dissolving 1 mg of PMA™ in 98 µL of dimethyl sulfoxide (DMSO; Sigma-Aldrich Co. St. Louis, MO) (PMA-DMSO) or 98 µL nuclease-free water (Promega Corp.) (PMA-water). The 12 mM working solution of EMA was made by dissolving 5.0 mg of EMA in 1 mL of DMSO (Sigma-Aldrich) (EMA-DMSO). During the development of the vPCR assay, the PMA-DMSO and PMA-water

solutions were used at a final concentration of 50 micromolar (µM) while the EMA-DMSO solution was used at a final concentration of 20 µM.[13] The surfactants used included: Triton® X100 (Molecular Biology Grade, Promega Corp.) and Tween®20 (Molecular Biology Grade, Promega Corp.). Triton® X100 was used at a final concentration of 0.5% while Tween®20 was used at final concentrations of 0.5% and 0.1%.[13] Tween®20 was used as a substitute for Span 20 used by Moreno.[13]

4.2.4.3 Viability dye treatment of viral suspensions

Once a vdye was added to the viral suspension (1 mL) the resultant mixture was gently mixed by inversion three times. If a surfactant was to be added to the same viral suspension, it was done before adding the vdye. The mixture was then incubated for 10 min in the dark at room temperature (25°C) on a microplate shaker (IKA® Schüttler Microplate Shaker Model MTS 4 [IKA, Janke and Kunkel, GmbH, Germany]) set to 150 rpm.[13] The incubation was followed by photoactivation for 15 min on a PMA-Lite™ LED Photolysis Device (Biotium) (Figure 4.1), following manufacturer's instructions. After photoactivation, nucleic acid was extracted from the treated viral suspension, the virus was quantified by RT-qPCR and the results were expressed as gc/mL.[13]

Figure 4.1: Photoactivation on a PMA-Lite™ LED Photolysis Device.

4.2.4.4 Assay 1: Efficacy of viability dye pretreatment for the selective quantification of potentially infectious CV-B6 in a cell culture suspension

In this assay the potential of vdyes to differentiate between non-inactivated and heat-inactivated CV-B6 particles was determined (Figure 4.2). Ten aliquots (1 mL each) of CV-B6 suspended at 3.16 x 10^4 $TCID_{50}$/mL in sterile serum-free E-MEM were prepared. Viruses in five of the 10 aliquots were heat inactivated (section 4.2.4.1) and the remaining five aliquots were not inactivated (Figure 4.2). Three out of five aliquots that have not been heat inactivated were treated with PMA-DMSO (50 µM), PMA-water (50 µM) and EMA-DMSO (20 µM), respectively (section 4.2.4.3) (Figure 4.2). Similarly, three out of five aliquots that have been heat inactivated were treated with PMA-DMSO (50 µM), PMA-water (50 µM) and EMA-DMSO (20 µM), respectively (Figure 4.2). After viability treatment, nucleic acid was extracted from the treated and untreated viral suspensions, along with one aliquot each of untreated non-inactivated (positive control) and untreated heat-inactivated (negative control) viral suspension (Figure 4.2). The extracted nucleic acid (5 µL) was tested in triplicate by RT-qPCR using the enterovirus@ceeramTools® Kit on the LightCycler® v2.0. The reduction (in Log) for each viral suspension was calculated from the initial concentration of CV-B6 suspension that was neither heat inactivated nor subjected to viability treatment. One aliquot each of the non-inactivated and heat-inactivated batch, that have not been treated, was used for $TCID_{50}$ as controls to monitor for infectivity (Figure 4.2).

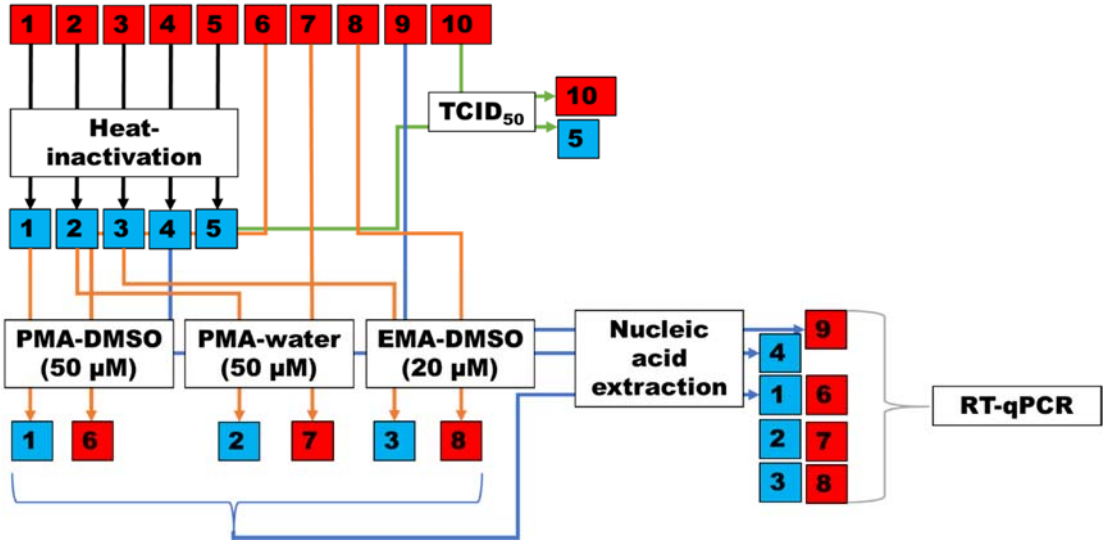


Figure 4.2: Schematic diagram outlining the viability treatment of CV-B6 suspensions. The red squares represent infectious aliquots; the blue squares represent heat-inactivated aliquots; the solid black arrows indicate aliquots that have been heat inactivated; the orange arrows indicate aliquots that have been treated with the vdyes; the green arrows indicate aliquots that have been used for $TCID_{50}$; the blue arrows indicate aliquots that have been used for nucleic acid extraction; the grey arrows represent aliquots from which CV-B6 has been quantified.

4.2.4.5 Assay 2: Efficacy of viability dyes pretreatment for the selective quantification of potentially infectious HAV suspended in PBS

In this assay, the potential of PMA-DMSO (50 µM), EMA-DMSO (20 µM) and PMA-water (50 µM) at differentiating between non-inactivated and heat-inactivated HAV particles was determined (Figure 4.3). Hepatitis A virus was suspended at a final concentration of 2.67 x 10^4 gc/mL in PBS (Sigma-Aldrich Co.). The protocol used for the HAV assay is essentially the same as the one used for CV-B6 assay (4.2.4.4). However, 14 aliquots (1 mL each) of HAV suspensions were used (Figure 4.3). After viability treatment, nucleic acid was extracted from the treated and untreated viral suspensions, along with one aliquot each of the untreated non-inactivated (positive control) and untreated heat-inactivated (negative control) HAV suspensions (Figure 4.3). For each extracted nucleic acid three aliquots (5 µL each) were tested by RT-qPCR using the QuantiFast® Kit on the QS5 (Applied Biosystems™). The reduction (in Log) for each HAV suspension was calculated from the initial concentration of HAV suspended in PBS that was neither heat inactivated nor subjected to viability treatment. Three aliquots each of the untreated non-inactivated and untreated

heat-inactivated HAV suspensions, were used for $TCID_{50}$ as controls to monitor for infectivity (Figure 4.3). This entire assay was performed in triplicate.

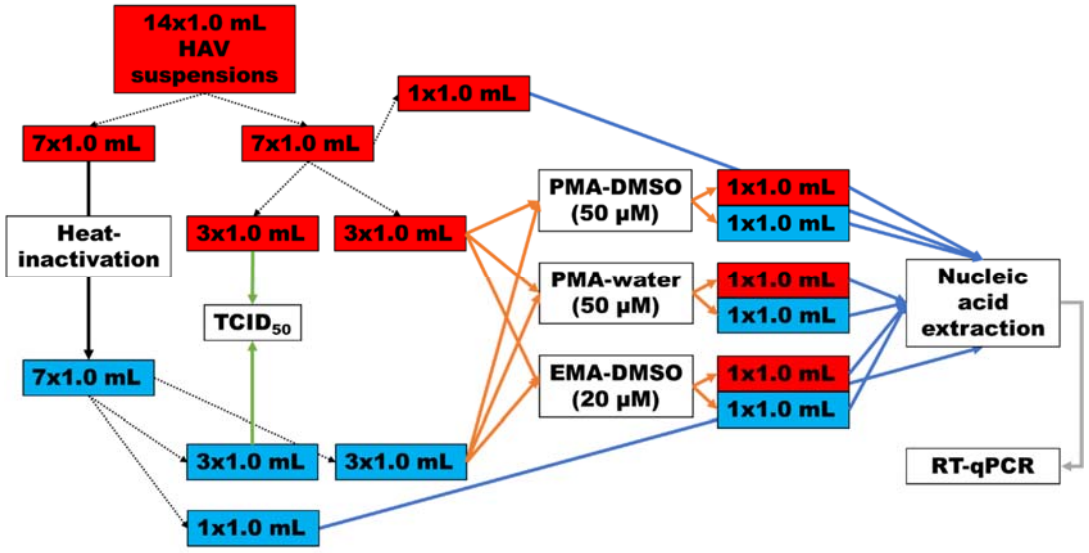


Figure 4.3: Schematic diagram outlining the viability treatment of HAV suspensions. The red squares represent infectious aliquots; the blue squares represent heat-inactivated aliquots; the solid black arrows indicate aliquots that have been heat inactivated; the orange arrows indicate aliquots that have been treated with the vdyes; the green arrows indicate aliquots that have been used for $TCID_{50}$; the blue arrows indicate aliquots that have been used for nucleic acid extraction; the grey arrows represent aliquots from which HAV has been quantified.

4.2.4.6 Assay 3: Efficacy of the combination of viability dye and surfactant pretreatment for the selective quantification of potentially infectious HAV suspended in PBS

In the third assay, the effect of combining vdyes and surfactants in the differentiation of non-inactivated and heat-inactivated HAV particles was determined (Figure 4.4). Hepatitis A virus was suspended at a final concentration of 2.67 x 10^4 gc/mL in PBS. A total of 26 aliquots (1 mL each) were used for the assay. Thirteen aliquots were heat inactivated (section 4.2.4.1) and 13 were not (Figure 4.4). Three aliquots each of the non-inactivated and heat-inactivated HAV suspensions were treated with PMA-DMSO (50 µM), or EMA-DMSO (50 µM), or PMA-water (50 µM) and a surfactant (section 4.2.4.2) (Figure 4.4). After viability treatment, nucleic acid was extracted along with one aliquot each of the untreated non-inactivated (positive control) and untreated heat-inactivated (negative control) viral suspensions (Figure 4.4). For each extracted nucleic acid three aliquots (5 µL each) were tested by RT-qPCR using the QuantiFast® Kit on the QS5 (Applied

Biosystems™). The reduction (in Log) for each HAV suspension was calculated from the initial concentration of HAV suspended in PBS that was neither heat inactivated nor subjected to viability treatment. Three aliquots each of the untreated non-inactivated and untreated heat-inactivated HAV suspensions, were used for $TCID_{50}$ as controls to monitor for infectivity (Figure 4.4). The entire assay was performed in triplicate.

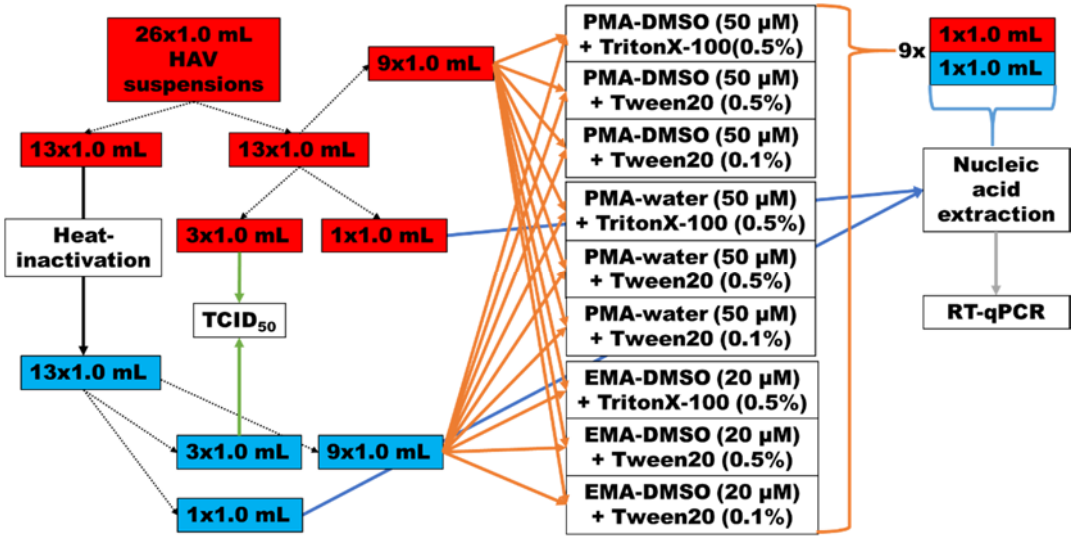


Figure 4.4: Schematic diagram outlining the viability treatment of HAV suspensions with a combination of vdye and surfactant. The red squares represent infectious aliquots; the blue squares represent heat-inactivated aliquots; the solid black arrows indicate aliquots that have been heat inactivated; the orange arrows indicate aliquots that have been treated with the vdyes; the green arrows indicate aliquots that have been used for $TCID_{50}$; the blue arrows indicate aliquots that have been used for nucleic acid extraction; the grey arrows represent aliquots from which HAV has been quantified.

4.2.4.7 Assay 4: Efficacy of the combination of viability dye and surfactant pretreatment for the selective quantification of potentially infectious HAV suspended in simulated water samples

The effect of the combination of vdyes and surfactants in differentiating between non-inactivated and heat-inactivated HAV particles in a simulated water sample was investigated (Figure 4.4). This assay was carried out exactly as described previously (4.2.4.6). Hepatitis A virus was suspended in a simulated water sample at a final concentration of 2.67 x 10^4 gc/mL. Aliquots (6 mL each) of a HAV-negative river water sample, previously tested for HAV, were treated with 2 mL chloroform and used as simulated water sample. Briefly, following addition of the chloroform, the mixture was vortexed, then centrifuged for 15 min

at 2000 rpm. A volume of 10 mL of the chloroformed river water was treated with 150 µL of Penicillin-Streptomycin-Neomycin Solution stabilized (Sigma-Aldrich) and 150 µL of Nystatin (Sigma-Aldrich). The resultant mixture was gently swirled, closed halfway and refrigerated overnight. Nucleic acid was extracted from an aliquot (1 mL) of the treated river water and retested for HAV. After quantifying the treated and untreated HAV suspensions, the reduction (in Log) for each suspension was calculated from the initial concentration of HAV suspended in simulated water that was neither heat inactivated nor subjected to viability treatment.

4.2.4.8 Assay 5: Sensitivity of the most efficient viability treatment for the selective quantification of potentially infectious HAV suspended in simulated water samples

In this assay, the sensitivity of the most efficient viability treatment was determined (Figure 4.5). A HAV viral stock of 2.67 x 10^5 gc/mL was used for the assay. Three sets of 10-fold serial dilutions were prepared in simulated water samples (4.2.4.7). The first set consisted of 8 x 1 mL HAV suspensions (from 10^0 to 10^{-6}; where 10^0 represents undiluted viral stock) (Figure 4.5). The second set of dilutions consisted of 11 x 1 mL HAV suspensions (from 10^0 to 10^{-6}) that were heat inactivated (Figure 4.5). The third set of dilutions consisted of 7 x 1 mL HAV suspensions (from 10^0 to 10^{-6}) (Figure 4.5). The dilutions from the third set together with 10^0 to 10^{-2} dilutions from the heat-inactivated batch were used for $TCID_{50}$ as controls to monitor for infectivity (Figure 4.5). The remaining dilutions from the first and second sets were subjected to the most efficient viability treatment as determined in 4.2.4.7. After viability treatment, nucleic acid was extracted along with one aliquot each of the untreated non-inactivated (positive control) and untreated heat-inactivated (negative control) viral suspensions (Figure 4.5). For each nucleic acid extract three aliquots (5 µL each) were tested by RT-qPCR using the QuantiFast® Kit on the QS5 (Applied Biosystems™). The entire assay was performed in triplicate. The reduction (in Log) for each HAV suspension was calculated from the initial concentration of HAV suspended in simulated water that was neither heat inactivated nor subjected to viability treatment.

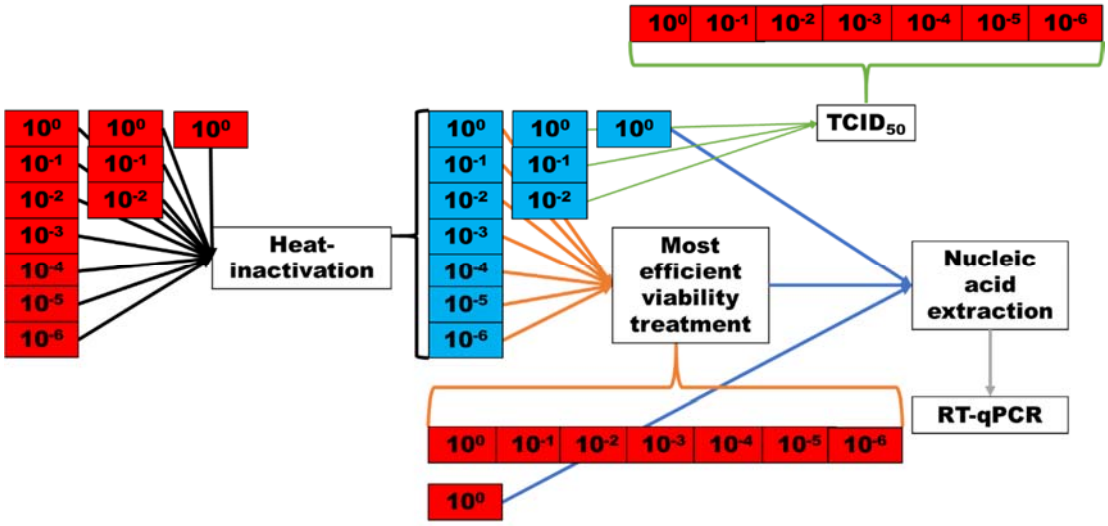


Figure 4.5: Schematic diagram outlining assay 5 – sensitivity of the most efficient viability treatment. The red squares represent infectious aliquots; the blue squares represent heat-inactivated aliquots; the solid black arrows indicate aliquots that have been heat inactivated; the orange arrows indicate aliquots that received viability treatment; the green arrows indicate aliquots that have been used for $TCID_{50}$; the blue arrows indicate aliquots that have been used for nucleic acid extraction; the grey arrows represent aliquots from which HAV has been quantified.

4.2.5 Application of the optimised viability PCR assay

The optimised vPCR assay was used to retest the recovered virus concentrates from 79 HAV-positive samples collected from the five WWTPs. The virus concentrates (1 mL) were treated with a combination of PMA-water (50 µM) and Tween®20 (0.5%).

Total nucleic acid was extracted from 1 mL of virus concentrates subjected to viability treatment and from 200 µL of virus concentrates that had not been subjected to viability treatment. Elution was done in 100 µL and in 50 µL, respectively. The extraction was done using the semi-automated platform of the NucliSENS® EasyMAG® (BioMérieux) (section 4.2.3.1). The extracted nucleic acid (5 µL) was tested by RT-qPCR on the QS5 (Applied Biosystems™) using the QuantiFast® Kit (Qiagen) and the hepatitisA@ceeramTools™ Kit (Ceeram s.a.s) for environmental samples. Viral titres obtained before and after viability treatment are indicated with Ci and Cf, respectively. The Log reduction was calculated using the following equation:

LogCi - LogCf

Due to sample availability, viability treatment for five of the 79 HAV positive samples was done on 200 µL of the virus concentrates.

4.2.6 Data analysis

Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA) was used to analyse the data generated during optimisation and application of the vPCR assay, and, to construct the graphs. Statistical analysis was performed on the results obtained from the vPCR assays 2, 3 and 4 (sections 4.2.4.5 to 4.2.4.7). Basic descriptive summary statistics were carried out in the form of proportions and associated 95% confidence intervals for the presence of infectious HAV strains. The different observations made were evaluated using the STATA® 15 software package (StataCorp LLC, College Station, TX). Correlation analyses between the different viability treatments measured by the molecular assays were performed using Microsoft Excel 2013 (Microsoft Corporation). P values < 0.0500 were considered statistically significant.

4.3 RESULTS

4.3.1 Construction of standard curves

4.3.1.1 CV-B6

The efficiency of the CV-B6 standard curve was 1.985 with an error of 0.008 (Figure 4.6) as determined on the LightCycler® v2.0. The detection limit of the assay was between 5 and 50 gc/reaction (User Manual supplied with kit, Ceeram s.a.s).

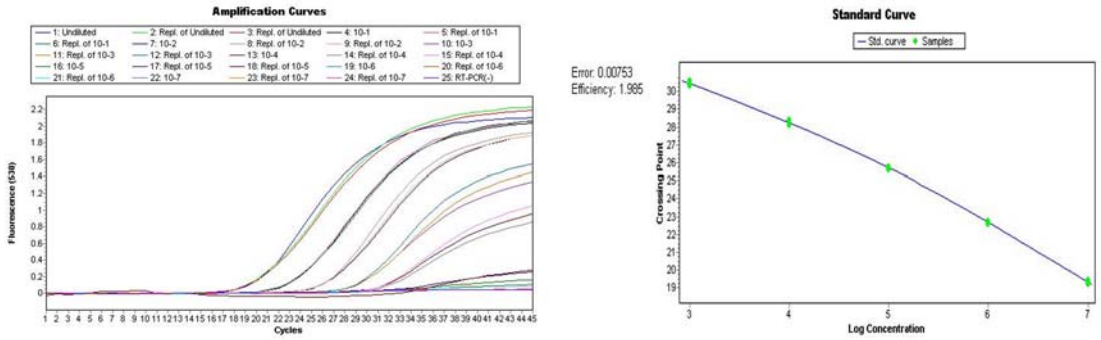


Figure 4.6: Enterovirus DNA standard curve for the quantification of CV-B6 on the LightCycler® v2.0. The standard curve was prepared from an enterovirus DNA plasmid standard and the entero@ceeramTools® Kit.

4.3.1.2 HAV

The two standard curves constructed for the quantification of HAV on the QS5 platform are presented on Figures 4.7 (A and B). The standard curve used to quantify HAV with a QuantiFast® Kit had slope of -3.44 and a correlation coefficient (R^2) of 0.999, which corresponds to an efficiency of 95.25% with an error of 0.020 (Figure 4.7-A). The HAV standard curve constructed using the hepatitisA@ceeramTools™ Kit had a slope of -3.41 and a R^2 value of 1.000, which corresponds to an efficiency of 96.37% with an error of 0.013 (Figure 4.7-B).

(A)

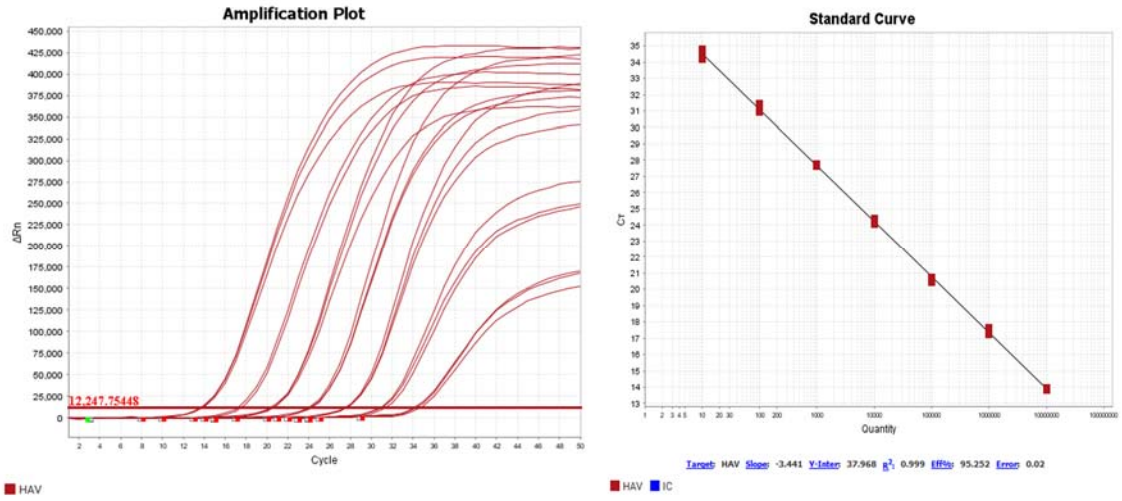


Figure 4.7: Hepatitis A DNA standard curve for the quantification of HAV on the QS5 (Applied Biosystems™). The standard curve was prepared from a HAV DNA plasmid standard and the QuantiFast® Kit (Qiagen) (A) and the hepatitisA@ceeramTools™ Kit (B).

(B)

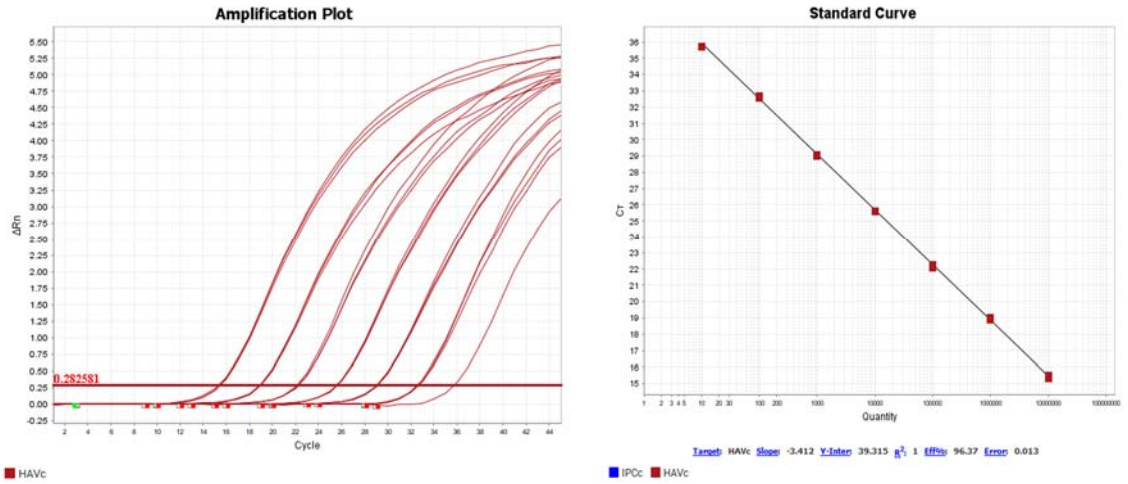


Figure 4.7: Hepatitis A DNA standard curve for the quantification of HAV on the QS5 (Applied Biosystems™). The standard curve was prepared from a HAV DNA plasmid standard and the QuantiFast® Kit (Qiagen) (A) and the hepatitisA@ceeramTools™ Kit (B).

4.3.2 Optimisation of viability assays

4.3.2.1 Virus stock concentration

CV-B6: The CV-B6 virus stock concentration expressed as $TCID_{50}$ was determined to be 3.16 x 10^7 $TCID_{50}$/mL.

HAV: The HAV virus stock concentration expressed as $TCID_{50}$ and as gc/mL was determined to be 4.87 x 10^5 $TCID_{50}$/mL and 2.67 x 10^5 gc/mL, respectively.

4.3.2.2 Efficacy of heat-inactivation

The presence of infectious CV-B6 and HAV in viral suspensions after heat-inactivation and as determined by $TCID_{50}$ is shown on Figure 4.8. Heat-inactivation was successful at reducing (>2.30) the concentration of infectious viruses in each viral suspension and for each assay.

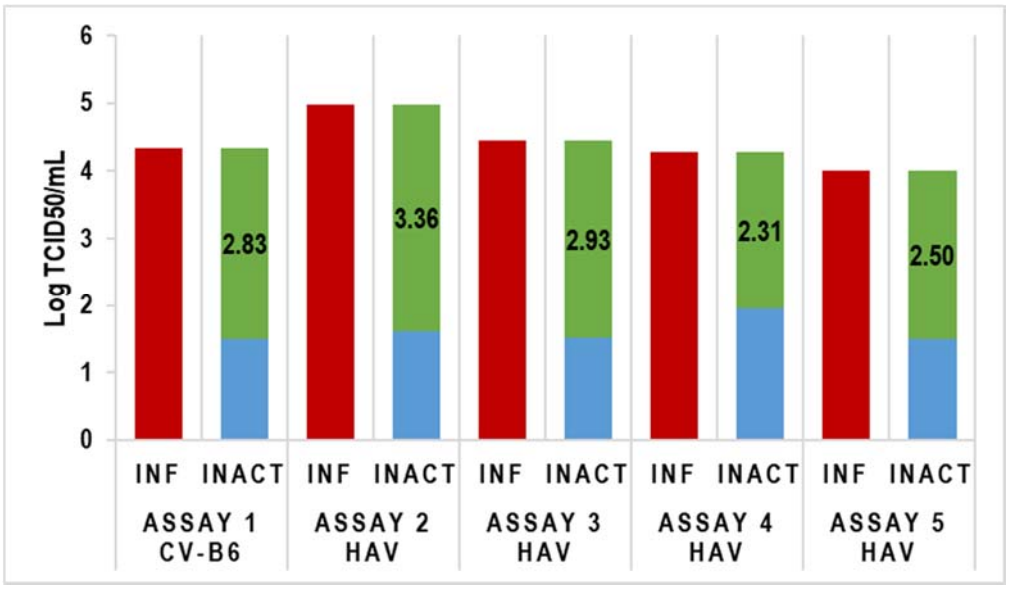


Figure 4.8: Bar graphs depicting the reduction of CV-B6 and HAV titres after heat-inactivation of viral suspensions. Infectivity was measured by $TCID_{50}$. Non-inactivated (Inf) and heat-inactivated (Inact) viral suspensions are represented in red and blue, respectively. Log reduction values are represented in green.

4.3.2.3 Assay 1: efficacy of viability dye pretreatment for the selective quantification of potentially infectious CV-B6 in cell culture suspension

The titre of CV-B6 in cell culture suspensions obtained after treatment with PMA-DMSO (50 µM), PMA-water (50 µM) and EMA-DMSO (20 µM) is illustrated in Figure 4.9. The non-inactivated (positive control) and inactivated (negative control), that have not been treated are indicated by 'Inf' and 'Inact', respectively. The treated suspensions are indicated by either 'Inf' or 'Inact' followed by the type of treatment (Figure 4.9).

From Figure 4.9, it can be seen that the reduction recorded for heat-inactivated CV-B6 suspensions, which have not been treated with the vdyes, was 1.53. This value is higher than the reductions of heat-inactivated CV-B6 suspensions that have been treated.

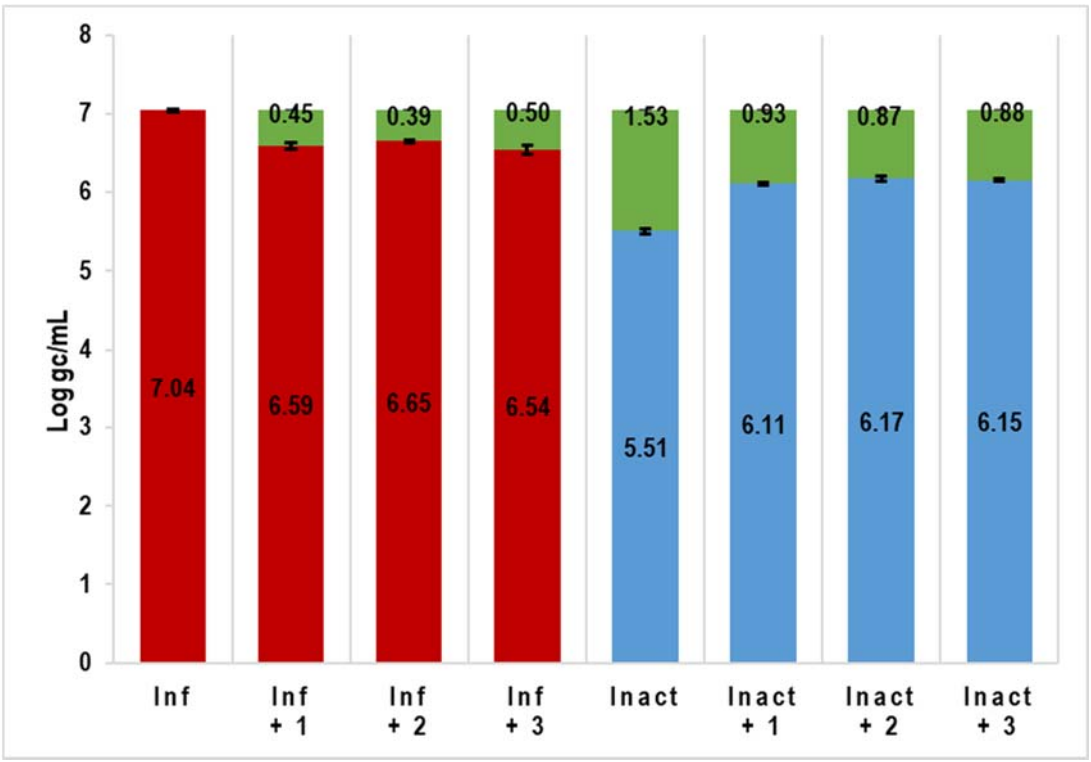


Figure 4.9: Bar graphs depicting the reduction of CV-B6 titres after heat-inactivation of viral suspensions. Non-inactivated (Inf) and heat-inactivated (Inact) CV-B6 suspensions are represented in red and blue, respectively. Log reduction values are represented in green. The Arabic numerals on the X-axis indicate the type of vdye used for the treatment: (1) PMA-DMSO (50 µM), (2) PMA-water (50 µM) and (3) EMA-DMSO (20 µM).

4.3.2.4 Assay 2: efficacy of viability dye pretreatment for the selective quantification of potentially infectious HAV suspended in PBS

Figure 4.10 illustrates the concentration of HAV in cell culture suspensions obtained after treatment with PMA-DMSO (50 µM), PMA-water (50 µM) and EMA-DMSO (20 µM). The reduction of concentrations recorded between infectious and heat-inactivated HAV suspensions that have been treated with a vdye or not, were statistically significant. From Figure 4.10, it is evident that high reductions (>1.0) were recorded after viability treatment of heat-inactivated HAV suspensions with the highest (2.73) recorded after treatment with EMA-DMSO (20 µM). The results suggest that treating partially purified HAV cell culture suspensions with EMA-DMSO (20 µM) prior to RT-qPCR improves the detection of intact HAV particles.

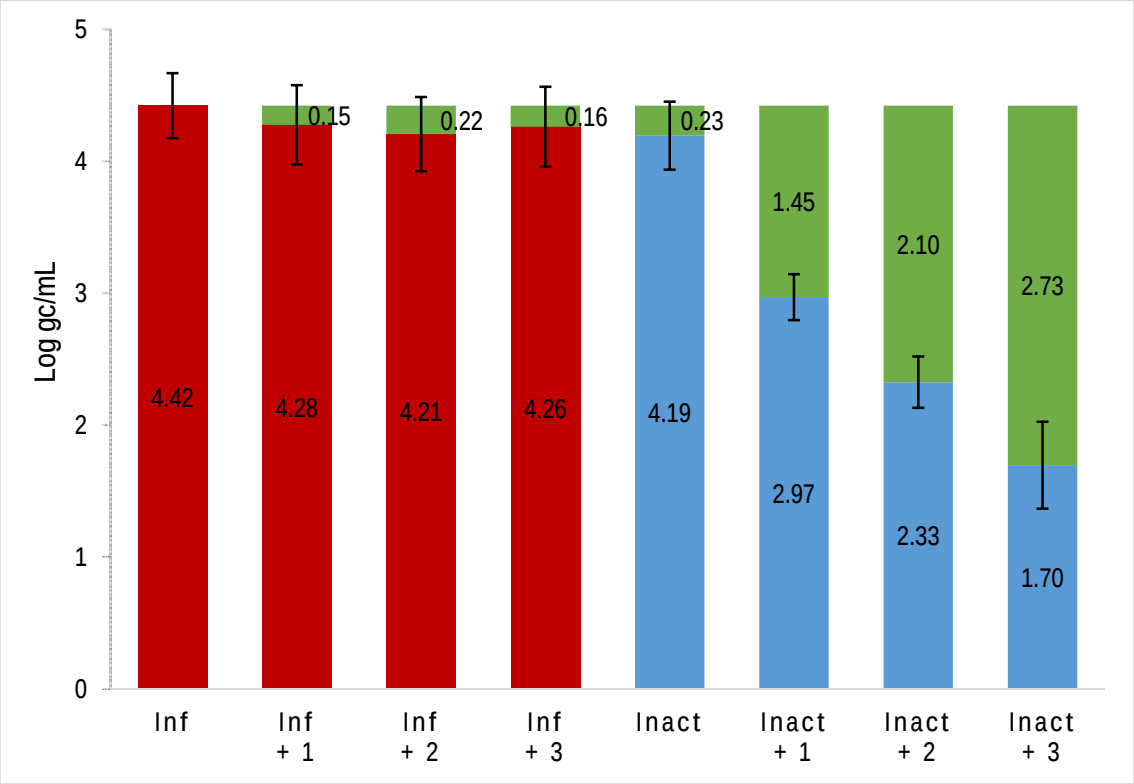


Figure 4.10: Bar graphs depicting the reduction of HAV titres after viability treatment of HAV suspensions using vdyes. Hepatitis A virus cell culture suspensions were treated before and after heat-inactivation. Non-inactivated (Inf) and heat-inactivated (Inact) HAV suspensions are represented in red and blue, respectively. Log reduction values are represented in green. The Arabic numerals on the X-axis indicate the type of vdye used for the treatment: (1) PMA-DMSO (50 µM), (2) PMA-water (50 µM) and (3) EMA-DMSO (20 µM).

4.3.2.5 Assay 3: efficacy of the combination of viability dye and surfactant pretreatment for the selective quantification of potentially infectious HAV suspended in PBS

Figure 4.11 illustrates the concentration of HAV in cell culture suspensions obtained after treatment with a combination of vdye and a surfactant. The recorded reduction in concentrations, between infectious and heat-inactivated HAV suspensions that have been treated or not, are statistically significant. Viability treatment of non-inactivated HAV suspensions showed <0.2 reduction (Figure 4.11). The heat-inactivated negative control (Inact) showed 0.38 reduction. The heat-inactivated treated HAV suspensions recorded high (>2.0) reductions, with the highest (3.91) recorded for treatment using a combination of EMA-DMSO (20 µM) and Triton® X100 (0.5%). From the data, it is evident that treatment of partially purified HAV cell culture suspensions with the

combination of EMA-DMSO (20 µM) and Triton® X100 (0.5%) was better at quantifying intact HAV particles.

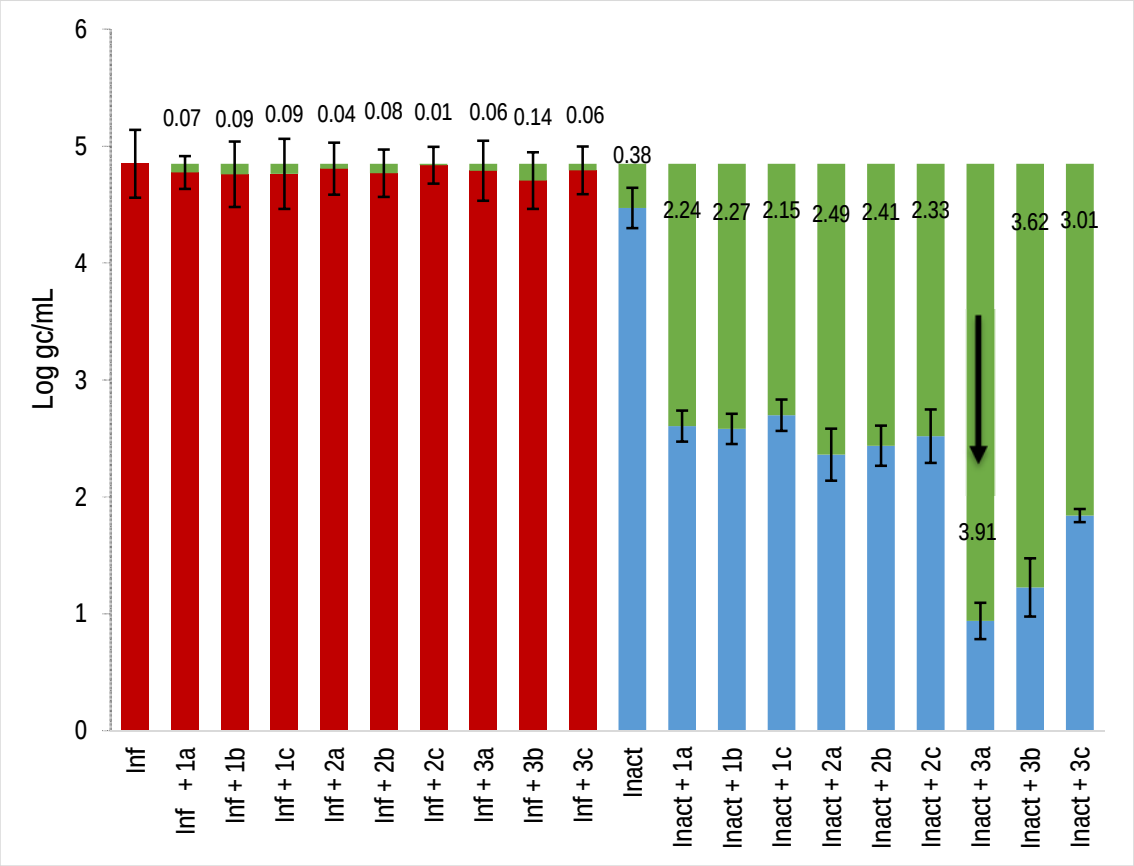


Figure 4.11: Bar graphs depicting the reduction of HAV titres after viability treatment of HAV cell culture suspension using a combination of vdye and surfactant. The viral suspensions were treated before and after heat-inactivation. Non-inactivated (Inf) and heat-inactivated (Inact) HAV suspensions are represented in red and blue, respectively. Log reduction values are represented in green. The Arabic numerals on the X-axis indicate the type of vdye used for the treatment: (1) PMA-DMSO (50 µM), (2) PMA-water (50 µM) and (3) EMA-DMSO (20 µM). The alphabet letters indicate the type of surfactant used: (a) Triton® X100 (0.5%), (b) Tween®20 (0.5%) and (c) Tween®20 (0.1%).

4.3.2.6 Assay 4: efficacy of the combination of viability dye and surfactant pretreatment for the selective quantification of potentially infectious HAV suspended in simulated water samples

The concentrations of HAV, suspended in simulated water samples, obtained after treatment with a combination of vdye and surfactant, are recorded in Table 4.1.

Table 4.1: Concentrations of HAV after the first replicate of assay 4 (4A).

	Sample name	Inactivated(iT)/Infectious(iF)	Treated or not(N)	Vol(µL) of RNA used for RT-qPCR	Mean Conc in 5 µL RNA	Conc in 100 µL eluate = Conc in 1mL sample	1 in 200 particles is infectious	Logs genome copies/mL
4A	4AiT 0	iT	N	5	3.15E+03	6.31E+04	3.15E+02	2.4989191
	4AiT 1	iT	1a	5	1.57E+02	3.14E+03	1.57E+01	1.19530101
	4AiT 2	iT	1b	5	8.42E+01	1.68E+03	8.42E+00	0.92519786
	4AiT 3	iT	1c	5	1.11E+02	2.21E+03	1.11E+01	1.04376159
	4AiT 4	iT	2a	5	2.35E+02	4.69E+03	2.35E+01	1.37037316
	4AiT 5	iT	2b	5	7.68E+01	1.54E+03	7.68E+00	0.88519161
	4AiT 6	iT	2c	5	1.97E+02	3.94E+03	1.97E+01	1.2942942
	4AiT 7	iT	3a	5	7.10E+01	1.42E+03	7.10E+00	0.85111902
	4AiT 8	iT	3b	5	5.42E+01	1.08E+03	5.42E+00	0.73414596
	4AiT 9	iT	3c	5	8.22E+01	1.64E+03	8.22E+00	0.91482532
	4AiF 0	iF	N	5	7.92E+05	1.58E+07	7.92E+04	4.89897218
	4AiF 1	iF	1a	5	5.61E+05	1.12E+07	5.61E+04	4.74874561
	4AiF 2	iF	1b	5	6.08E+05	1.22E+07	6.08E+04	4.78379129
	4AiF 3	iF	1c	5	-extr failed	#VALUE!	#VALUE!	#VALUE!
	4AiF 4	iF	2a	5	8.69E+05	1.74E+07	8.69E+04	4.93926609
	4AiF 5	iF	2b	5	8.43E+05	1.69E+07	8.43E+04	4.92582886
	4AiF 6	iF	2c	5	6.99E+05	1.40E+07	6.99E+04	4.8444049
	4AiF 7	iF	3a	5	9.12E+05	1.82E+07	9.12E+04	4.95994171
	4AiF 8	iF	3b	5	6.48E+05	1.30E+07	6.48E+04	4.81166581
	4AiF 9	iF	3c	5	6.81E+05	1.36E+07	6.81E+04	4.8330585

For the purpose of the statistical analyses three groups were defined, namely 'Mean', 'One_100' and 'One_200'. The 'Mean' group represents the mean concentration calculated after quantification in triplicate. The 'One_100' group represents the concentration in 1 mL suspension. The 'One_200' group represents the concentration in 1 mL suspension adjusted after application of the '1 in 200 quantified particles were infectious' assumption. The data obtained for the three groups was transformed with Log on the STATA® 15 software package (StataCorp LLC) and Microsoft Excel 2013 (Microsoft Corporation).

Results from Log transformed data in STATA® 15: The reduction of concentrations recorded for infectious and heat-inactivated HAV suspensions, whether they have been treated or not, are statistically significant (Table 4.2). Furthermore, the differences between the three groups with respect to the degree of reduction is also statistically significant (Table 4.2).

The Log transformed data for each group is illustrated on Figure 4.12 according to the treatment used for infectious or heat-inactivated suspensions (Figure 4.12).

Table 4.2: Statistical analysis of Log reduction values recorded for assay 4 as obtained from the STATA® 15 software package (StataCorp LLC). P-values are highlighted in red.

```
      Number of obs =       179    R-squared     =  0.9820
      Root MSE      =  .273192    Adj R-squared =  0.9785

------------------------+----------------------------------------------------------
                 Source | Partial SS         df         MS        F     Prob>F
------------------------+----------------------------------------------------------
                  Model |  605.48431         29   20.878769    279.75   0.0000
                        |
                grp_nw |  148.37069          2   74.185345    993.99   0.0000
           Inactd_in~t |  360.87997          1   360.87997   4835.34   0.0000
           Treat_UNt~t |   13.27164          9   1.4746266     19.76   0.0000
  grp_nw#Inactd_in~t |  16.016062          2   8.0080311    107.30   0.0000
       grp_nw#pma_ema |  5.4170312          6   .90283854     12.10   0.0000
Inactd_in~t#Treat_UNt~t |  19.265866          9   2.1406518     28.68   0.0000
                        |
                Residual |   11.12044        149   .07463383
------------------------+----------------------------------------------------------
                   Total |  616.60475        178   3.4640716
```

(A)

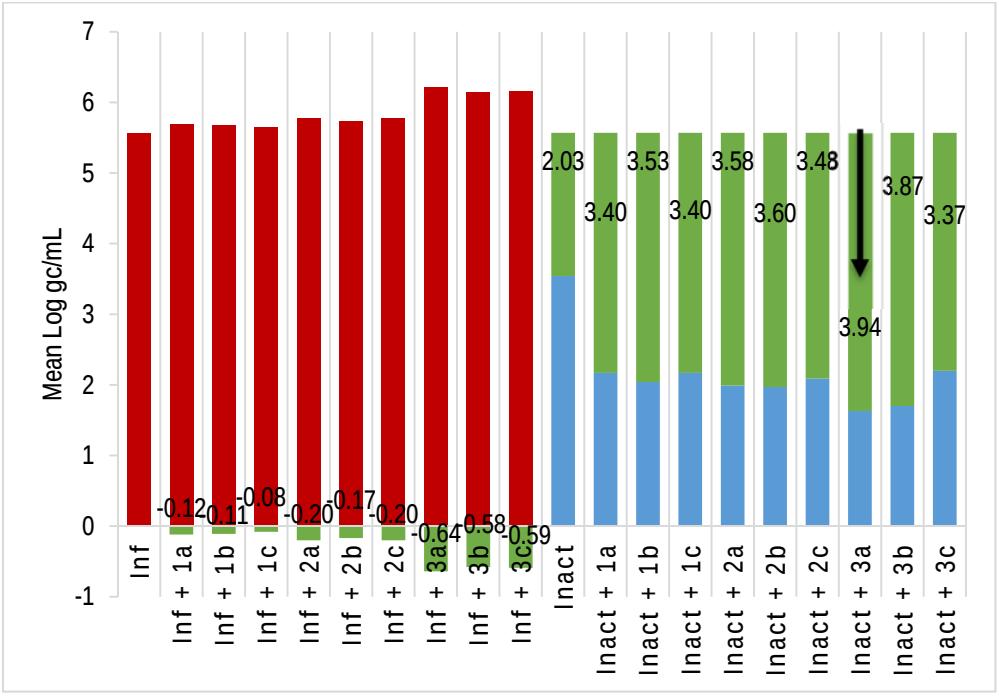


Figure 4.12: Bar graphs depicting the reduction of HAV titres as calculated by STATA® 15 software for the groups 'Mean' (A), 'One_100' (B) and 'One_200' (C). The HAV suspended in simulated water were treated using a combination of vdye and surfactant. The viral suspensions were treated before and after heat-inactivation. Non-inactivated (Inf) and heat-inactivated (Inact) HAV suspensions are represented in red and blue, respectively. Log reduction values are represented in green. The Arabic numerals on the X-axis indicate the type of vdye used for the treatment: (1) PMA-DMSO (50 µM), (2) PMA-water (50 µM) and (3) EMA-DMSO (20 µM). The alphabet letters indicate the type of surfactant used: (a) Triton® X100 (0.5%), (b) Tween®20 (0.5%) and (c) Tween®20 (0.1%).

(B)

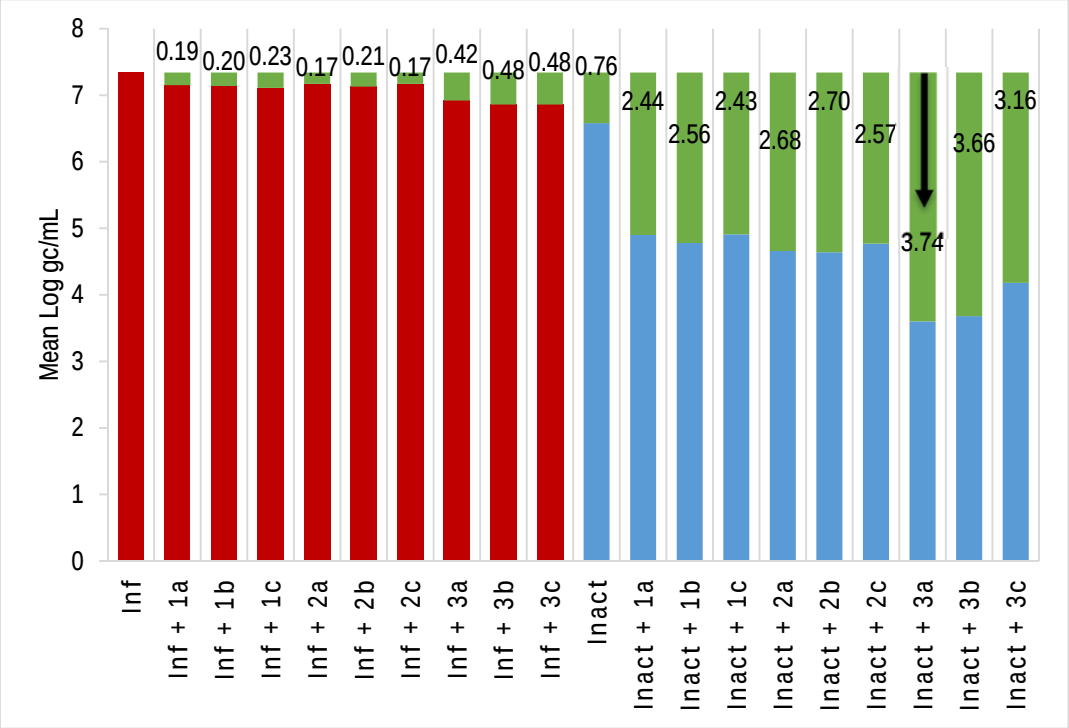


Figure 4.12: Bar graphs depicting the reduction of HAV titres as calculated by STATA® 15 software for the groups 'Mean' (A), 'One_100' (B) and 'One_200' (C). The HAV suspended in simulated water were treated using a combination of vdye and surfactant. The viral suspensions were treated before and after heat-inactivation. Non-inactivated (Inf) and heat-inactivated (Inact) HAV suspensions are represented in red and blue, respectively. Log reduction values are represented in green. The Arabic numerals on the X-axis indicate the type of vdye used for the treatment: (1) PMA-DMSO (50 µM), (2) PMA-water (50 µM) and (3) EMA-DMSO (20 µM). The alphabet letters indicate the type of surfactant used: (a) Triton® X100 (0.5%), (b) Tween®20 (0.5%) and (c) Tween®20 (0.1%).

From Figure 4.12 it is clear that the highest reduction (3.94 and 3.74 for Figures 4.12-A, and, B and C, respectively) was obtained by treating heat-inactivated HAV, suspended in simulated water, with treatment 3a which corresponds to a combination of EMA-DMSO (20 µM) and Triton® X100 (0.5%). This reduction is closely followed (3.87 and 3.66 for Figures 4.12-A, and, B and C, respectively) by the reduction obtained after treating heat-inactivated suspensions with 3b which corresponds to a combination of EMA-DMSO (20 µM) and Tween®20 (0.5%) (Figure 4.12). The Log reduction recorded for non-inactivated HAV suspended in simulated water was negative when considering the data from the group 'Mean' (Figure 4.12-A), while it was positive when calculated through the data obtained for the groups 'One_100' and 'One_200' (Figure 4.12-B and C).

(C)

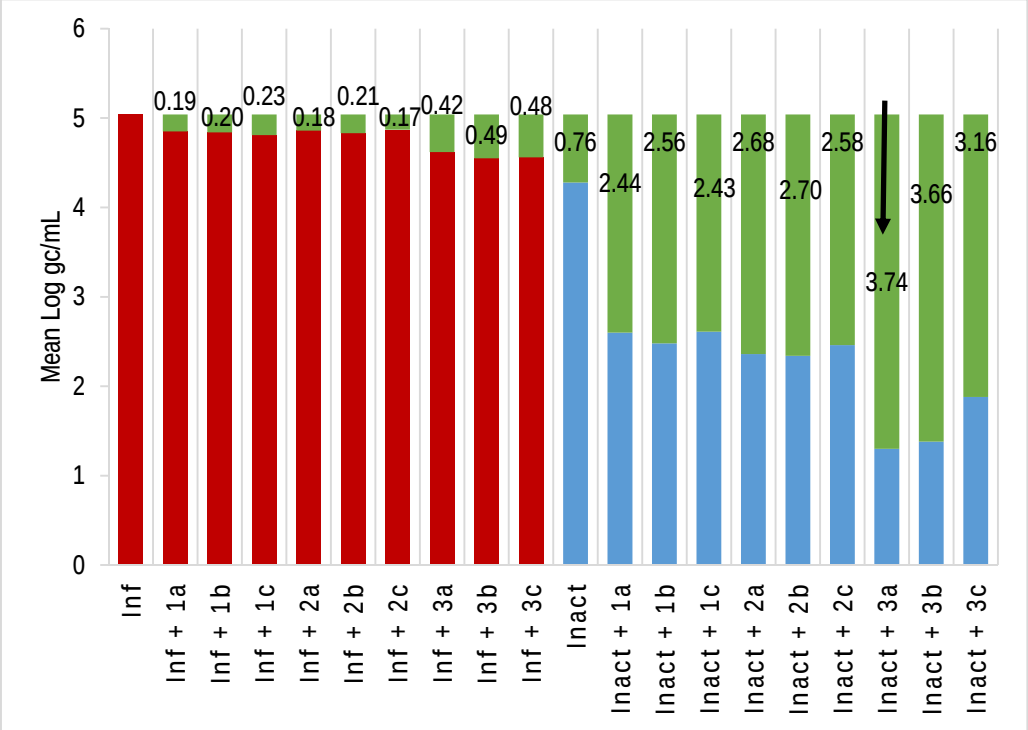


Figure 4.12: Bar graphs depicting the reduction of HAV titres as calculated by STATA® 15 software for the groups 'Mean' (A), 'One_100' (B) and 'One_200' (C). The HAV suspended in simulated water were treated using a combination of vdye and surfactant. The viral suspensions were treated before and after heat-inactivation. Non-inactivated (Inf) and heat-inactivated (Inact) HAV suspensions are represented in red and blue, respectively. Log reduction values are represented in green. The Arabic numerals on the X-axis indicate the type of vdye used for the treatment: (1) PMA-DMSO (50 µM), (2) PMA-water (50 µM) and (3) EMA-DMSO (20 µM). The alphabet letters indicate the type of surfactant used: (a) Triton® X100 (0.5%), (b) Tween®20 (0.5%) and (c) Tween®20 (0.1%).

Results from Log transformed data in Microsoft Excel 2013: The same reductions values were obtained for all three groups, using Microsoft Excel 2013 (Microsoft Corporation). Non-inactivated HAV suspended in simulated water showed <0.30 reduction after viability treatment (Figure 4.13). The heat-inactivated negative control (Inact) showed a 2.80 reduction. The viability treatment of heat-inactivated HAV suspensions recorded high reductions (>3.50), with the highest reduction (4.17) recorded for treatment using a combination of PMA-water (50 µM) and Tween®20 (0.5%) (Figure 4.13). It should be noted that treatment with PMA-DMSO (50 µM) and Tween®20 (0.5%) gave the second highest reduction (4.12) (Figure 4.13). The combination of PMA-water (50 µM) and Tween®20 (0.5%) was better than that of PMA-water (50 µM) and Triton® X100 (0.5%) at reducing PCR signal (4.17 and 3.98

reduction, respectively) (Figure 4.13). The combination of PMA-DMSO (50 µM) and Tween®20 (0.5%) was also better than that of PMA-DMSO (50 µM) and Triton® X100 (0.5%) at reducing PCR signal (4.12 and 3.80 reduction, respectively) (Figure 4.13). Viability treatment of heat-inactivated HAV suspensions with the remaining combinations of vdyes and surfactants gave slightly lower reductions values (between 3.60 and 4.00) (Figure 4.13). These values are higher than those obtained for non-inactivated treated HAV suspensions (Figure 4.13). The data, as analysed using Microsoft Excel 2013 (Microsoft Corporation), suggests that the combination of PMA-water (50 µM) and Tween®20 (0.5%) is best suited for the quantification, by RT-qPCR, of potentially infectious HAV particles in simulated water samples.

4.3.2.7 Assay 5: sensitivity of the most efficient viability treatment for the selective quantification of potentially infectious HAV suspended in simulated water samples

Figure 4.14 illustrates the reductions recorded after viability treatment of HAV suspended at different concentrations in simulated water samples. The combination of PMA-water (50 µM) and Tween®20 (0.5%) was used for the treatment. The viability treatment of non-inactivated HAV suspension showed a gradual increase in reduction (from 0.14 to 5.26) with each dilution (Figure 4.14). The viability treatment of heat-inactivated HAV suspensions also showed gradual increase in reduction but from 10^{-1} dilution, with negative values recorded for 10^{-5} and 10^{-6} dilutions (Figure 4.14). The reduction recorded after viability treatment of concentrated and heat-inactivated HAV stock was higher (4.06) than the reduction recorded after viability treatment of the 10^{-1} dilution (2.96). Viability treatment was able to improve RT-qPCR signal up to 10^{-4} dilution in heat-inactivated HAV suspensions which corresponds to 6.63 gc/mL. Thus, the limit of quantification of potentially infectious HAV in simulated water samples using the RT-qvPCR optimised in this study is 6.63 gc.

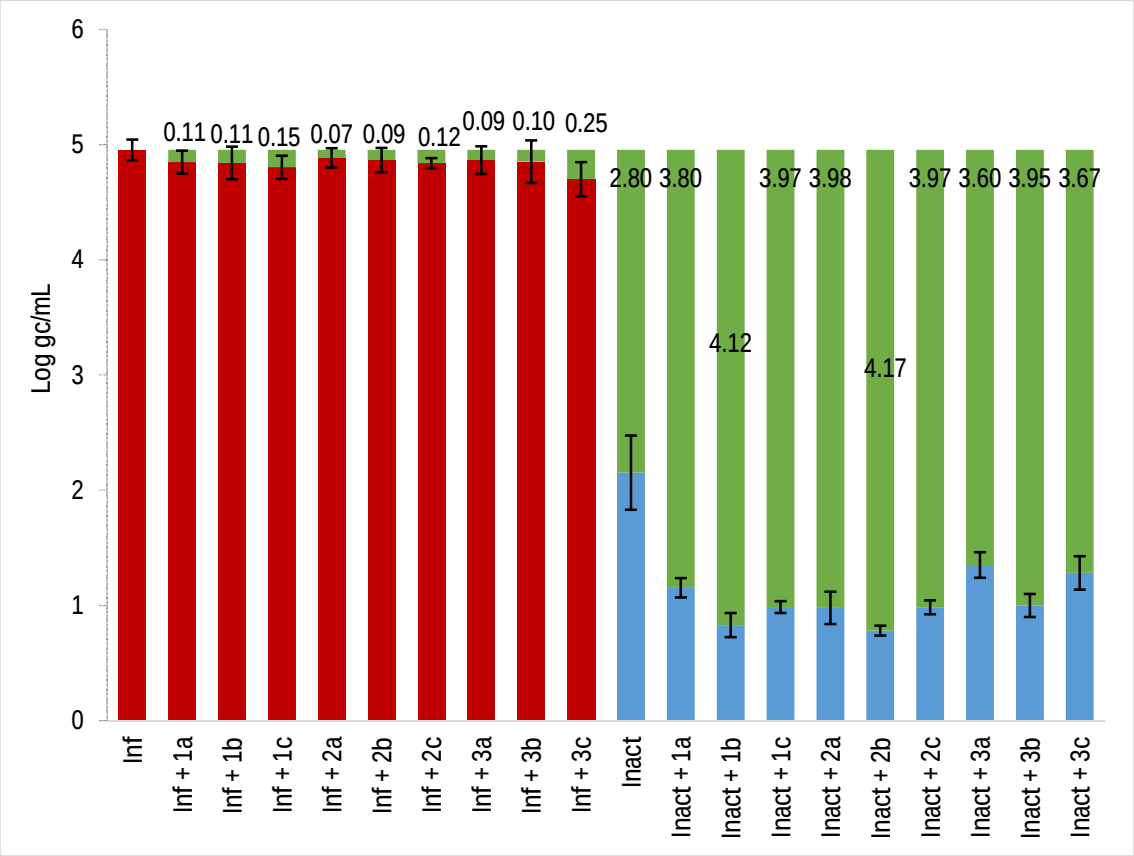


Figure 4.13: Bar graphs depicting the reduction of HAV titres after viability treatment of HAV suspended in simulated water samples using a combination of vdye and surfactant. The viral suspensions were treated before and after heat-inactivation. Non-inactivated (Inf) and heat-inactivated (Inact) HAV suspensions are represented in red and blue, respectively. Log reduction values are represented in green. The Arabic numerals on the X-axis indicate the type of vdye used for the treatment: (1) PMA-DMSO (50 µM), (2) PMA-water (50 µM) and (3) EMA-DMSO (20 µM). The alphabet letters indicate the type of surfactant used: (a) Triton® X100 (0.5%), (b) Tween®20 (0.5%) and (c) Tween®20 (0.1%).

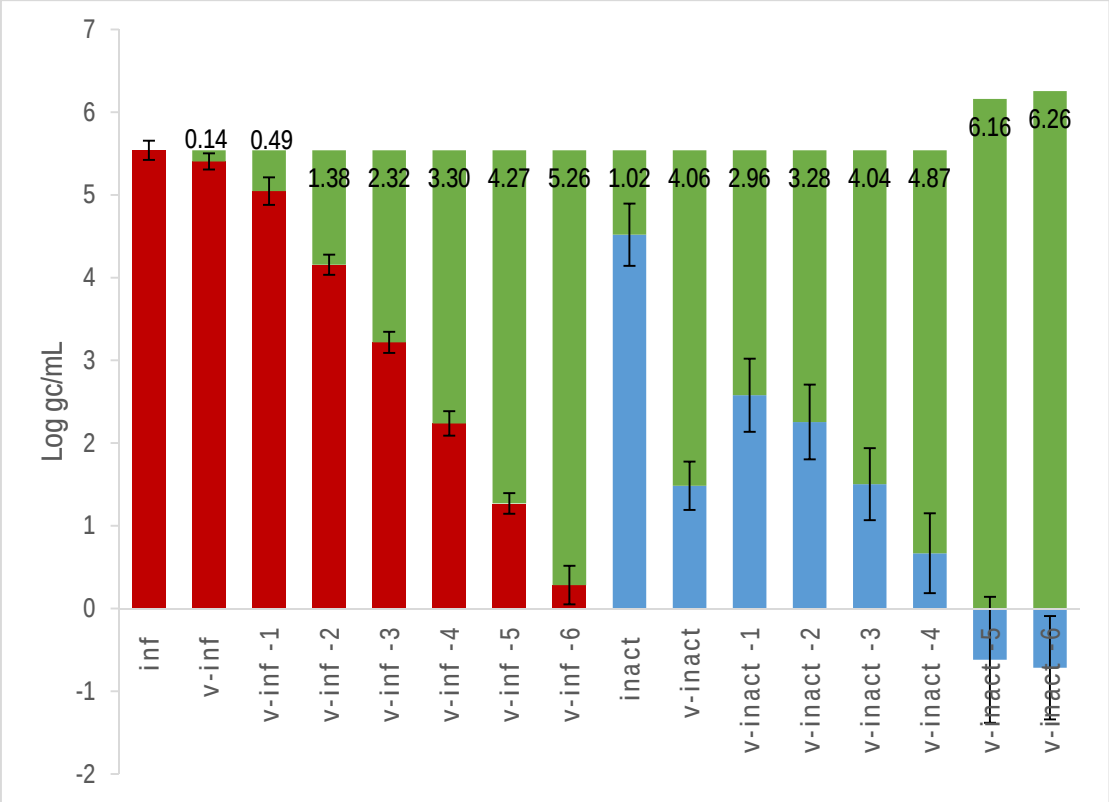


Figure 4.14: Bar graphs depicting the reduction of HAV titres after viability treatment of HAV stocks and HAV suspended at different concentrations in simulated water samples. The stocks and suspensions were treated before and after heat-inactivation using a combination of PMA-water (50 µM) and Tween®20 (0.5%). Non-inactivated (inf) and heat-inactivated (inact) HAV stocks or suspensions are indicated in red and blue, respectively. Log reduction values are represented in green. Undiluted non-inactivated and undiluted heat-inactivated HAV stock that received viability treatment are indicated by 'v-inf' and 'v-inact', respectively. The Arabic numerals preceded by (-) on the X-axis indicate the dilutions, i.e. -1 indicates 10^{-1} dilution.

4.3.3 Quantification of potentially infectious HAV from WWTPs 1 to 5

The validity of the RT-qPCR assays was confirmed as the positive controls were positive and the negative controls were negative. No inhibition of amplification was detected using either the QuantiFast® Kit (Qiagen) or the hepatitisA@ceeramTools™ Kit (Ceeram s.a.s), as the IC was amplified for all assays.

Eight result scenarios were observed which could be grouped into four main categories: failed, successful, ambiguous and unclassified (Table 4.3). From Table 4.3, quantification of intact HAV particles failed (Ci=0, Cf=0) for nine (4R and 5E) and four (3R and 1E) WWTP samples using the QuantiFast® Kit

(Qiagen) and the hepatitisA@ceeramTools™ Kit (Ceeram s.a.s), respectively. Viability treatment successfully reduced PCR signals (Ci>Cf) in 56 (71% [56/79], [26R and 30E]) and 65 (81% [65/79]; [28R and 37E]) WWTP samples using the QuantiFast® Kit (Qiagen) and the hepatitisA@ceeramTools™ Kit (Ceeram s.a.s), respectively (Table 4.3). Quantification of intact HAV particle was ambiguous (Ci=0 and Cf>0, or Ci>0 and Cf=0) for eight and seven WWTP samples using the QuantiFast® Kit (Qiagen) and the hepatitisA@ceeramTools™ Kit (Ceeram s.a.s), respectively (Table 4.3). The detection of potentially infectious HAV particles was unclassified (Ci=Cf, or Ci<Cf, or Ci=ND [not done] and Cf=0, or Ci=ND and Cf>0) for 23 (11R and 12E) and 14 (9R and 5E) WWTP samples using the QuantiFast® Kit (Qiagen) and the hepatitisA@ceeramTools™ Kit (Ceeram s.a.s), respectively (Table 4.3).

Table 4.3: Categories observed after quantification of potentially infectious HAV in naturally contaminated wastewater. Quantification was done using the QuantiFast® Kit or the hepatitisA@ceeramTools™ Kit

Categories		Pathogen Kit		Ceeram kit	
		[c]R	[d]E	R	E
Failed	[a]Ci=0, [b]Cf=0	4R	5E	3R	1E
Successful	Ci>Cf	26R	30E	28R	37E
Ambiguous	Ci=0; Cf>0	2R	1E	2R	1E
	Ci>0; Cf=0	2R	3E	1R	3E
Unclassified	Ci=Cf	4R	5E	3R	1E
	Ci<Cf	6R	4E	5R	1E
	Ci=[e]ND; Cf=0	0R	0E	0R	0E
	Ci=ND; Cf>0	1R	3E	1R	3E

a: Ci = viral titre before viability treatment
b: Cf = viral titre after viability treatment
c: R = sewage
d: E = treated wastewater discharge
e: ND = not done/tested

The quantification of HAV before and after viability treatment of naturally contaminated WWTPs samples, using either the QuantiFast® Kit (Qiagen) or the hepatitisA@ceeramTools™ Kit (Ceeram s.a.s), is illustrated on Figure 4.15.

The dashed red and blue lines indicate trends observed during quantification before and after viability treatment, respectively (Figure 4.15). Trends observed during quantification before and after viability treatment are maintained for both kits for all WWTPs over the sampling period (April 2015 to March 2016) (Figure 4.15). No apparent seasonal pattern was recorded. Potentially infectious HAVs were detected before and after wastewater treatment, as illustrated by the quantification of HAV in sewage and treated discharge samples. Quantification data was missing for a number of months, because either the sample was not available for retesting (WWTP1 –Sewage in November and December; Figure 4.15 - A-i and iii) or the amplification (RT-qPCR and/or RT-qvPCR) failed (WWTP1 – Treated wastewater in August; Figure 4.15 - A-ii and A-iv).

Viral titres recorded before and after viability treatment are represented in red and blue blocks, respectively (Figure 4.15). The WWTPs 1, 2, 4 and 5 discharged potentially infectious HAV into downstream water sources (Figure 4.15 – A-ii, A-iv, B-ii, B-iv, D-ii, D-iv, E-ii and E-iv). With regard to the sewage samples collected from WWTP3, HAV was detected in June, September and February using the QuantiFast® Kit (Qiagen), and, in June and February using the hepatitisA@ceeramTools™ Kit (Ceeram s.a.s) (Figure 4.15 – C-i and C-iii). However, potentially infectious HAV could only be detected in February (Figure 4.15 – C-i and C-iii). Using the QuantiFast® Kit (Qiagen), HAV was not detected in the treated wastewater samples (Figure 4.15 – C-ii), while using the hepatitisA@ceeramTools™ Kit (Ceeram s.a.s) HAV was detected in the June and July treated wastewater discharge samples (Figure 4.15 – C-iv). But, the detected strains may not have been potentially infectious, as illustrated by the absence of blue blocks on the figure (Figure 4.15 – C-iv).

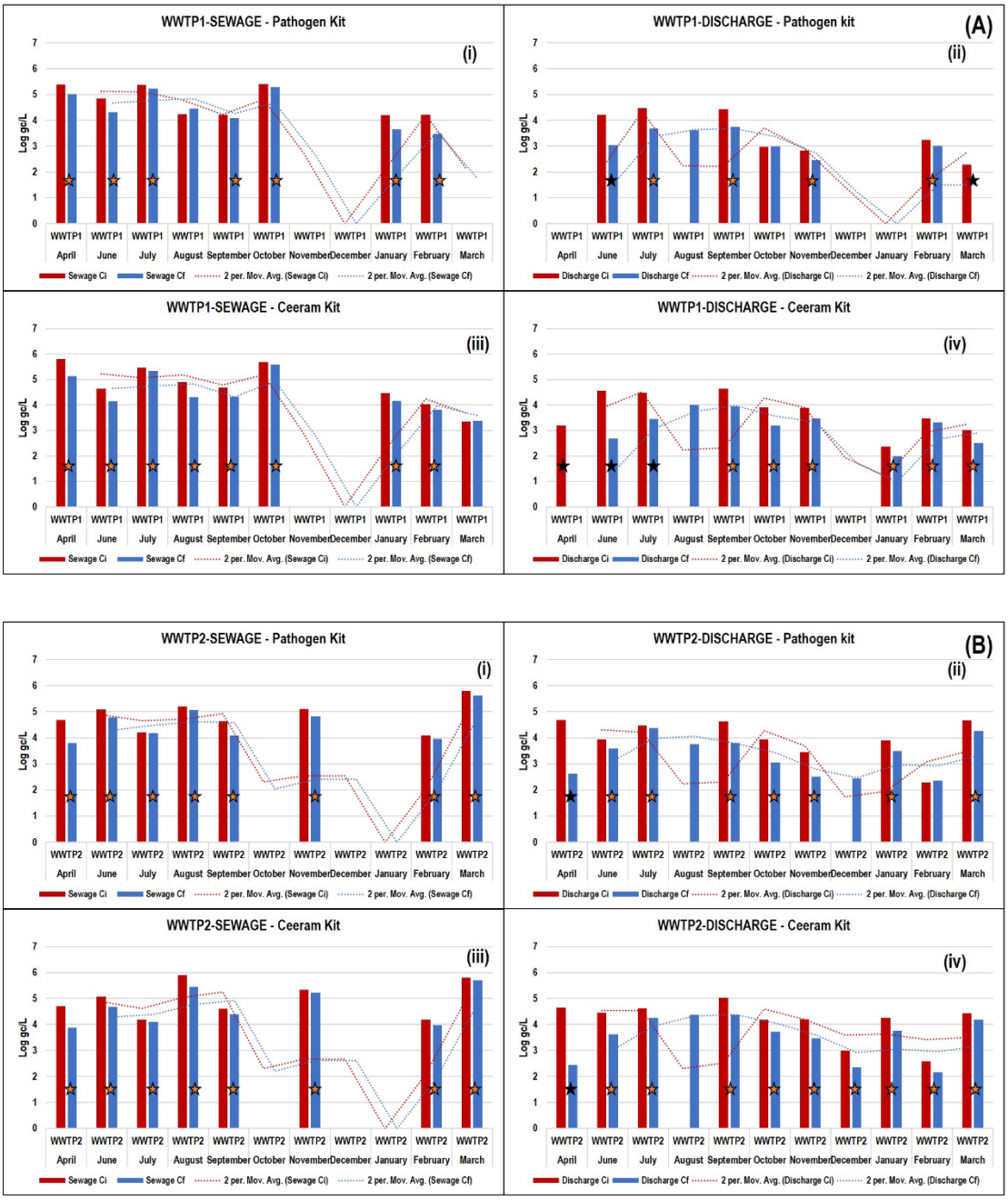


Figure 4.15: Quantification of HAV before and after viability treatment of naturally contaminated WWTP samples. The QuantiFast® Kit (Qiagen) and the Ceeram Kit (Ceeram s.a.s) were used for the quantification. Viral titres recorded before and after viability treatment are represented in red and blue, respectively; the dashed red and blue lines indicate trends observed during quantification before and after viability treatment, respectively; the orange stars indicate Log reduction values <1.0; the black stars indicate Log reduction values ≥1.0.

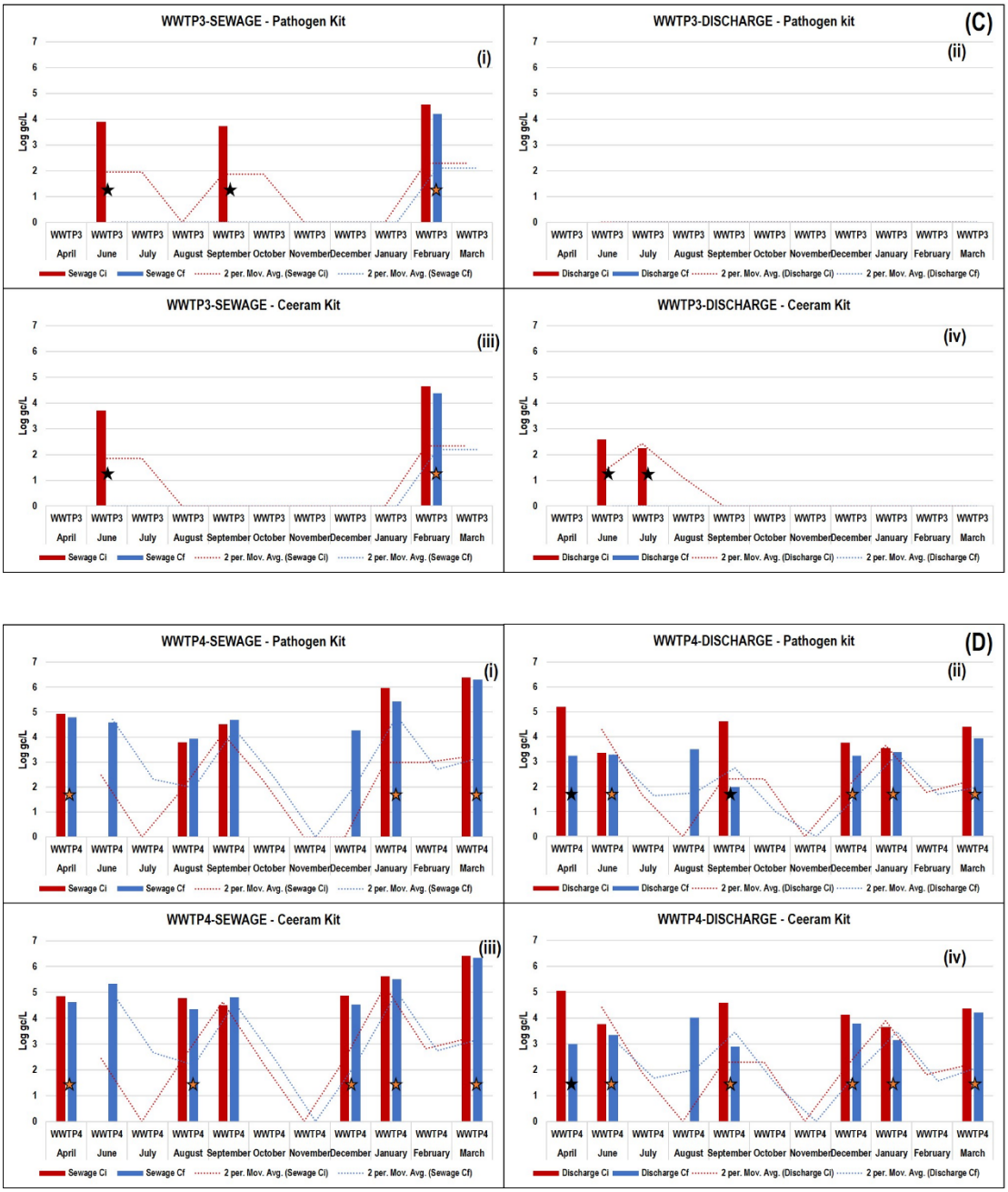


Figure 4.15: Quantification of HAV before and after viability treatment of naturally contaminated WWTP samples. The QuantiFast® Kit (Qiagen) and the Ceeram Kit (Ceeram s.a.s) were used for the quantification. Viral titres recorded before and after viability treatment are represented in red and blue, respectively; the dashed red and blue lines indicate trends observed during quantification before and after viability treatment, respectively; the orange stars indicate Log reduction values <1.0; the black stars indicate Log reduction values ≥1.0.

The successful reduction in titres obtained after viability treatment of sewage and treated wastewater discharge samples are summarised (Tables 4.4 to 4.7). These reductions are also indicated by orange and black stars which represent positive reduction values <1.0, and, ≥1.0, respectively (Figure 4.15). Reduction

values recorded for sewage samples were less than 1.0, except for samples from WWTP3 (Figure 4.15 – C-i and C-iii).

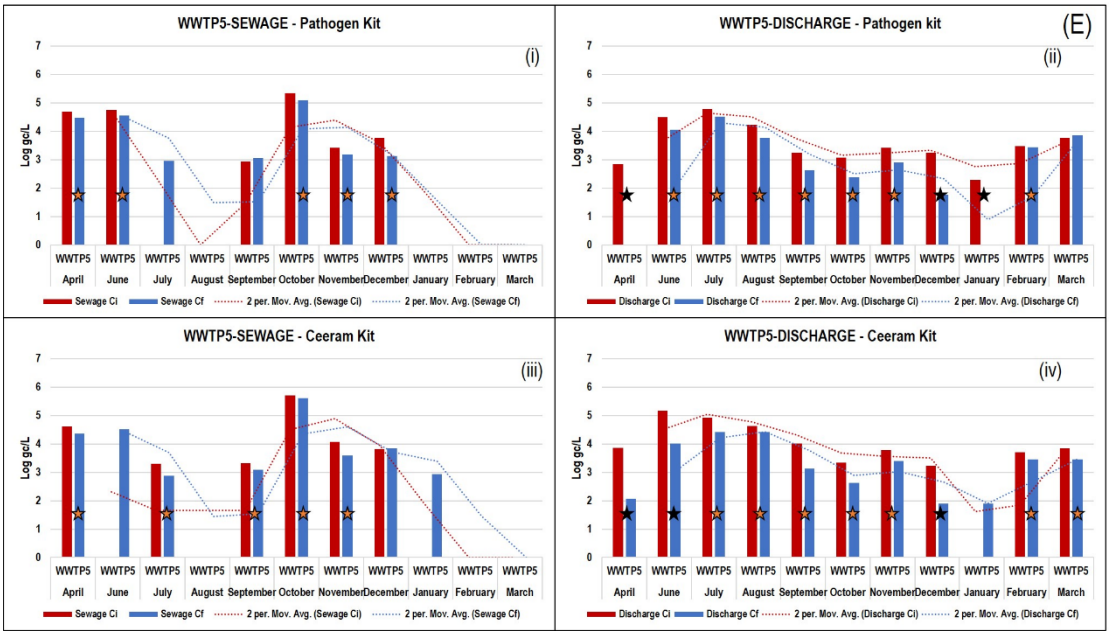


Figure 4.15: Quantification of HAV before and after viability treatment of naturally contaminated WWTP samples. The QuantiFast® Kit (Qiagen) and the Ceeram Kit (Ceeram s.a.s) were used for the quantification. Viral titres recorded before and after viability treatment are represented in red and blue, respectively; the dashed red and blue lines indicate trends observed during quantification before and after viability treatment, respectively; the orange stars indicate Log reduction values <1.0; the black stars indicate Log reduction values ≥1.0.

The reduction in titres recorded using the QuantiFast® Kit (Qiagen) are summarised in Tables 4.4 and 4.5. The range of concentrations does not decrease after treatment sewage samples (Table 4.4), but only after treatment of treated discharge samples (Table 4.5). The sewage sample collected from WWTP3 in June had the highest reduction value (3.90) (Table 4.4). On the other hand, the treated discharge sample collected from WWTP5 in April, recorded the highest reduction value (2.84) (Table 4.5). In addition, Table 4.4 indicates that between 0% and 94% of HAV quantified in the sewage samples had intact capsids and are potentially infectious. Slightly lower percentages (between 0% and 93%) of potentially infectious HAV have been detected in treated wastewater discharge samples (Table 4.5).

Table 4.4: Summary of successful reduction in titres obtained after viability treatment of sewage samples, using the QuantiFast® Kit (Qiagen). Red and yellow highlights indicate highest and lowest values for each column, respectively.

			[a]Ci	Log Ci	[b]Cf	Log Cf	Log reduction	% intact capsid
1	April	WWTP1	2.49E+05	5.40	1.06E+05	5.03	0.37	43%
2	April	WWTP2	4.92E+04	4.69	6.33E+03	3.80	0.89	13%
3	April	WWTP4	8.34E+04	4.92	6.28E+04	4.80	0.12	75%
4	April	WWTP5	4.86E+04	4.69	2.97E+04	4.47	0.21	61%
5	June	WWTP1	7.04E+04	4.85	2.04E+04	4.31	0.54	29%
6	June	WWTP2	1.23E+05	5.09	6.02E+04	4.78	0.31	49%
7	June	WWTP3	7.93E+03	3.90	0.00E+00	0.00	3.90	0%
8	June	WWTP5	5.59E+04	4.75	3.58E+04	4.55	0.19	64%
9	July	WWTP1	2.35E+05	5.37	1.72E+05	5.23	0.14	73%
10	July	WWTP2	1.60E+04	4.20	1.51E+04	4.18	0.03	94%
11	August	WWTP2	1.60E+05	5.21	1.17E+05	5.07	0.14	73%
12	September	WWTP1	1.66E+04	4.22	1.19E+04	4.07	0.15	71%
13	September	WWTP2	4.33E+04	4.64	1.21E+04	4.08	0.55	28%
14	September	WWTP3	5.43E+03	3.73	0.00E+00	0.00	3.73	0%
15	October	WWTP1	2.52E+05	5.40	1.93E+05	5.29	0.12	77%
16	October	WWTP5	2.21E+05	5.34	1.26E+05	5.10	0.24	57%
17	November	WWTP2	1.27E+05	5.10	6.47E+04	4.81	0.29	51%
18	November	WWTP5	2.66E+03	3.42	1.55E+03	3.19	0.23	58%
19	December	WWTP5	5.89E+03	3.77	1.33E+03	3.12	0.65	23%
20	January	WWTP1	1.62E+04	4.21	4.56E+03	3.66	0.55	28%
21	January	WWTP4	9.23E+05	5.97	2.62E+05	5.42	0.55	28%
22	February	WWTP1	1.67E+04	4.22	2.95E+03	3.47	0.75	18%
23	February	WWTP2	1.22E+04	4.09	9.11E+03	3.96	0.13	75%
24	February	WWTP3	3.82E+04	4.58	1.61E+04	4.21	0.38	42%
25	March	WWTP2	6.33E+05	5.80	4.29E+05	5.63	0.17	68%
26	March	WWTP4	2.44E+06	6.39	2.02E+06	6.30	0.08	82%
Range			2.66E+03 - 2.44E+06	-	0 - 2.02E+06	-	0.03 - 3.90	0% - 94%

a: Ci = viral titre (gc/L) before viability treatment
b: Cf = viral titre (gc/L) after viability treatment

The results obtained with the Ceeram Kit (Ceeram s.a.s) are similar to those obtained with the QuantiFast® Kit (Qiagen) (Tables 4.6 and 4.7). The range of concentrations recorded before and after viability treatment only decreases after treatment of treated wastewater discharge samples (Table 4.7). Using the Ceeram Kit (Ceeram s.a.s) the highest reduction in titres recorded from sewage (3.71) (Table 4.6) and treated discharge (3.20) (Table 4.7) were obtained from samples collected from WWTP3 in June and from WWTP1 in April, respectively. The percentages of potentially infectious viruses are higher in the sewage samples (between 0% and 85%) compared to the treated discharge samples (between 0% and 69%).

Table 4.5: Summary of successful reduction in titres obtained after viability treatment of treated wastewater discharge samples, using the QuantiFast® Kit (Qiagen). Red and yellow highlights indicate highest and lowest values for each column, respectively.

			[a]Ci	Log Ci	[b]Cf	Log Cf	Log reduction	% intact capsid
1	April	WWTP2	4.80E+04	4.68	4.27E+02	2.63	2.05	1%
2	April	WWTP4	1.59E+05	5.20	1.74E+03	3.24	1.96	1%
3	April	WWTP5	6.96E+02	2.84	0.00E+00	0.00E+00	2.84	0%
4	June	WWTP1	1.67E+04	4.22	1.12E+03	3.05	1.17	7%
5	June	WWTP2	8.77E+03	3.94	3.94E+03	3.60	0.35	45%
6	June	WWTP4	2.33E+03	3.37	1.91E+03	3.28	0.09	82%
7	June	WWTP5	3.14E+04	4.50	1.15E+04	4.06	0.44	37%
8	July	WWTP1	3.00E+04	4.48	4.82E+03	3.68	0.79	16%
9	July	WWTP2	2.99E+04	4.48	2.35E+04	4.37	0.10	79%
10	July	WWTP5	6.11E+04	4.79	3.35E+04	4.53	0.26	55%
11	August	WWTP5	1.67E+04	4.22	5.94E+03	3.77	0.45	35%
12	September	WWTP1	2.66E+04	4.42	5.59E+03	3.75	0.68	21%
13	September	WWTP2	4.16E+04	4.62	6.38E+03	3.80	0.81	15%
14	September	WWTP4	4.19E+04	4.62	9.76E+01	1.99	2.63	0%
15	September	WWTP5	1.80E+03	3.25	4.26E+02	2.63	0.62	24%
16	October	WWTP2	8.62E+03	3.94	1.15E+03	3.06	0.87	13%
17	October	WWTP5	1.18E+03	3.07	2.42E+02	2.38	0.69	21%
18	November	WWTP1	6.69E+02	2.83	2.88E+02	2.46	0.37	43%
19	November	WWTP2	2.90E+03	3.46	3.23E+02	2.51	0.95	11%
20	November	WWTP5	2.60E+03	3.41	7.85E+02	2.89	0.52	30%
21	December	WWTP4	5.79E+03	3.76	1.77E+03	3.25	0.52	30%
22	December	WWTP5	1.73E+03	3.24	5.80E+01	1.76	1.48	3%
23	January	WWTP2	7.74E+03	3.89	3.07E+03	3.49	0.40	40%
24	January	WWTP4	3.47E+03	3.54	2.44E+03	3.39	0.15	70%
25	January	WWTP5	1.89E+02	2.28	0.00E+00	0.00E+00	2.28	0%
26	February	WWTP1	1.72E+03	3.23	9.99E+02	3.00	0.24	58%
27	February	WWTP5	3.03E+03	3.48	2.81E+03	3.45	0.03	93%
28	March	WWTP1	1.96E+02	2.29	0.00E+00	0.00E+00	2.29	0%
29	March	WWTP2	4.68E+04	4.67	1.84E+04	4.27	0.41	39%
30	March	WWTP4	2.47E+04	4.39	8.73E+03	3.94	0.45	35%
Range			1.89E+02 - 1.59E+05	-	0 - 3.35E+04	-	0.03 - 2.84	0% - 93%

a: Ci = viral titre (gc/L) before viability treatment
b: Cf = viral titre (gc/L) after viability treatment

The titres of potentially infectious HAV in the virus concentrates of sewage and paired treated discharge are summarised in Table 4.8. For samples with successful quantification using both the QuantiFast® Kit (Qiagen) and the hepatitisA@ceeramTools™ Kit (Ceeram s.a.s), titres corresponding to the highest reduction was chosen. In addition, titres recorded only after viability treatment, were included in the summary table. For four (grey highlights) and one (green highlight) samples, quantification, before viability treatment, was either not done or equal to zero (Table 4.8). Potentially infectious HAV was quantified from the virus concentrates of 81% (30/37) sewage and 90% (38/42)

treated discharge samples. The WWTPs 1, 2, 4 and 5 discharged potentially infectious HAV into the dam or rivers feeding into the dam, while WWTP3 did not discharge any potentially infectious HAV (Table 4.8). The range of titres between sewage and treated discharge samples decreased for all five WWTPs.

Table 4.6: Summary of successful reduction in titres obtained after viability treatment of sewage samples, using the Ceeram Kit (Ceeram s.a.s). Red and yellow highlights indicate highest and lowest values for each column, respectively.

			[a]Ci	Log Ci	[b]Cf	Log Cf	Log reduction	% intact capsid
1	April	WWTP1	6.53E+05	5.81	1.35E+05	5.13	0.68	21%
2	April	WWTP2	5.13E+04	4.71	7.57E+03	3.88	0.83	15%
3	April	WWTP4	7.14E+04	4.85	4.23E+04	4.63	0.23	59%
4	April	WWTP5	4.09E+04	4.61	2.33E+04	4.37	0.24	57%
5	June	WWTP1	4.35E+04	4.64	1.41E+04	4.15	0.49	32%
6	June	WWTP2	1.18E+05	5.07	4.78E+04	4.68	0.39	41%
7	June	WWTP3	5.11E+03	3.71	0.00E+00	0.00	3.71	0%
8	July	WWTP1	2.98E+05	5.47	2.13E+05	5.33	0.14	72%
9	July	WWTP2	1.50E+04	4.18	1.27E+04	4.10	0.07	85%
10	July	WWTP5	2.00E+03	3.30	7.57E+02	2.88	0.42	38%
11	August	WWTP1	7.85E+04	4.90	2.06E+04	4.31	0.58	26%
12	August	WWTP2	7.94E+05	5.90	2.78E+05	5.44	0.46	35%
13	August	WWTP4	5.85E+04	4.77	2.29E+04	4.36	0.41	39%
14	September	WWTP1	4.83E+04	4.68	2.10E+04	4.32	0.36	43%
15	September	WWTP2	4.09E+04	4.61	2.51E+04	4.40	0.21	61%
16	September	WWTP5	2.08E+03	3.32	1.23E+03	3.09	0.23	59%
17	October	WWTP1	4.87E+05	5.69	3.81E+05	5.58	0.11	78%
18	October	WWTP5	5.06E+05	5.70	4.18E+05	5.62	0.08	83%
19	November	WWTP2	2.14E+05	5.33	1.70E+05	5.23	0.10	79%
20	November	WWTP5	1.18E+04	4.07	3.96E+03	3.60	0.47	34%
21	December	WWTP4	7.47E+04	4.87	3.28E+04	4.52	0.36	44%
22	January	WWTP1	2.94E+04	4.47	1.45E+04	4.16	0.31	49%
23	January	WWTP4	4.23E+05	5.63	3.15E+05	5.50	0.13	75%
24	February	WWTP1	1.05E+04	4.02	6.51E+03	3.81	0.21	62%
25	February	WWTP2	1.55E+04	4.19	9.21E+03	3.96	0.22	60%
26	February	WWTP3	4.55E+04	4.66	2.45E+04	4.39	0.27	54%
27	March	WWTP2	6.25E+05	5.80	5.06E+05	5.70	0.09	81%
28	March	WWTP4	2.62E+06	6.42	2.16E+06	6.34	0.08	83%
Range			2.00E+03 - 2.62E+06	-	0 - 2.16E+06	-	0.07 - 3.71	0% - 85%

a: Ci = viral titre (gc/L) before viability treatment
b: Cf = viral titre (gc/L) after viability treatment

Table 4.7: Summary of successful reduction in titres obtained after viability treatment of treated wastewater discharge samples, using the Ceeram Kit (Ceeram s.a.s). Red and yellow highlights indicate highest and lowest values for each column, respectively.

			[a]Ci	Log Ci	[b]Cf	Log Cf	Log reduction	% intact capsid
1	April	WWTP1	1.57E+03	3.20	0.00E+00	0.00	3.20	0%
2	April	WWTP2	4.36E+04	4.64	2.75E+02	2.44	2.20	1%
3	April	WWTP4	1.14E+05	5.06	1.01E+03	3.01	2.05	1%
4	April	WWTP5	7.10E+03	3.85	1.18E+02	2.07	1.78	2%
5	June	WWTP1	3.60E+04	4.56	4.76E+02	2.68	1.88	1%
6	June	WWTP2	2.78E+04	4.44	4.12E+03	3.62	0.83	15%
7	June	WWTP3	3.92E+02	2.59	0.00E+00	0.00	2.59	0%
8	June	WWTP4	5.86E+03	3.77	2.26E+03	3.35	0.41	39%
9	June	WWTP5	1.47E+05	5.17	1.05E+04	4.02	1.14	7%
10	July	WWTP1	2.96E+04	4.47	2.83E+03	3.45	1.02	10%
11	July	WWTP2	4.19E+04	4.62	1.82E+04	4.26	0.36	43%
12	July	WWTP3	1.76E+02	2.25	0.00E+00	0.00	2.25	0%
13	July	WWTP5	8.18E+04	4.91	2.66E+04	4.43	0.49	33%
14	August	WWTP5	4.19E+04	4.62	2.68E+04	4.43	0.20	64%
15	September	WWTP1	4.28E+04	4.63	8.93E+03	3.95	0.68	21%
16	September	WWTP2	1.07E+05	5.03	2.41E+04	4.38	0.65	23%
17	September	WWTP4	3.93E+04	4.59	7.96E+02	2.90	1.69	2%
18	September	WWTP5	1.05E+04	4.02	1.34E+03	3.13	0.89	13%
19	October	WWTP1	7.94E+03	3.90	1.55E+03	3.19	0.71	19%
20	October	WWTP2	1.52E+04	4.18	5.16E+03	3.71	0.47	34%
21	October	WWTP5	2.15E+03	3.33	4.33E+02	2.64	0.70	20%
22	November	WWTP1	7.66E+03	3.88	2.93E+03	3.47	0.42	38%
23	November	WWTP2	1.55E+04	4.19	2.96E+03	3.47	0.72	19%
24	November	WWTP5	5.97E+03	3.78	2.47E+03	3.39	0.38	41%
25	December	WWTP2	9.90E+02	3.00	2.25E+02	2.35	0.64	23%
26	December	WWTP4	1.35E+04	4.13	6.04E+03	3.78	0.35	45%
27	December	WWTP5	1.70E+03	3.23	8.01E+01	1.90	1.33	5%
28	January	WWTP1	2.31E+02	2.36	9.81E+01	1.99	0.37	42%
29	January	WWTP2	1.82E+04	4.26	5.66E+03	3.75	0.51	31%
30	January	WWTP4	4.43E+03	3.65	1.41E+03	3.15	0.50	32%
31	February	WWTP1	3.02E+03	3.48	2.05E+03	3.31	0.17	68%
32	February	WWTP2	3.84E+02	2.58	1.44E+02	2.16	0.43	38%
33	February	WWTP5	5.19E+03	3.72	2.83E+03	3.45	0.26	55%
34	March	WWTP1	1.02E+03	3.01	3.25E+02	2.51	0.50	32%
35	March	WWTP2	2.65E+04	4.42	1.51E+04	4.18	0.25	57%
36	March	WWTP4	2.34E+04	4.37	1.60E+04	4.20	0.16	69%
37	March	WWTP5	6.92E+03	3.84	2.90E+03	3.46	0.38	42%
Range			1.47E+05 - 1.76E+02	-	0 - 2.68E+04	-	0.16 - 3.20	0% - 69%

a: Ci = viral titre (gc/L) before viability treatment
b: Cf = viral titre (gc/L) after viability treatment

Table 4.8: Summary of titres (gc/L) of potentially infectious HAV in sewage and wastewater discharge for WWTPs 1 to 5. The samples were collected between April 2015 and March 2016. The range, the mean of titres, the fraction and percentage of positive samples are also given. Grey and green highlight titres obtained only after viability treatment. Red highlights a sample with a higher titre obtained after viability treatment.

	WWTP1		WWTP2		WWTP3		WWTP4		WWTP5	
	Sewage	Wastewater discharge	Sewage	Wastewater discharge	Sewage	Wastewater discharge	Sewage	Wastewater discharge	Sewage	Wastewater discharge
April	1.35E+05	0.00E+00	7.57E+03	4.27E+02	0.00E+00	ND	6.28E+04	1.74E+03	2.97E+04	1.18E+02
May	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
June	2.04E+04	4.76E+02	6.02E+04	4.12E+03	0.00E+00	0.00E+00	2.16E+05	2.26E+03	3.58E+04	1.15E+04
July	2.13E+05	4.82E+03	1.51E+04	2.35E+04	ND	0.00E+00	ND	ND	7.57E+02	3.35E+04
August	2.06E+04	1.03E+04	2.78E+05	2.36E+04	ND	ND	2.29E+04	1.05E+04	ND	2.68E+04
September	2.10E+04	8.93E+03	2.51E+04	2.41E+04	0.00E+00	ND	Neg	7.96E+02	1.23E+03	1.34E+03
October	3.81E+05	1.55E+03	ND	5.16E+03	ND	ND	ND	ND	4.18E+05	4.33E+02
November	ND	2.93E+03	1.70E+05	2.96E+03	ND	0.00E+00	ND	ND	3.96E+03	2.47E+03
December	ND	ND	ND	2.25E+02	ND	ND	3.28E+04	6.04E+03	1.33E+03	8.01E+01
January	1.45E+04	9.81E+01	ND	5.66E+03	ND	ND	2.62E+05	2.44E+03	0.00E+00	7.83E+01
February	6.51E+03	2.05E+03	9.21E+03	1.44E+02	2.45E+04	ND	ND	ND	ND	2.83E+03
March	0.00E+00	3.25E+02	5.06E+05	1.84E+04	0.00E+00	ND	2.16E+06	1.60E+04	ND	2.90E+03
Range	0 - 3.81E+05	0 - 1.03E+04	7.57E+03 - 5.06E+05	1.44E+02 - 2.41E+04	0 - 2.45E+04	0.00	2.29E+04 - 2.16E+06	7.96E+02 - 1.60E+04	0 - 4.18E+05	7.83E+01 - 3.35E+04
Mean	9.02E+04	3.15E+03	1.34E+05	9.85E+03	4.90E+03	0.00E+00	4.60E+05	5.68E+03	6.14E+04	7.46E+03
Fraction of positive samples	8/9	9/10	8/8	11/11	1/5	0/4	6/7	7/7	7/8	11/11
Percentage of positive samples	89%	90%	100%	100%	20%	0%	86%	100%	88%	100%

4.4 DISCUSSION

In a published report, the risk of HAV infection from contaminated surface waters in Gauteng, SA was determined.[82] However, the titres of HAV were obtained using an ICC-PCR assay.[82] Given that wild-type HAV grows poorly on cell culture, risk assessment studies based on quantification data obtained using RT-qPCR would be more accurate. On the other hand, RT-qPCR is fast, accurate and sensitive, but it does not indicate the infectivity of the detected virus.[110] Pretreatment of wastewater samples with a vdye, such as PMA or EMA, aids the amplification of potentially infectious viruses by preventing PCR amplification from damaged viral particles.[109,125] However, vPCR assays need to be optimised before being applied to specific samples.[109,125] The present study set out to quantify HAV in wastewater samples, by pretreating the corresponding virus concentrates with PMA or EMA in combination with or without surfactant. The results showed that pretreating river water samples, with a combination of PMA-water (50 µM) and Tween®20 (0.5%) can decrease PCR amplification signals from heat damaged HAV particles up to 6.63 gc/mL of samples. The optimised vPCR assay detected potentially infectious HAV in 86% of the virus concentrates from sewage and treated wastewater discharge samples. This is the first time, in SA, that a molecular assay is used to quantify potentially infectious HAV in wastewater, without using cell culture assays. In addition it is the first time that potentially infectious HAV have been quantified from sewage and associated treated wastewater.

In the present study the use of PMA-DMSO (50 µM), PMA-water (50 µM) and EMA-DMSO (20 µM) combined with or without Triton® X100 (0.5%), Tween®20 (0.5%) or Tween®20 (0.1%), for the selective detection of infectious HAV in river water was tested. The results showed that pretreatment with PMA-water (50 µM) and Tween®20 (0.5%) was better at quantifying potentially infectious HAV. The outcomes of the present study agree with previous studies on the use of PMA but differ about the surfactant used. Since the development of vdye pretreatment, PMA and EMA have been used to prevent PCR amplification of damaged HAV particles in food concentrates and lettuce wash water,[13] river water and sewage samples.[2] These studies found that PMA (dissolved in water

or DMSO) used in combination with Triton® X100 (0.5%) is the best pretreatment to reduce PCR amplification from heat-inactivated HAV particles in selected food concentrates and in lettuce wash water,[13] in river water and sewage samples.[2] The use of surfactants has proven to enhance entry of vdyes in a viral particle with a damaged capsid.[2,13] Moreno et al[13] used a similar protocol to this study for vdye pretreatment and found that Triton® X100 (0.5%) was better than Span®20 at increasing efficiency of PMA pretreatment of lettuce wash water. However, in the present study, Tween®20 (0.5%) outperformed Triton® X100 (0.5%) in decreasing PCR signal (Figure 4.13). Fuster et al[2] only tested the effect of Triton® X100 (0.5%). The matrices of the river water used during optimisation of the vPCR could be more favourable to the use of Tween®20 rather than Triton® X100. In addition, the virus concentrates of the wastewater samples analysed in this study may also be favourable to the used of Tween®20 as shown by the high percentage of virus concentrates from which potentially infectious HAV were quantified.

The efficiency of a vPCR at quantifying potentially infectious viruses is usually reflected by a reduction of virus concentration recorded before and after viability treatment, since amplification from naked nucleic acid and damaged viral particles are prevented.[2,13,134,136-137,181] The present study found that 81% of the virus concentrates of HAV positive sewage samples harboured potentially infectious HAV particles. The recorded reduction in virus concentration before and after viability treatment was <1.0 for all sewage samples with potentially infectious HAV, except for a sewage sample collected from WWTP3 in June. Lower reductions in the analysis of sewage samples have been reported.[2,136] From both studies, a total of six sewage samples were pretreated with a combination of PMA-water (50 µM) and Triton® X100 (0.5%) or PMAxx and Triton® X100. The low reduction values indicate that the majority of HAV strains quantified in sewage samples are infectious.[2] That is why the present study recorded up to 94% of the HAV detected in sewage samples had intact capsid and therefore were potentially infectious. In contrast, the high reduction, recorded for the WWTP3 June sewage sample, indicates absence of potentially infectious HAV strains in the sample. This is reflected by the recorded 0% intact HAV particle (Tables 4.4 and 4.6).

The retesting of HAV positive treated wastewater discharge using the optimised RT-qvPCR assay revealed that up to 93% of the HAV detected were potentially infectious. This is similar to the percentage of potentially infectious HAV detected in sewage. The detection of high proportions of intact HAV capsid in sewage and corresponding treated wastewater discharge samples may be unprecedented, however it has been noted for other enteric viruses. A recent study used a PEMAX-RT-qPCR assay to quantify potentially infectious NoV GI and GII from wastewater samples in New Zealand.[182] The study found that up to 89% and up to 59% of NoV GI and GII detected in influent and effluent samples, respectively, were potentially infectious.

The viability PCR developed in this study used the capsid integrity theory to infer the infectivity of thermally inactivated HAV in PBS and unchlorinated river water. However, in the environment, HAV particles could be exposed to different forms of inactivation processes that could cause loss of infectivity without affecting the capsid. It has been shown that the naturally occurring compound epigallocatechin gallate can decrease HAV infectivity without disrupting the HAV capsid and allowing entry of the vdye PMAxx.[183] The successful reduction of PCR signal after applying the PMA-water (50 µM) and Tween®20 (0.5%) in the RT-qPCR assay indicate that the wastewater samples analysed in this study did contain heat damaged HAV particles. A second limitation to the study is that nucleic acid extraction controls were not used while retesting the virus concentrates of wastewater samples. Therefore, wastewater samples with potentially infectious HAV particles might have been missed out. Nevertheless, the results did show that four out of the five WWTPs, analysed in this study, discharged potentially infectious HAV into downstream water sources and that is a cause for concern from a public health perspective. Despite these limitations, a number of observations were made during optimisation and application of the PMA-water (50 µM) and Tween®20 (0.5%) RT-qPCR assay that could impact future development of vPCR assays.

The successful outcome of a vPCR assay depends on several factors. The protocol followed in the present study, to optimise the PMA-water (50 µM) and Tween®20 (0.5%) RT-qPCR assay, showed previously known and potential new

factors could influence the outcome of the assay. Intercalating dye pretreatment must be validated for each virus target.[109,125,138] During optimisation, viability treatment did not improve PCR amplification of heat damaged CV-B6 particles. On the other hand the use of PMA and EMA pretreatment successfully reduced PCR amplification of heat damaged HAV particles. The efficiency of the pretreatment was increased by adding surfactants. This confirms the results of previous studies.[13] Unlike these studies, pretreatment with EMA reduced PCR amplification of heat damaged HAV particles in PBS, better than treatment with PMA. However, once HAV was suspended in river water, treatment with PMA gave better results.

The data obtained during optimisation of the vPCR assay was analysed using Microsoft Excel 2013 (Microsoft Corporation). The STATA® 15 software package (StataCorp LLC) was used to infer statistical significance and perform a second analysis following the pretreatment of simulated water samples. Both software agreed on the vdye pretreatment used to detect potentially infectious HAV in PBS, but they disagreed on the type of pretreatment and surfactant to use in river water. While analysis with Microsoft Excel 2013 (Microsoft Corporation) revealed that pretreatment with PMA-water (50 µM) and Tween®20 (0.5%) is the most appropriate, analysis with STATA® 15 software package (StataCorp LLC) suggest that pretreatment with EMA-DMSO (20 µM) and Triton® X100 (0.5%) would give better results. However, given that the analysis obtained with Microsoft Excel 2013 (Microsoft Corporation) was consistent throughout the optimisation, pretreatment with PMA-water (50 µM) and Tween®20 (0.5%) was chosen.

The kit used for RT-qPCR after vdye pretreatment could influence the outcome of a vPCR assay. The QuantiFast® Kit (Qiagen) and the hepatitisA@ceeramTools™ Kit (Ceeram s.a.s) were used to quantify potentially infectious HAV in the virus concentrates of wastewater samples after viability treatment. Even though both kits gave similar quantification trends before and after viability treatment (Figure 4.15), with the hepatitisA@ceeramTools™ Kit (Ceeram s.a.s) potentially infectious HAV were quantified from a larger number of wastewater samples compared to quantification with the QuantiFast® Kit

(Qiagen) (65 and 56, respectively). In addition, the proportion of HAV with intact capsid detected using the hepatitisA@ceeramTools™ Kit (Ceeram s.a.s) was lower (0%-85% in sewage; 0%-69% in treated discharge) when compared to the QuantiFast® Kit (Qiagen) (0%-94% in sewage; 0%-93% in treated discharge). The data on comparison of kits need further investigation, especially given that the wastewater samples were not tested in triplicate. The different results scenario obtained after retesting the virus concentrates of the wastewater samples indicate that the application of vPCR assay is not always straightforward.

For a number of wastewater samples (at least 9) HAV quantification was null before and after viability treatment. There was no PCR inhibitors detected by both the QuantiFast® Kit (Qiagen) and the hepatitisA@ceeramTools™ Kit (Ceeram s.a.s). This suggests sample degradation, since the virus concentrates of all wastewater samples subjected to viability treatment previously tested positive for HAV. The 'ambiguous' and 'unclassified' results obtained suggest that at least four of the wastewater samples analysed did not harbour potentially infectious HAV (Ci>0 and Cf=0) or that all the HAV strains quantified from at least 9 wastewater samples were potentially infectious (Ci=Cf). For a number (at least 14) of wastewater samples, potentially infectious HAV strains were quantified but the quantification performed before viability treatment was either not done or the recorded titres were null or less than the concentration obtained after viability treatment. Hepatitis A virus can be associated with organic matter in the environment.[83] This association could potentially prevent amplification of the virus. The results obtained after viability treatment of at least 10 of the 14 wastewater samples indicate that the pretreatment optimised and applied in this study could potentially dissociate HAV particles from organic matter and allow quantification.

The successful quantification of potentially infectious HAV, in the virus concentrates of treated discharge samples from WWTPs 1, 2, 4 and 5, suggest that HAV inactivation at the treatment plants is ineffective. However, the results presented here may still be an overestimation of the concentration of potentially infectious HAV in the wastewater samples analysed, because the assay was

optimised for heat damaged HAV particles. However, the recorded reduction in concentrations does indicate that potentially infectious HAV have been discharged into downstream water sources. Potentially infectious HAV could be quantified from 90% to 100% of the virus concentrates of treated discharge samples collected from WWTPs 1, 2, 4 and 5 (Table 4.8). None of the virus concentrates of treated discharge samples collected from WWTP3 harboured potentially infectious HAV (Table 4.8). The absence of potentially infectious HAV in the treated discharge samples from WWTP3 (Table 4.8) could reflect an effective wastewater treatment process, given that the treatment plant is the only one using activated sludge and diffused air to treat sewage.

This is the first report of the quantification of potentially infectious HAV in sewage and associated treated wastewater discharge samples in SA. The PMA-water (50 µM) and Tween®20 (0.5%) RT-qPCR assay can detect potentially infectious HAV without pre-amplification in cell culture. However, the assay still overestimates the concentration of HAV and needs to be improved before it can be applied to other sample matrices and to other viruses. Additional evaluation and optimisation of the assay will bring us closer to developing a vPCR assay for routine use. The results presented here demonstrated that WWTPs 1, 2, 4 and 5 discharged potentially infectious HAV into the dam water or rivers feeding into the dam. Hence, using water from the dam or rivers feeding into the dam could potentially expose its users to infection with HAV. Hepatitis A is a vaccine preventable disease, but potential vaccine escape mutant could be emerging. In order to better assess the health risk, the genetic identity of quantified potentially infectious HAV strains needs to be determined.

CHAPTER 5

GENETIC DIVERSITY OF HEPATITIS A VIRUS STRAINS IN WASTEWATER

5.1 INTRODUCTION

Nucleotide sequence analysis of HAV strains has played an important role in epidemiological investigations of foodborne and waterborne outbreaks of hepatitis A.[9,27,29,78-79,90-91,184] Knowledge of the genetic identity of HAV strains has enabled the identification of common risk factors, transmission routes or vehicles in localised or multistate outbreaks of hepatitis A.[184] However, prior knowledge of HAV strains circulating in a region is needed to determine whether recorded hepatitis A cases are locally acquired or imported. The molecular characterisation of HAV strains detected in sewage provides an overview of the strains circulating in a community, as it includes asymptomatically and symptomatically shed viruses. Hepatitis A is a notifiable disease in SA. The number of anti-HAV IgM positive cases recorded onto the National Health Laboratory Service information system is reported by the National Institute for Communicable Diseases (NICD), on a monthly basis, to the Multisector National Outbreak Response Team. With the potential epidemiological shift recorded in the country,[25-26] the number of hepatitis A cases is expected to increase as populations exposed to HAV and those not exposed to HAV come into contact.[6] The hepatitis A vaccine offers immunity to people that have not been exposed to HAV, but it could be ineffective in the presence of antigenic escape variants of the virus.[15,55]

Nucleotide sequence analysis is the gold standard for typing HAV strains.[111] It is usually performed by PCR amplification of subgenomic regions followed by Sanger or NGS sequencing.[19,139,143-145] Given that PCR detects both infectious and non-infectious viruses, genetic characterisation of HAV strains following viability treatment could be more valuable from a public health perspective,

defining infectious viruses genotypes in the environment. It is for these reasons that the aim of this part of the study was to genetically characterise potentially infectious HAV strains detected in selected SAn wastewater samples. The HAV strains detected in sewage and treated wastewater discharge, before and after viability treatment, were subjected to Sanger sequencing. Full genome analysis was used after viability treatment to further characterise potential new variants of HAV strains. Pairwise, nucleotide sequence, protein sequence and phylogenetic analyses were performed to determine the genetic identity and clinical relevance of HAV strains circulating in the sampling region.

5.2 MATERIALS AND METHODS

Clinical specimens (stool and serum) and wastewater samples (sewage and treated discharge samples) were processed and analysed for the purpose of this study. To prevent cross-contamination, clinical specimens and wastewater samples were processed in different dedicated laboratories on different dates. Nucleic acid extraction and HAV characterisation were also carried out in separate laboratories on different days for each sample type. For Sanger sequencing, sewage and treated discharge samples were processed on the same day, with treated discharge samples processed first. During sample preparation for NGS the serum specimen, sewage and treated discharge samples were each processed on different dates.

5.2.1 Samples

5.2.1.1 Wastewaters

Sanger sequencing: Hepatitis A virus positive wastewater samples were selected for further analysis. Nucleic acid previously extracted (Chapter 3, section 3.2.3) from the virus concentrates of sewage (*n=43*) and treated wastewater discharge (*n=43*) samples, collected from WWTPs 1, 2, 3, 4 and 5, were subjected to Sanger sequencing.

Nucleic acid previously extracted (Chapter 4, section 4.2.3.1) after successful viability treatment of the virus concentrates of sewage (*n=3*) and treated discharge (*n=3*) samples collected from three of the five WWTPs investigated, were subjected to Sanger sequencing. These samples were selected, because Sanger sequencing performed prior to viability treatment revealed the presence of potential vaccine escape mutants of HAV.

NGS: Following viability treatment, the nucleic acid extracted from the treated virus concentrates of sewage (*n=2*) and treated discharge (*n=2*) samples were subjected to NGS. These samples were collected from WWTPs 1 and 4. The samples were chosen because Sanger sequencing performed before and after viability treatment revealed the presence of potential new variants of HAV.

5.2.1.2 Clinical specimens

Sanger sequencing: Suspensions (10% w/v in nuclease-free water [Promega Corp.]) of HAV positive stool specimens of two previously characterised strains (JVR and VDM)[185] were used as positive controls during characterisation of HAV strains by Sanger sequencing. Based on nucleotide sequence analysis of the VP1 and VP1/P2B subgenomic regions, the strains were classified as subgenotypes IA (VDM) and IB (JVR).[21,185] Nucleic acid from the stool suspensions were extracted using the QIAamp® Viral RNA Mini Kit (Qiagen) as per manufacturer's instructions. Extracted RNA was eluted into 60 µL elution buffer, aliquoted and stored at -70°C.

NGS: An anti-HAV IgM positive serum, collected in the Western Cape for the purpose of a previous study,[21] was subjected to NGS. The specimen was chosen because in the previous study two consecutive serum specimens were collected from the same patient and phylogenetic analyses revealed minor genetic difference between the strains detected in the two sera. Due to specimen availability, the HAV strain from the second serum specimen could not be characterised by NGS. Nucleic acid was extracted from 140 µL of the first serum specimen and eluted in 60 µL using the QIAamp® Viral RNA Mini Kit (Qiagen) following manufacturer's instructions. The extracted RNA was

aliquoted and stored at -70°C until complementary DNA (cDNA) synthesis. Two additional nucleic acid extractions were performed on 400 µL sera each and eluted in 40 µL. The second extraction was performed using the MagMAX™ Viral RNA Isolation Kit (Applied Biosystems™), following the manufacturer's instructions. Nucleic acid from the second extraction were immediately pooled, purified, concentrated and used for double-stranded cDNA (ds-cDNA) synthesis.

5.2.2 Viral characterisation

5.2.2.1 Sanger sequencing

The JVR strain nucleic acid and nuclease-free water (Promega Corp.) were used as positive and negative controls, respectively. They were used throughout characterisation by Sanger sequencing, from cDNA synthesis to amplification of subgenomic regions and visualisation of amplicons by gel electrophoresis.

Synthesis of cDNA: Complementary DNA (20 µL) was synthesised from 10 µL of nucleic acid extracted before and after viability treatment of the HAV positive wastewater samples. The synthesis was performed using random hexamers primers (Roche Diagnostics) and the Protoscript® II Reverse Transcriptase (New England Biolabs®, Ipswich, MA). The following modifications were made to the original cDNA synthesis protocol given by the manufacturer: 30 µM random hexamers primers were used instead of 6 µM, 50 U Protoscript® II Reverse Transcriptase were used instead of 200 U and 20 U RNase inhibitor were used instead of 8 U. The synthesised cDNA was used immediately for the amplification, by PCR, of the VP1 and VP1/P2B genomic regions.

Amplification of subgenomic regions: The entire VP1 region (900 nt) and a portion (350 nt) of the VP1/P2B junction were each amplified in two rounds of conventional PCR using the EmeraldAmp® MAX HS PCR Master Mix (Takara Bio Inc. Shiga, Japan) as well as published primers (Table 5.1).[14,29]

Table 5.1: Nucleotide sequences of primers used to amplify the VP1 and VP1/P2B genomic regions[14,29]

Genomic region	PCR	Primer's name	Nucleotide sequence (5'-3')
VP1	First round	HAV1 (Forward)	gTT TTg CTC CTC TTT ATC ATg CTA Tg
		HAV2 (Reverse)	AgT CAC ACC TCT CCA ggA AAA CTT
	Second round	2172P (Forward)	gCT CCT CTT TAT CAT gCT ATg gAT
		3125N (Reverse)	CCT gCA TTC TAT ATg ACT CT
VP1/P2B	First round	2870P (Forward)	gAC AgA TTC TAC ATT Tgg ATT ggT
		3381N (Reverse)	CCA TTT CAA gAg TCC ACA CAC T
	Second round	2896P (Forward)	CTA TTC AgA TTg CAA ATA CAA T
		3289N (Reverse)	AAC TTC ATT ATT TCA TgC TCC T

The reaction mix used for the first round amplification of the VP1 region was 50 µL and contained the following: 5 µL cDNA, 25 µL EmeraldAmp® MAX HS PCR Master Mix (2x Premix) (Takara Bio Inc.), 1 µL HAV1 forward primer (0.2 µM final concentration), 1 µL HAV2 reverse primer (0.2 µM final concentration) and 18 µL nuclease-free water (Promega Corp.). The conditions for amplification were: 30 cycles of 98°C for 10 s, 50°C for 30 s, 72°C for 1 min, and, final extension at 72°C for 5 min. The second round PCR was performed using 2172P and 3125N primers (Table 5.1) and 1 µL of the completed first round PCR. The amplification conditions of the second round PCR were the same as the first round PCR except for the annealing temperature that was decreased from 50°C to 48°C.

The reaction mix used to amplify the VP1/P2B junction had a total volume of 50 µL and included the following: 5 µL cDNA, 25 µL EmeraldAmp® MAX HS PCR Master Mix (2x Premix) (Takara Bio Inc.), 1 µL 2870P (0.2 µM final concentration), 1 µL 3381N (0.2 µM final concentration) and 18 µL nuclease-free water (Promega Corp.). The first round PCR conditions were: 30 cycles of 98°C for 10 s, 45°C for 45 s, 72°C for 30 s, and, final extension at 72°C for 5 min. The primers 2896P and 3289N (Table 5.1) together with 1 µL of the completed first round PCR were used for the second round of amplification of the VP1/P2B junction. The amplification conditions were the same as the second round PCR conditions used for the VP1 region.

Cloning and nucleotide sequencing of positive amplicons: The results of the VP1 and VP1/P2B amplifications were analysed using a 2% agarose gel (SeaKem® LE Agarose, Lonza) electrophoresis. Amplicons were visualised after staining the gel with ethidium bromide followed by UV illumination. Positive amplicons were cleaned up using the Zymogen DNA Clean & Concentrator-25™ Kit (Zymo Research, Irvine, CA).

The positive amplicons were cloned using the CloneJET PCR cloning Kit (Thermo Fisher Scientific). The only modification made to the manufacturer's instructions was that the ligation reaction mix was halved. In the case where the cloning failed the process was repeated using a full ligation reaction mix. A minimum of seven colonies were randomly selected for colony PCR using the One *Taq*® Quick-Load® 2X Master Mix with Standard Buffer (New England Biolabs). The 20 µL reaction mix for colony PCR contained the following: 10 µL of One *Taq*® Quick-Load® 2X Master Mix with Standard Buffer (New England Biolabs), 0.4 µL pJET1.2 forward primer (10 µM final concentration) (Thermo Fisher Scientific), 0.4 µL pJET1.2 reverse primer (10 µM final concentration) (Thermo Fisher Scientific) and 9.2 µL nuclease-free water (Promega Corp.). The amplification conditions of the colony PCR assay were: pre-denaturation at 94°C for 30 s, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, 68°C for 1 min 30 s, and, final extension at 68°C for 5 min. The expected size of the colony PCR products was 1025 bp (125 bp from the vector and 900 bp of the insert) and 475 bp (125 bp from the vector and 350 bp of the insert) corresponding to the VP1 and VP1/P2B genomic regions, respectively. After analysing the amplicons using a 2% agarose gel electrophoresis, approximately 5 clones containing the correct insert were cleaned up using the Zymogen DNA Clean & Concentrator-25™ Kit (Zymo Research). Additional clones, with inserts that were approximately 200 bp larger or smaller than the expected size of the colony PCR products, were also cleaned up and sequenced.

The positive amplicons obtained after amplification of the VP1 and VP1/P2B genomic regions and the purified products from colony PCR were subjected to Sanger sequencing. The second round PCR primers (Table 5.1) were used to sequence the amplified VP1 and VP1/P2B genomic regions, while the pJET

primers (Thermo Fisher Scientific) were used to sequence the purified products from colony PCR. The sequencing was performed in both the forward and reverse direction using the ABI Prism BigDye® Terminator v3.1 Cycle sequencing Kit on an ABI 3130 automated analyser (Applied Biosystems, Foster City, CA). The 20 µL sequencing reaction contained the following: 3 µL BigDye™ Terminator v3.1 5X Sequencing Buffer (1X final concentration), 1 µL BigDye™ Terminator v3.1 Ready Reaction Mix, 1 µL forward or reverse primer (3.2 µM final concentration), 3 µL clean PCR product and 12 µL nuclease-free water (Promega Corp.).

5.2.2.2 NGS

Preparation of wastewater samples: Due to sample availability pools of RNA (100 to 200 µL total volume), extracted after viability treatment were created for WWTP1 samples (Table 5.2). For WWTP4, RNA was only pooled from the sewage samples. A single treated discharge sample from WWTP4, collected in August, was submitted for NGS (Table 5.2).

Table 5.2: Names of wastewater samples pooled for NGS.

	Sample name	**Sample type**	**Name of pooled samples**	**Sampling month**
WWTP1	DEc	[a]E	DE4, DE6, DE7, DE8, DE9, DE10, DE11, DE1, DE2, DE3	April, June, July, August, September, October, November, January, February, March
	DR	[b]R	DR4, DR6, DR7, DR8, DR9, DR10, DR1, DR2, DR3	April, June, July, August, September, October, January, February, March
WWTP4	FE8	E	FE8	August
	FR	R	FR6, FR3, FR1	June, March, January

a: E = treated wastewater discharge
b: R = sewage

The pooled RNA was purified and concentrated using Agencourt® RNAClean™ XP (Beckman Coulter™ Life Sciences, Beckman Coulter Inc. CA) according to manufacturer's instructions. Concentrated RNA (13 µL for each synthesis) was used to perform multiple (two to four) synthesis of ds-cDNA per pool of RNA. The HAVRS (5'- TAT TTA CTg ATA AAA gAA ATA AAC-3') reverse transcription primer[186] and the Maxima H Minus Double-stranded cDNA Synthesis Kit (Thermo Fisher Scientific) were used for the syntheses, which were performed according to manufacturer's instructions. The reverse transcriptase step was performed at 50°C and at 60°C for WWTP1 and WWTP4 samples, respectively. The synthesised ds-cDNAs were purified and concentrated with Agencourt® AMPure XP beads (Beckman Coulter™ Life Sciences) at a ratio of 1.8X according to manufacturer's instructions. The newly purified and concentrated ds-cDNA were pooled by sample and stored at -20°C until further analysis. The pool of ds-cDNA corresponding to the treated discharge sample of WWTP1 was further concentrated with Agencourt® AMPure XP beads (Beckman Coulter™ Life Sciences) at a ratio of 0.75X.

The single treated discharge sample from WWTP4 was prepared for NGS similarly to the pooled wastewater samples. The RNA was also purified and concentrated with Agencourt® RNAClean™ XP (Beckman Coulter™ Life Sciences), but a single ds-cDNA synthesis reaction was performed. The synthesised ds-cDNA was also purified and concentrated using Agencourt® AMPure XP beads (Beckman Coulter™ Life Sciences) at a ratio of 1.8X.

Four overlapping fragments, encompassing a portion of the 5'UTR and the entire ORF of the HAV genome, were amplified in two rounds of PCR using the EmeraldAmp® MAX HS PCR Master Mix (Takara Bio Inc.) and published primers (Table 5.3).[187] The reaction volume of the first round PCR was 50 µL and contained the following: 5 µL ds-cDNA, 25 µL EmeraldAmp® MAX HS PCR Master Mix (2x Premix) (Takara Bio Inc.), 1 µL forward primer (0.2 µM final concentration), 1 µL reverse primer (0.2 µM final concentration) and 18 µL nuclease-free water (Promega Corp.). The components of the second round PCR were essentially the same as the first round, except that 1 µL of the completed first round PCR was used as template and the volume of nuclease-

free water (Promega Corp.) was adjusted accordingly to obtain the 50 µL reaction volume. The cycling conditions for both rounds of PCR were the same. These amplifications and the expected size of each fragment are indicated on Table 5.3.

Table 5.3: Primers used to amplify the four overlapping fragments and their amplification conditions.[187]

Fragment number	Primer's name	Nucleotide sequence (5'-3')	PCR cycling conditions		Expected fragment size
1	HAV-For4	TAC CTC ACC gCC gTT TgC CTA ggC	98°C – 10 s 54°C - 30 s 72°C - 2 min	30 cycles	1906 bp
	HAV-1969-Rev	ATR TCC ATC ACT gCA CAA ggA gC	72°C - 5 min		
2	HAV-1000-For	TgA TTC ATT CTg CAg ATT ggC TTA CT	98°C – 10 s 52°C - 30 s 72°C - 3 min	30 cycles	2981 bp
	HAV-3980-Rev	WTC TCA RCT CCA TCA TTC TAg AgT CC	72°C - 5 min		
3	HAV-For2	gCC gWT gAT ACT CCT Tgg gT	98°C – 10 s 48°C - 30 s 72°C - 4 min 10 s	30 cycles	4001 bp
	HAV-Rev2	CTA gCA TCA RAA gCA gAg AAA TC	72°C - 5 min		
4	HAV-For3	ACg CTT TTT AgA AAg AgT CCM AT	98°C – 10 s 42°C - 30 s 72°C - 1 min 30 s	30 cycles	1471 bp
	HAV-Rev3	ATA AAA gAA ATA AAC AAA CCT CA	72°C - 5 min		

The amplified four overlapping fragments were analysed using a 2% agarose gel electrophoresis. The positive amplicons were sequenced using NGS.

Preparation of clinical specimens: Single-stranded cDNA was synthesised with random hexamers primers (Roche Diagnostics) and with the HAVRS reverse transcription primer[186] using nucleic acid extracted from 140 µL sera. The cDNA synthesised using random hexamers primers (Roche Diagnostics) was performed as previously described (section 5.2.2.1). The only difference between the protocols for cDNA synthesised with random hexamers primers and with HAVRS reverse transcription primer, is the concentration of primer used. With the HAVRS primer, 20 pmol was used for the synthesis. The cDNA synthesised was used immediately to amplify the four overlapping fragments

The pool of nucleic acid, from the second extraction of the clinical specimen was purified and concentrated to 40 µL using Agencourt® RNAClean™ XP (Beckman Coulter™ Life Sciences). Two ds-cDNA synthesis were performed

using 13 µL of the concentrated RNA each. The reverse transcriptase step for the two synthesis was performed at 50°C. The two synthesis were then pooled, purified and concentrated with Agencourt® AMPure XP beads (Beckman Coulter™ Life Sciences) at a ratio of 1.8X. The purified and concentrated ds-cDNA was stored at -20°C until further analysis.

Amplification of the four overlapping fragments from the cDNA and ds-cDNA were performed as mentioned during preparation of wastewater samples for NGS, with one difference. Before being used as a template, the first round of PCR was concentrated with Agencourt® AMPure XP beads (Beckman Coulter™ Life Sciences) at a ratio of 0.75X. After elution in 10 µL nuclease-free water (Promega Corp.), 5 µL of the concentrated first round PCR was used as template for the second round PCR. Following analysis with a 2% agarose gel electrophoresis, the positive amplicons were sent for NGS.

Amplicon sequencing: A total of 22 amplicons, amplified from five samples were submitted for NGS (Table 5.4) to the Sequencing Core Facility, NICD, Sandringham, SA. The amplicons were prepared and sequenced in three main steps: construction of DNA libraries, construction of sequencing libraries and sequencing run.

At the sequencing facility, the amplicons were first quantified using the Qubit® dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific) on a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific). The DNA libraries were constructed using the Nextera XT Sample Preparation Kit (Illumina, CA). Firstly, amplicons were fragmented and ligated to Illumina-compatible adapter sequences. The tagged and fragmented (tagmented) amplicons were amplified by a limited cycle PCR, to add index primers and common adapters. After amplification, Agencourt® AMPure XP beads (Beckman Coulter™ Life Sciences) was used to purify the DNA libraries two times and size select fragments at a ratio of 0.575X, then at a ratio of 0.75X. The constructed DNA libraries were further quantified on a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific) and their quality was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, CA). To construct the sequencing libraries, the DNA libraries were normalised to 2 nanomolar and pooled accordingly at equimolar concentration. Before being

loaded onto the reagent cartridge, the sequencing libraries were denatured with sodium hydroxide. Sequencing was performed using the 600 cycles MiSeq® Reagent Kit v3 (Illumina) on an Illumina MiSeq® instrument (Illumina).

Table 5.4: Samples submitted for NGS and the amplicons amplified from each sample.

	Sample name	Amplicon name	Amplicon size (bp)
Serum specimen	44S	A1cN	1906
		A2cN	2981
		A4cN	1471
		Ads3cN	4001
		Ads4cN	1474
		44S1N	[a]1500
		44S3N	4001
		44S4N	1471
WWTP1	DEc	DEc1N	1906
		DEc2N	2981
		DEc3N	4001
		DEc4N	1471
	DR	DR1N	2981
		DR2N	4001
		DR3N	1906
		DR4N	2981
WWTP4	FE8	FE83N	4001
		FE84N	1471
	FR	FR1N	1906
		FR2N	2981
		FR3N	4001
		FR4N	1471

a: amplicon is 406 bp less than the expected 1906 bp for the first overlapping fragment

5.2.3 Genetic analyses

5.2.3.1 Sanger sequencing

Processing raw sequence data: Raw sequences were analysed using Sequencher™ v4.10.1 (Gene Codes Corporation, Ann Arbor, MI) and BioEdit Sequence Alignment Editor (v6.0.5.2). The contigs were constructed from the forward and corresponding reverse sequences of each strain in Sequencher™ v4.10.1 (Gene Codes Corporation). BioEdit Sequence Alignment Editor (v6.0.5.2) was used to create two FASTA files of the contigs, one for the sequences representing the VP1 region and one for sequences representing the VP1/P2B junction. The identity of edited sequences was verified using the Basic Local Alignment Search Tool (BLAST) program[188] of GenBank and the

Hepatitis A Virus Genotyping tool v1.0 (available at https://www.rivm.nl/mpf/typingtool/hav/job/1899775064/).

Sequence alignments and Pairwise analyses: Multiple alignments of the verified nucleic acid sequences, together with reference sequences of HAV and closely matched sequences from the output BLAST search were created in MAFFT v7.110 (http://mafft.cbrc.jp/alignment/server/). South African HAV strains previously characterised from clinical and water sources, that were available in GenBank, were also included in the alignment. After being aligned, the nucleic acid sequences were checked manually, translated into protein in BioEdit Sequence Alignment Editor (v6.0.5.2) and compared to reference strains (Table 5.5) in order to detect any novel or previously described amino acid mutation. The position of recorded amino acid changes was relative to HAV HM175 strain (M14707). Pairwise comparison of the nucleotide sequences and amino acid sequences of characterised strains with that of HAV reference types and top hits BLAST search retrieved from GenBank were performed using the two-parameter model of Kimura[189] in MEGA X.[190] Nucleotide sequence identities expressed in percentage allowed identification of the genotype and/or subgenotype of characterised HAV strains.

Table 5.5: HAV strains used during nucleotide sequence analysis.

Genotype	Strain name	Geographical location	GenBank Accession number	Genomic region
IA	GBM	Germany	X75215	Full [e]CDS
IA	HAV5	Uruguay	EU131373	Full CDS
IA	[a,c]VDM	SA	U66489	VP1, VP1/P2B
IA	LU38/WT	China	AF357222	VP1, VP1/P2B
IA	H2	China	EF406357	VP1, VP1/P2B
IA	M2	Cuba	AY974170	VP1, VP1/P2B
IA	BCN31	Spain	HQ401230	VP1
IA	BCN143	Spain	HQ401264	VP1
IA	MSM08-09-StrainE	Spain	HQ401258	VP1
IA	MSM08-09-219	Spain	HQ401259	VP1
IA	[a]923200	SA	-	VP1/P2B
IA	[a]923359	SA	-	VP1, VP1/P2B
IA	USA/2018/V18S02010	USA	MN062165	Full CDS
-	USA/2018/V18S00348	USA	MH577314	Full CDS
-	USA/2017/V17S06806	USA	MH577313	Full CDS
IA, IB	FG	Italy	X83302	Full CDS
IA, IB	HAV-Arg/06	Argentina	HM769724	Full CDS
IB, IIB	9F94	France	AJ519487	CDS
-	AH2	-	AB020565	Full CDS
	E0-198	Uganda	MH685714	CDS
	Uru-3	Uruguay	AJ437166	VP1
IB	HM175	Australia	M14707	Full [a]CDS
IB	CFH-HAV	Sierra Leone	HQ246217	Full CDS
IB	MBB	North Africa	M20273	Full CDS
IB	HAF203	Brazil	AF268396	Full CDS
IB	Banglane2000	Thailand	LC128713	Full CDS
IB	HAV/Egy/BI-11/2015	Egypt	KX228694	Full CDS
IB	ETH/2016/P5	Ethiopia	MF621612	CDS
IB	L-A-1	China	AF314208	VP1, VP1/P2B
IB	BCN02	Spain	DQ504423	VP1
IB	BCN60	Spain	HQ401237	VP1
IB	BCN147	Spain	HQ401265	VP1
IB	BCN152	Spain	HQ401266	VP1
IB	[a,c]JVR	SA	U68692	VP1, VP1/P2B
IB	126	Germany	EU416264	VP1/P2B
IB	166	Germany	EU416265	VP1/P2B
IB	034	Germany	EU416266	VP1/P2B
IB	[b]SA-2009-Tom	SA	-	VP1/P2B
IB	[b]SA-2009-Let	SA	-	VP1/P2B
IB	[c]GP_PT14S, 23S, 25S, 26S, 39S, 107S, 117S, 118S, 120S, 129S, 133S,	Gauteng, SA	-	VP1

a: Taylor[185]
b: Netshikweta[17]
c: Saïd[21]
d: Rachida[22]
e: Coding sequence

Table 5.5: HAV strains used during nucleotide sequence analysis.

IB	[c]GP_PT15S, 39S, 107S, 108S, 117S, 118S, 120S, 129S, 132S, 133S,	Gauteng, SA	-	VP1/P2B
IB	[c]GP_66S-1, 67S-1, 106S-1, 109S-1, 110S-1, 111S-1, 112S-1, 112S2-1, 116S-1, 119S-1, 122S-1, 134S-1	Gauteng, SA	KJ433581 to KJ433592	VP1
IB	[c]GP_131-S1	Gauteng, SA	KJ207022	VP1
IB	[c]GP_PT66S, 67S, 106S, 109S, 110S, 111S, 112S, 112S2, 115S, 116S, 119S, 122S, 130S, 131S, 134S	Gauteng, SA	KJ492621 to KJ492635	VP1/P2B
IB	[c]GP_RV2-20120618-col1	Gauteng, SA	KJ492595	VP1
IB	[c]GP_RV2-20120618-col9	Gauteng, SA	-	VP1
IB	[c]GP_RV2-20120618-col2	Gauteng, SA	-	VP1
IB	[c]GP_RV2-20120618-col6	Gauteng, SA	-	VP1
IB	[c]GP_RV2-20120618-col8	Gauteng, SA	-	VP1
IB	[c]GP_RV2-20120723	Gauteng, SA	-	VP1
IB	[c]GP_RV2 20121112	Gauteng, SA	-	VP1
IB	[c]GP_RV2_20130218	Gauteng, SA	-	VP1
IB	[c]GP_11/1085-col6	Gauteng, SA	-	VP1
IB	[c]GP_11.1085-col7	Gauteng, SA	KJ492598	VP1
IB	[c]GP_11.1085-col8	Gauteng, SA	KJ492599	VP1
IB	[c]GP_11.1145-col1	Gauteng, SA	KJ492600	VP1
IB	[c]GP_11.1145-col2	Gauteng, SA	KJ492601	VP1
IB	[c]GP_11/1145-col7	Gauteng, SA	-	VP1
IB	[c]GP_RV2-20121112	Gauteng, SA	KJ492654	VP1/P2B
IB	[c]GP_RV2-20130318	Gauteng, SA	KJ492655	VP1/P2B
IB	[c]GP_K19-20130225	Gauteng, SA	KJ492656	VP1/P2B
IB	[c]GP_11.1051	Gauteng, SA	KJ492657	VP1/P2B
IB	[c]GP_11.1145	Gauteng, SA	KJ492658	VP1/P2B
IB	[c]GP_11.1147	Gauteng, SA	KJ492659	VP1/P2B
IB	[c]GP_11.1085	Gauteng, SA	KJ492660	VP1/P2B
IB	[c]SZ_PT126S	Swaziland	KJ492643	VP1/P2B
IB	[c]SZ_PT29S-1, PT126S-1	Swaziland	KJ433605, KJ433606	VP1
IB	[c]K_KD-FEB, [c]K_KD-NOV, [c]K_KD-DEC, [c]K_MB-JUL, [c]K_NR-AUG	Kenya	KJ492671 to KJ492675	VP1/P2B
IB	[c]K_PT24S	Kenya	KJ433603	VP1
IB	[c]K_KD-DEC-1, [c]K_KD-Feb-1	Kenya	KJ492607, KJ492608	VP1
IB	[c]T_PT34S-1	Tanzania	KJ433604	VP1
IC	BCN70	Spain	HQ401240	
IC	Shellfish08-106	Spain	HQ401253	
IIA	CF53/Berne	France	AY644676	Full CDS
IIB	SLF88	Sierra Leone	AY644670	Full CDS

a: Taylor[185]
b: Netshikweta[17]
c: Saïd[21]
d: Rachida[22]
e: Coding sequence

Table 5.5: HAV strains used during nucleotide sequence analysis.

IIIA	SIM27	India	FJ227135	Full CDS
IIIB	HA-JNG06-90F	Japan	AB258387	Full CDS
IV	Cy145	Philippines	M59286	CDS
V	AGM-27	Kenya	D00924	Full CDS
V	[d]KZN_Irr-20130530-1	KwaZulu-Natal, SA	KP875236	VP1
V	[d]KZN_Irr-20130530-2B	KwaZulu-Natal, SA	KP875241	VP1/P2B

a: Taylor[185]
b: Netshikweta[17]
c: Saïd[21]
d: Rachida[22]
e: Coding sequence

Phylogenetic analyses: The evolutionary history of detected HAV strains was inferred using the Neighbor-Joining method[191] in MEGA X.[190] The evolutionary distance between the detected strains and previously characterised HAV strains was computed using the Kimura 2-parameter method.[189] The constructed phylogenetic trees were assessed by bootstrap analyses (1000 replicates)[192] and a cut-off of 75% was considered significant. In addition to nucleotide identity percentages, the genotype of each strain was confirmed by clustering pattern(s) with HAV reference sequences.

5.2.3.2 *NGS*

The paired sequence reads were analysed using the Viral genome-targeted assembly pipeline (VirusTAP) which is a web-based platform that performs quality trimming and *de novo* assembly of viral genomes.[193] When *de novo* assembly failed, VirusTAP constructed contigs from the sequence reads. The identity and genomic position of each contig was verified using the HAV Genotyping Tool v1.0 (https://www.rivm.nl/mpf/typingtool/hav/how-to-cite). Using the BioEdit Sequence Alignment Editor (v6.0.5.2), a FASTA file containing all the contigs corresponding to a sample was created. Thereafter, the contigs were sorted by genomic regions and aligned using MAFFT v7.110. Consensus sequences were created for each genomic region, then aligned to obtain the nucleotide sequence encompassing the entire coding region of the HAV strain. To verify nucleotide sequence gaps and positions at which more than one base was recorded, a second consensus sequence was assembled

using the Unipro UGENE software v1.32.0.[194] For the second consensus sequence, the Burrows-Wheeler Alignment mapping tool[195] from the Unipro UGENE software v1.32.0[194] was used to map the raw sequence reads, without quality trimming, to the CFH-HAV strain (GenBank Accession number: HQ246217). The CFH-HAV strain was one of the top BLAST hits obtained during Sanger sequence analysis. The identity of the assembled genomes was further verified using the HAV Genotyping Tool v1.0 and BLAST-n tool from NCBI, and the protein sequence was manually checked using the BioEdit Sequence Alignment Editor (v6.0.5.2). After genome assembly, multiple sequence alignments using the coding sequence (CDS), the P1, VP1, VP1/P2B and 3CD genomic regions were generated as previously described (section 5.2.3.1). Pairwise and phylogenetic analysis of the CDS and subgenomic regions were performed using the generated sequence alignments (section 5.2.3.1). Potential recombinants were detected based on clustering pattern with different HAV reference strains (Table 5.5) over different genomic regions.

5.3 RESULTS

5.3.1 Sanger sequencing analyses

5.3.1.1 Pairwise analyses

Hepatitis A virus strains could be genotyped from 79% (68/86) and 84% (72/86) of wastewater samples, by nucleotide sequence analysis of the VP1 and VP1/P2B genomic regions, respectively. A total of 337 and 356 HAV strains were characterised by Sanger sequencing based on the VP1 and VP1/P2B genomic regions, respectively. Pairwise analyses, performed over the VP1 region, demonstrated that the strains detected in this study were >97% similar to the JVR strain at the nucleotide and amino acid levels (Table 5.6). However, analyses based on the VP1/P2BN genomic regions, recorded highest similarity to the HM175 strain at the nucleotide level (Table 5.7). Further analysis of the VP1/P2BN junction revealed that at the amino acid level, genetic identities, between the strains detected in this study and HAV IB strains (HM175, MBB, CFH-HAV and JVR strains), were similar (Table 5.7). Lowest genetic identities,

at the nucleotide and amino acid levels, were recorded during comparison to HAV genotype IV type strain Cy145 (Tables 5.6 and 5.7).

Table 5.6: Range of percentages of genetic identities over the VP1 region. Highest (green) and lowest (yellow) ranges are highlighted.

Genotype	Strain name	Nucleotide (%)	Amino acid (%)
IA	GBM	87.7 - 90.0	96.3 - 98.7
	VDM	87.6 - 90.2	97.0 - 99.3
IB	HM175	91.5 - 93.6	97.6 - 100
	MBB	93.5 - 91.5	97.3 - 99.7
	CFH-HAV	95.2 - 97.4	97.6 - 100
	JVR	97.3 - 99.2	97.6 - 100
IC	BCN70	88.2 - 89.9	97.6 - 100
IIA	CF53/Berne	80.6 - 84.7	96.6 - 99.0
IIB	SLF88	78.7 - 81.5	96.3 - 98.7
IIIA	SIM27	79.6 - 76.6	92.9 - 96.8
IIIB	HAJNG0690F	77.4 - 80.2	92.9 - 97.2
IV	Cy145	73.8 - 77.5	88.6 - 95.2
V	AGM27	75 - 81.3	91.4 - 95.2

Table 5.7: Range of percentages of genetic identities over the VP1/P2B junction. Highest (green) and lowest (yellow) ranges are highlighted.

Genotype	Strain name	Nucleotide (%)	Amino acid (%)
IA	GBM	86.9 - 90.8	93.8 - 97.4
	VDM	86.1 - 89.0	93.8 - 97.4
IB	HM175	90.3 - 95.6	94.7 - 99.1
	MBB	90.3 - 94.3	94.7 - 99.1
	CFH-HAV	89.7 - 96.2	94.7 - 99.1
	JVR	89.4 - 98.8	94.7 - 99.1
IIA	CF53/Berne	75.8 - 80.8	92.0 - 97.4
IIB	SLF88	78.8 - 82.0	92.0 - 97.4
IIIA	SIM27	69.7 - 73.5	84.3 - 89.2
IIIB	HAJNG0690F	73.5 - 77.9	86.3 - 91.1
IV	Cy145	68.3 - 75.3	78.3 - 82.6
V	AGM27	68.5 - 72.6	84.3 - 88.2

5.3.1.2 Phylogenetic analyses

Phylogenetic trees, based on nucleotide sequence analysis of the VP1 region, were constructed for HAV strains characterised from samples collected from WWTPs 1 (Figure 5.1), 2 (Figure 5.2), 3 (Figure 5.3), 4 (Figure 5.4) and 5 (Figure 5.5). The results of the analysis showed that the strains characterised from all five WWTPs formed a unique cluster ('SAn Major cluster') within genotype IB (Figures 5.1A to 5.5A). None of the strains detected clustered with HAV IA strains, e.g. VDM, or any other genotypes or subgenotypes. The 'SAn Major cluster' groups the strains detected in this study (indicated with [▲] or [Δ] on Figures 5.1B to 5.5B), together with HAV strains previously characterised from water and clinical sources collected in Gauteng (Figures 5.1B to 5.5B). The JVR strain is included in the 'SAn Major cluster', but it does not group with any of the strains analysed (Figures 5.1B to 5.5B). Hepatitis A virus characterised from samples collected in Swaziland (SZ_PT126S and SZ_PT29S) cluster within the 'SAn Major cluster', while strains from Kenya (K_PT24S, K_KD-Feb and K_KD-Dec) and Tanzania (T_PT34S) do not (Figures 5.1A and B to 5.5A and B). Within the SAn Major cluster, the strains characterised from samples collected from WWTPs 2, 3, 4 and 5 tend to group together and not with previously characterised HAV strains from Gauteng (Figures 5.2 to 5.5). The exception is a cluster of HAV strains from WWTP1 (highlighted in red on Figure 5.1B and supported with a 75% bootstrap value) that includes the strain GP_PT106 (highlighted in orange on Figure 5.1B), previously detected in a clinical specimen.

Based on nucleotide sequence analysis of the VP1/P2B junction, phylogenetic trees were constructed for HAV strains characterised from samples collected from WWTPs 1 (Figure 5.6), 2 (Figure 5.7), 3 (Figure 5.8), 4 (Figure 5.9) and 5 (Figure 5.10). The analysis also recorded SAn major clusters for HAV strains characterised from samples collected from all five WWTPs (Figures 5.6A to 5.8A, 5.9 and 5.10). Within these major clusters, the strains detected in this study do not cluster with previously characterised HAV strains (Figures 5.7, 5.8, 5.11A and 5.12A) except for HAV strains characterised from samples collected from WWTP1 (highlighted in red on Figure 5.6B) and three strains from WWTP4

(highlighted in red on Figure 5.11A). Several HAV strains, characterised from samples collected from WWTPs 2, 4 and 5, form minor clusters outside the 'SAn Major cluster', but still within subgenotype IB (Figures 5.7A, 5.9 and 5.10). All three minor clusters are supported by >75% bootstrap values (Figures 5.7A, 5.9 and 5.10) and include the clinical strains GP_PT66S, GP_PT130S and GP_PT134S from Gauteng (highlighted in orange on Figures 5.7A, 5.11B and 5.12B).

Following viability treatment, HAV was characterised from the virus concentrates of sewage and treated discharge samples collected from WWTP4. Phylogenetic analyses based on the VP1 region group the strains detected after viability treatment within the 'SAn Major cluster-WWTP4' (indicated with green and orange triangles on Figure 5.4B). However, analyses based on the VP1/P2B junction revealed that most strains detected in the sewage and treated discharge samples cluster within the 'SAn minor cluster-WWTP4' (Figure 5.10B).

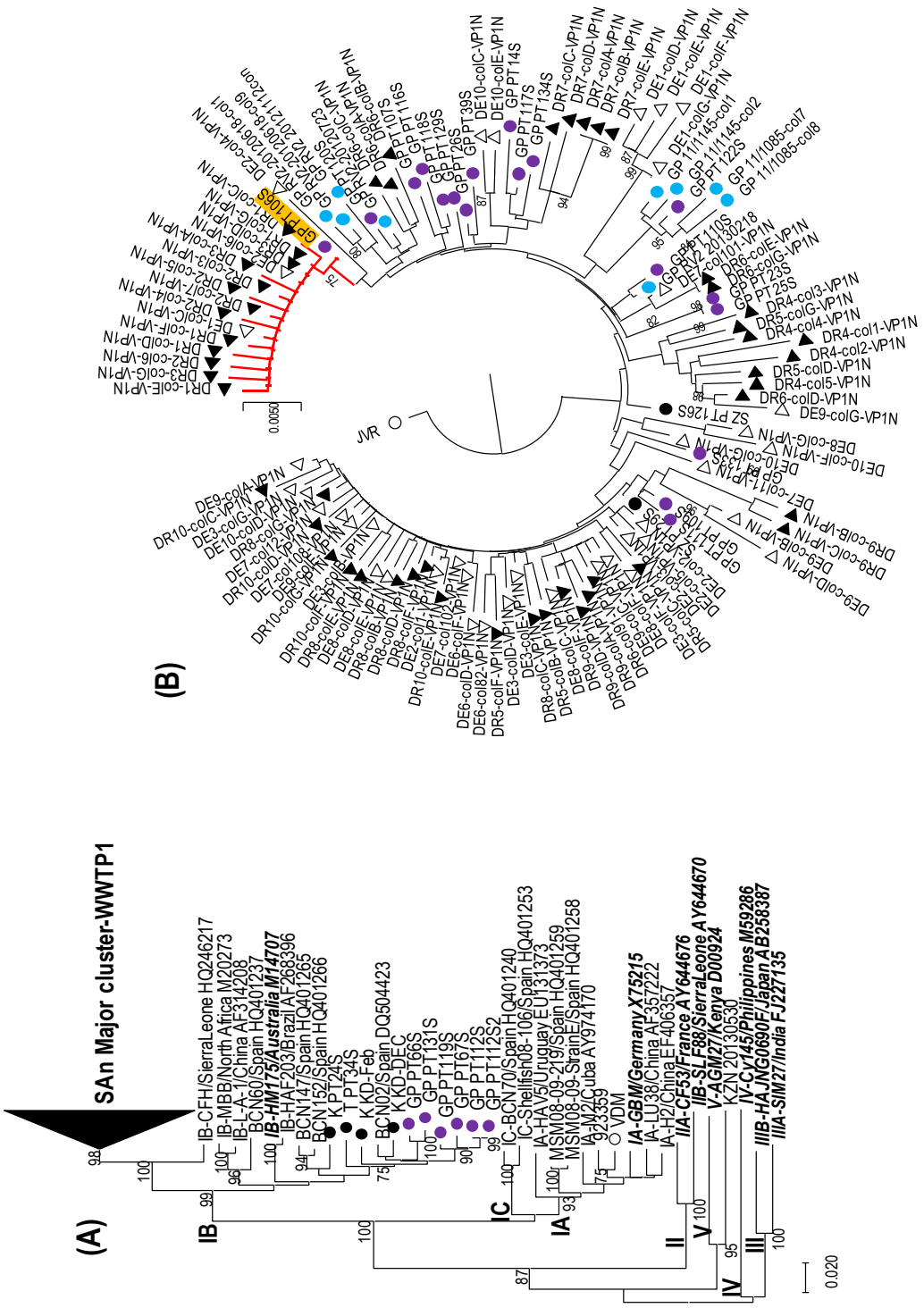


Figure 5.1: Phylogenetic analysis targeting the VP1 region of HAV strains detected in WWTP1 samples. The main tree (A) and 'SAn Major cluster-WWTP1' subtree (B) are shown. The strains analysed from sewage and treated discharge samples are indicated with ▲ and ∆, respectively. Previously characterised HAV strains from water (●) and clinical (●) sources in Gauteng and from other countries (●) are indicated. The VDM (○) and JVR (○) strains are shown. Reference strains retrieved from GenBank have accession number next to their name. The name of type strains for each genotype and subgenotype are bolded and italicised. The neighbour-joining tree was constructed using the Kimura two-parameter model. Bootstrap values greater or equal to 75% are shown at the nodes.

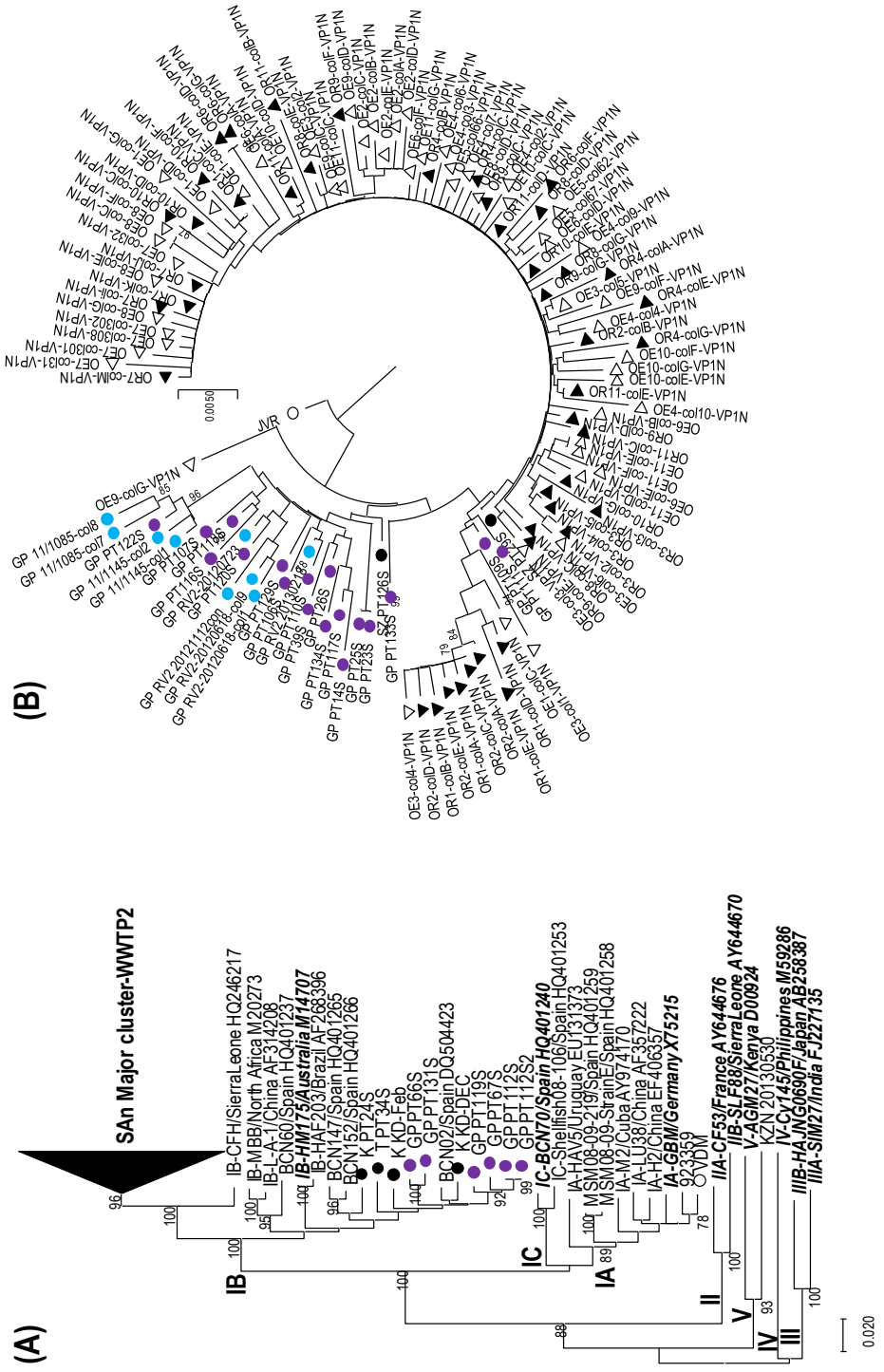


Figure 5.2: Phylogenetic analysis targeting the VP1 region of HAV strains detected in WWTP2 samples. The main tree (A) and 'SAn Major cluster-WWTP2' subtree (B) are shown. The strains analysed from sewage and treated discharge samples are indicated with ▲ and ∆, respectively. Previously characterised HAV strains from water (●) and clinical (●) sources in Gauteng and from other countries (●) are indicated. The VDM (○) and JVR (○) strains are shown. Reference strains retrieved from GenBank have accession number next to their name. The name of type strains for each genotype and subgenotype are bolded and italicised. The neighbour-joining tree was constructed using the Kimura two-parameter model. Bootstrap values greater or equal to 75% are shown at the nodes.

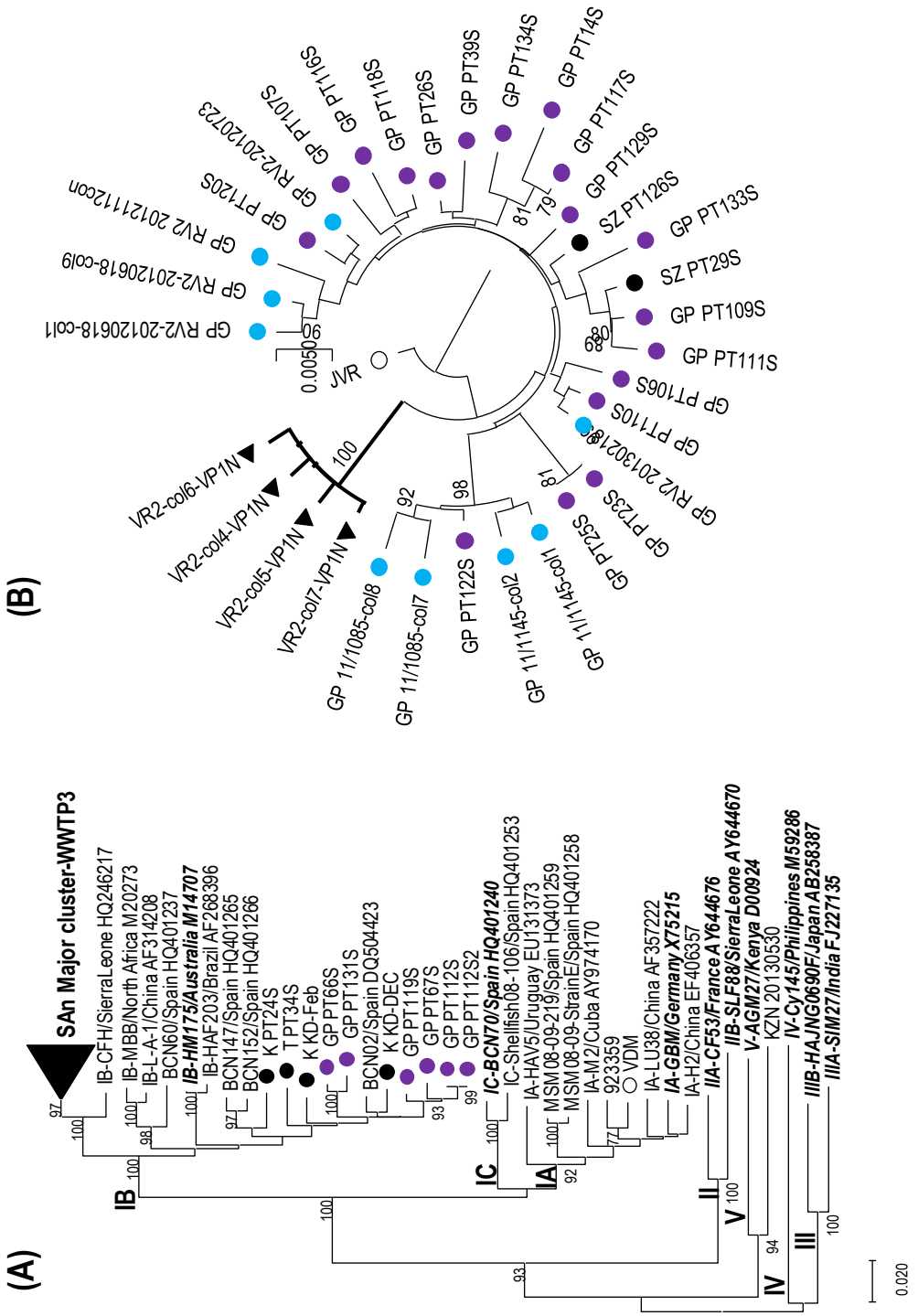


Figure 5.3: Phylogenetic analysis targeting the VP1 region of HAV strains detected in WWTP3 samples. The main tree (A) and 'SAn Major cluster-WWTP3' subtree (B) are shown. The strains analysed from sewage are indicated with ▲. Previously characterised HAV strains from water (●) and clinical (●) sources in Gauteng and from other countries (●) are indicated. The VDM (○) and JVR (○) strains are shown. Reference strains retrieved from GenBank have accession number next to their name. The name of type strains for each genotype and subgenotype are bolded and italicised. The neighbour-joining tree was constructed using the Kimura two-parameter model. Bootstrap values greater or equal to 75% are shown at the nodes.

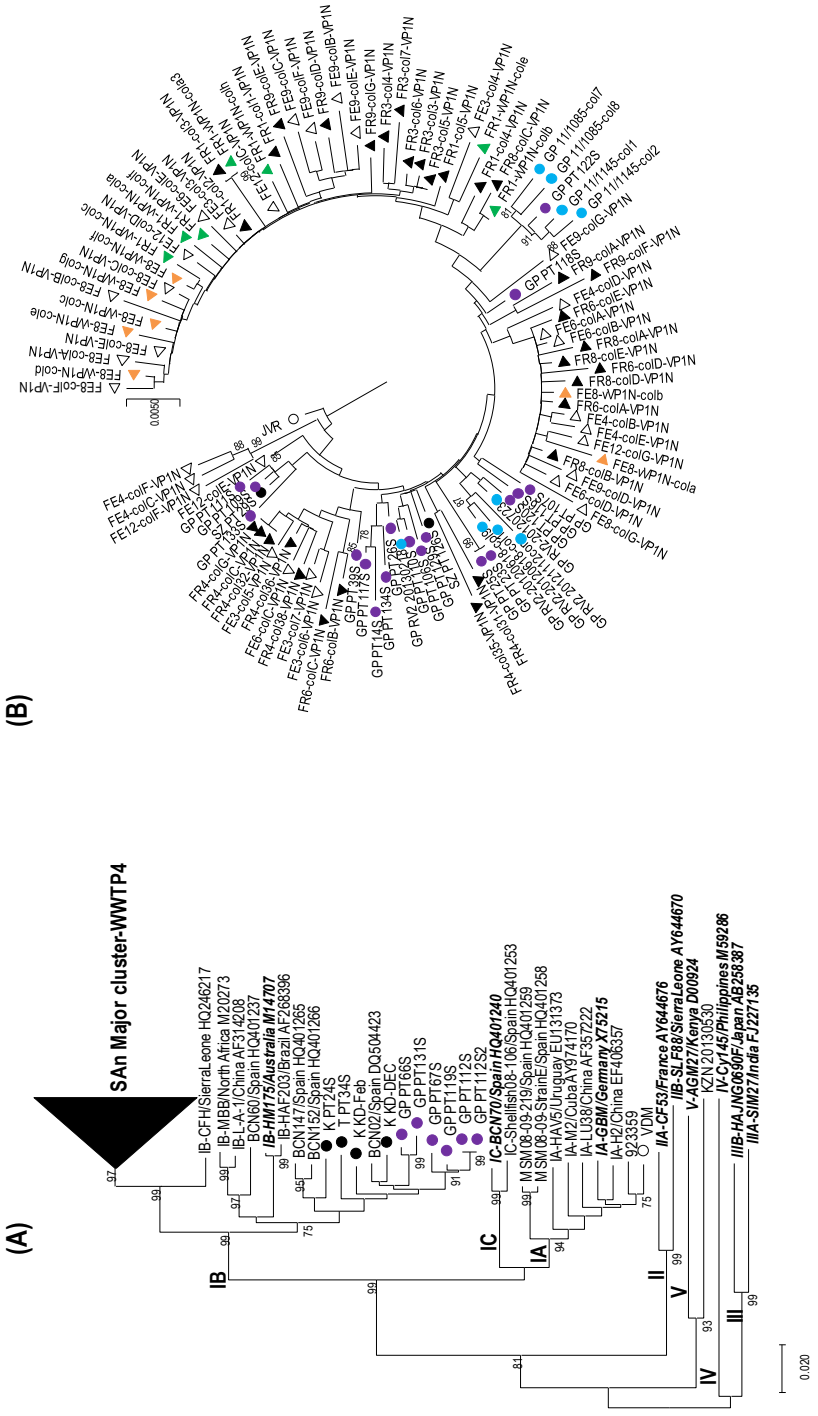


Figure 5.4: Phylogenetic analysis targeting the VP1 region of HAV strains detected in WWTP4 samples. The main tree (A) and 'SAn Major cluster-WWTP4' subtree (B) are shown. The strains analysed from sewage and treated discharge samples are indicated with ▲ and ∆, respectively. HAV strains detected after viability treatment are indicated with ▲ (sewage) and ▲ (treated discharge). Previously characterised HAV strains from water (●) and clinical (●) sources in Gauteng and from other countries (●) are indicated. The VDM (○) and JVR (○) strains are shown. Reference strains retrieved from GenBank have accession number next to their name. The name of type strains for each genotype and subgenotype are bolded and italicised. The neighbour-joining tree was constructed using the Kimura two-parameter model. Bootstrap values greater or equal to 75% are shown at the nodes.

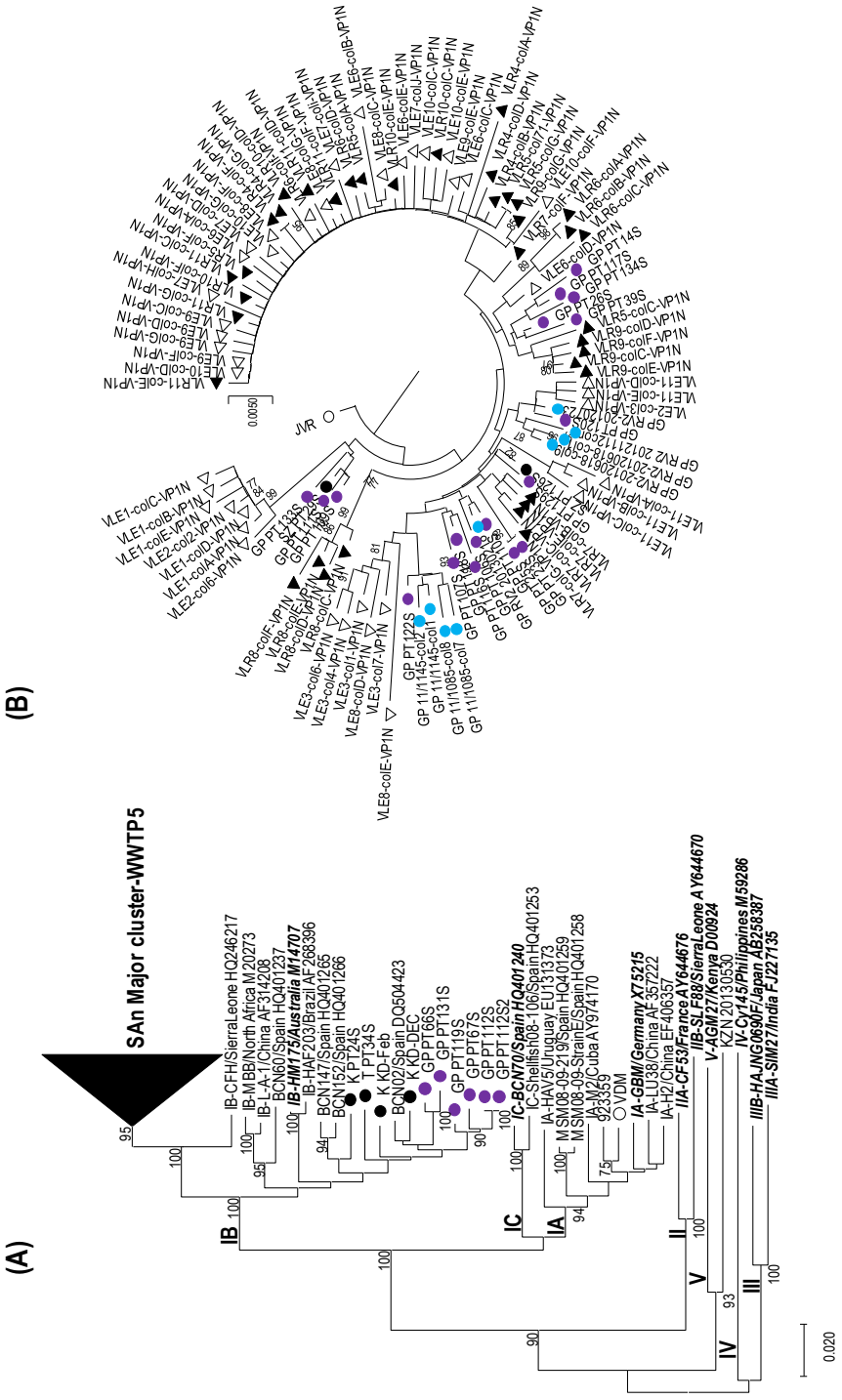


Figure 5.5: Phylogenetic analysis targeting the VP1 region of HAV strains detected in WWTP5 samples. The main tree (A) and 'SAn Major cluster-WWTP5' subtree (B) are shown. The strains analysed from sewage and treated discharge samples are indicated with ▲ and ∆, respectively. Previously characterised HAV strains from water (●) and clinical (●) sources in Gauteng and from other countries (●) are indicated. The VDM (○) and JVR (○) strains are shown. Reference strains retrieved from GenBank have accession number next to their name. The name of type strains for each genotype and subgenotype are bolded and italicised. The neighbour-joining tree was constructed using the Kimura two-parameter model. Bootstrap values greater or equal to 75% are shown at the nodes.

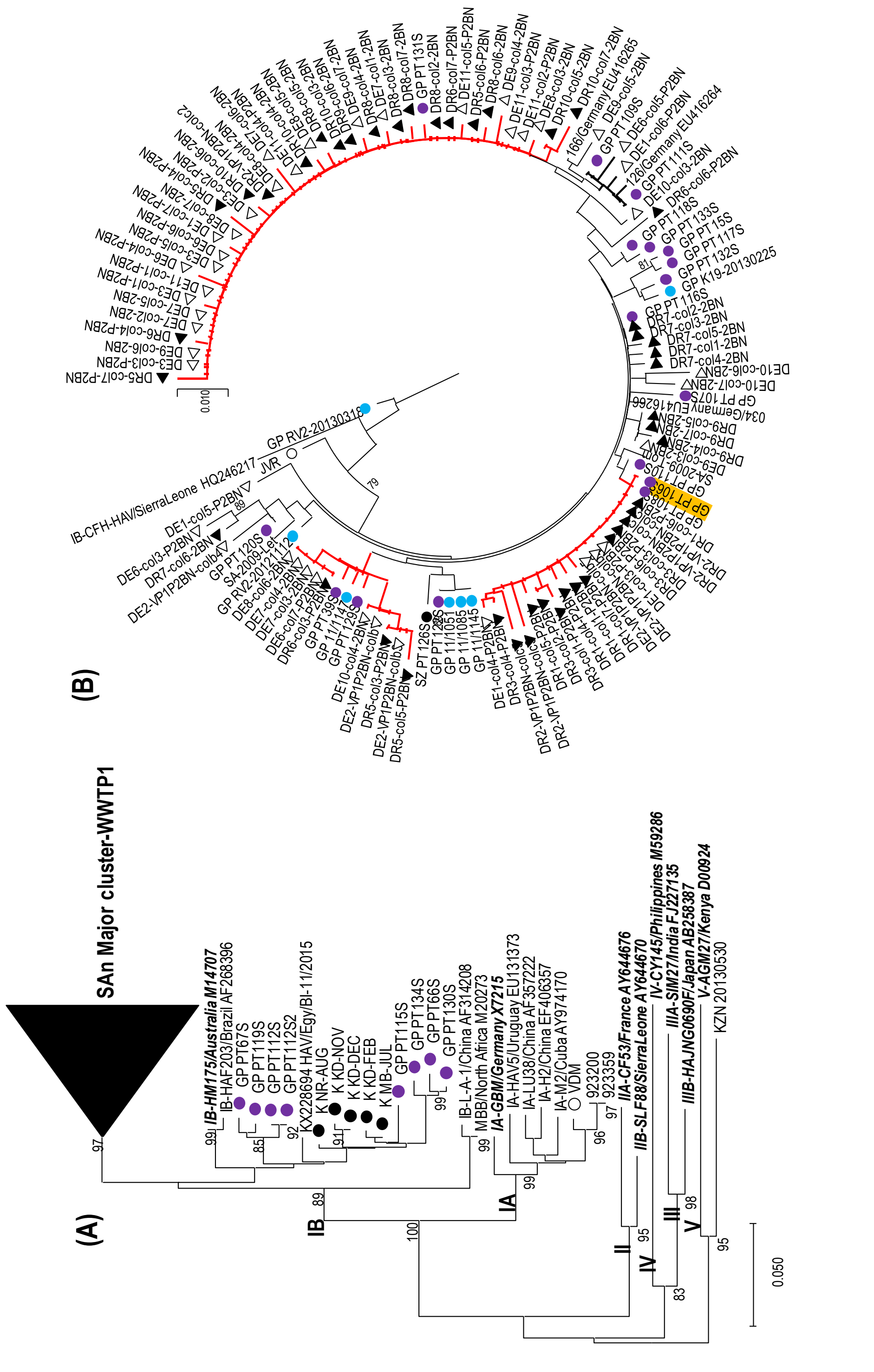


Figure 5.6: Phylogenetic analysis targeting the VP1/P2B junction of HAV strains detected in WWTP1 samples. The main tree (A) and ‘SAn Major cluster-WWTP1’ subtree (B) are shown. The strains analysed from sewage and treated discharge samples are indicated with ▲ and ∆, respectively. Previously characterised HAV strains from water (●) and clinical (●) sources in Gauteng and from other countries (●) are indicated. The VDM (○) and JVR (○) strains are shown. Reference strains retrieved from GenBank have accession number next to their name. The name of type strains for each genotype and subgenotype are bolded and italicised. The neighbour-joining tree was constructed using the Kimura two-parameter model. Bootstrap values greater or equal to 75% are shown at the nodes.

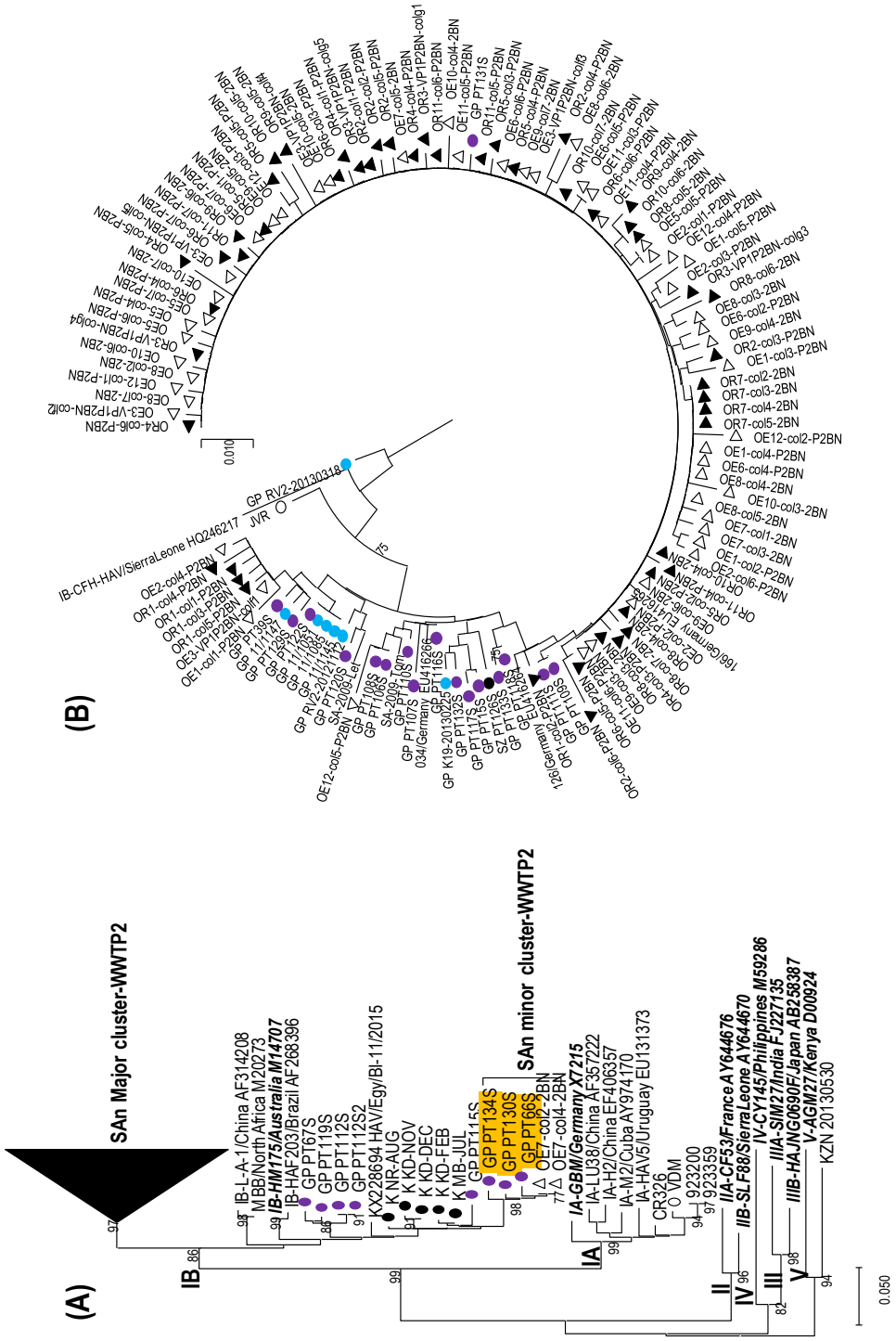


Figure 5.7: Phylogenetic analysis targeting the VP1/P2B junction of HAV strains detected in WWTP2 samples. The main tree (A) and 'SAn Major cluster-WWTP2' subtree (B) are shown. The strains analysed from sewage and treated discharge samples are indicated with ▲ and ∆, respectively. Previously characterised HAV strains from water (●) and clinical (●) sources in Gauteng and from other countries (●) are indicated. The VDM (○) and JVR (○) strains are shown. Reference strains retrieved from GenBank have accession number next to their name. The name of type strains for each genotype and subgenotype are bolded and italicised. The neighbour-joining tree was constructed using the Kimura two-parameter model. Bootstrap values greater or equal to 75% are shown at the nodes.

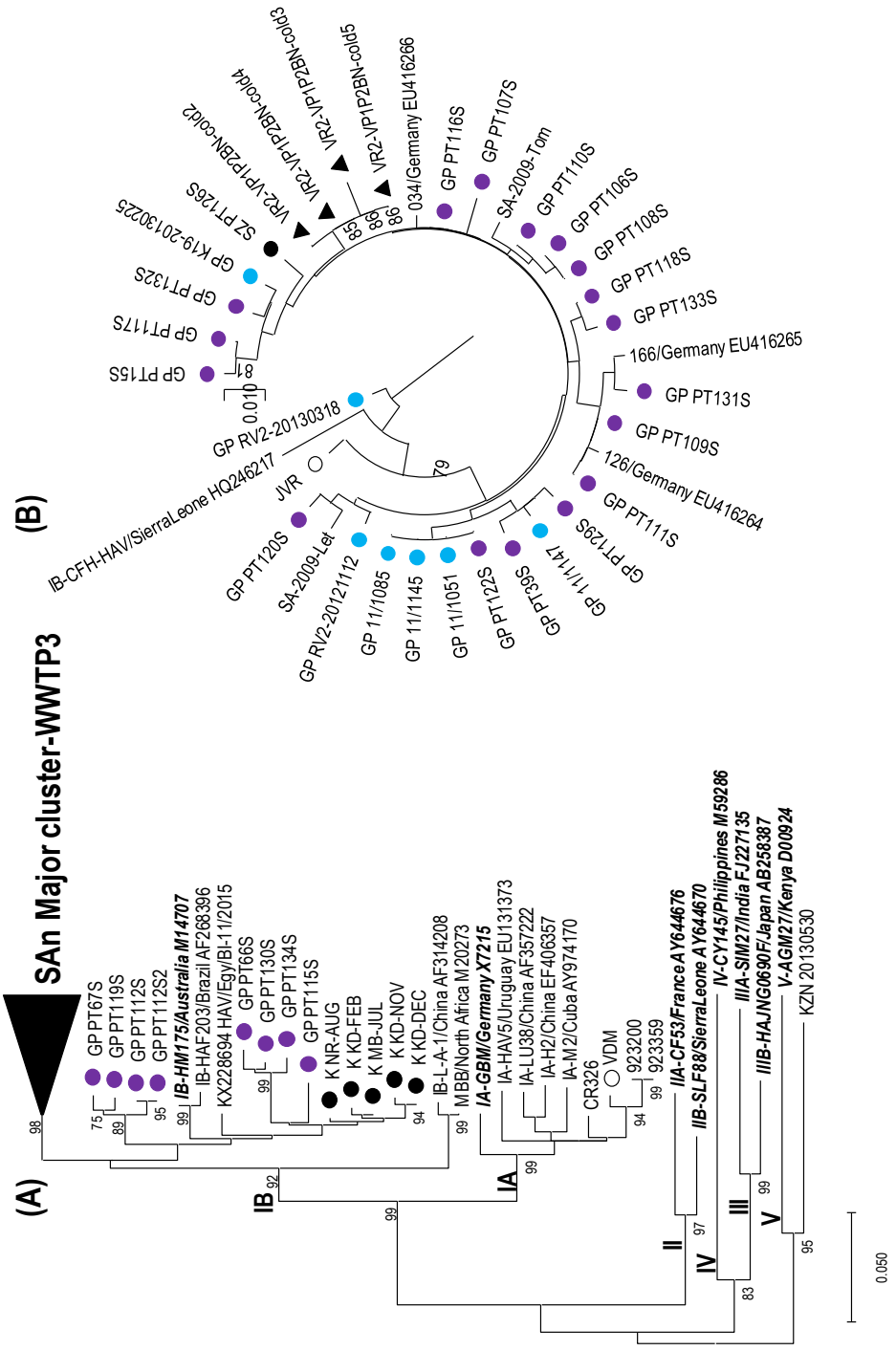


Figure 5.8: Phylogenetic analysis targeting the VP1/P2B junction of HAV strains detected in WWTP3 samples. The main tree (A) and 'SAn Major cluster-WWTP3' subtree (B) are shown. The strains analysed from sewage are indicated with ▲. Previously characterised HAV strains from water (●) and clinical (●) sources in Gauteng and from other countries (●) are indicated. The VDM (○) and JVR (○) strains are shown. Reference strains retrieved from GenBank have accession number next to their name. The name of type strains for each genotype and subgenotype are bolded and italicised. The neighbour-joining tree was constructed using the Kimura two-parameter model. Bootstrap values greater or equal to 75% are shown at the nodes.

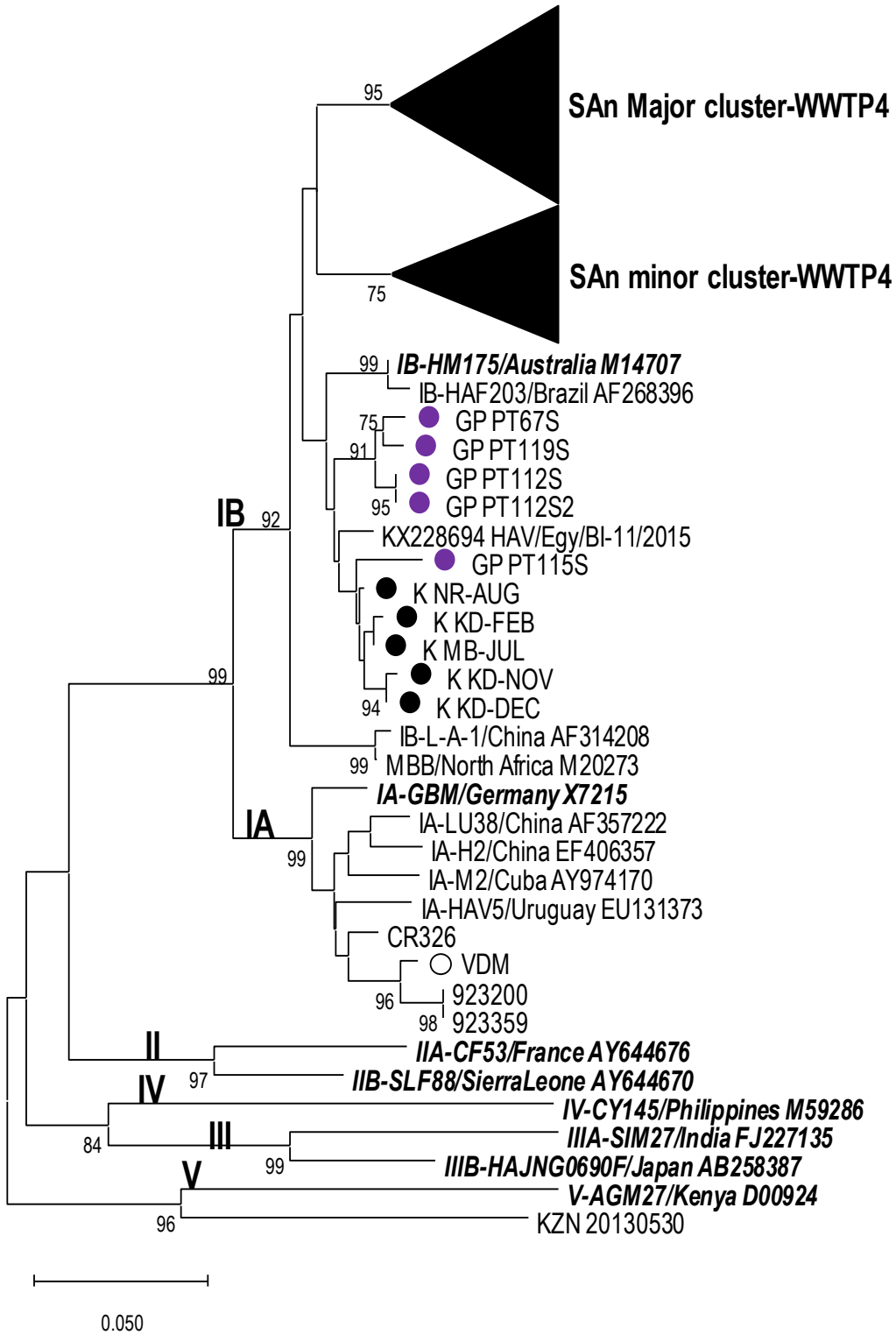


Figure 5.9: Phylogenetic analysis targeting the VP1/P2B junction of HAV strains detected in WWTP4 samples. Previously characterised HAV strains from clinical (●) sources in Gauteng and from other countries (●) are indicated. The VDM (○) strain is shown. Reference strains retrieved from GenBank have accession number next to their name. The name of type strains for each genotype and subgenotype are bolded and italicised. The neighbour-joining tree was constructed using the Kimura two-parameter model. Bootstrap values greater or equal to 75% are shown at the nodes.

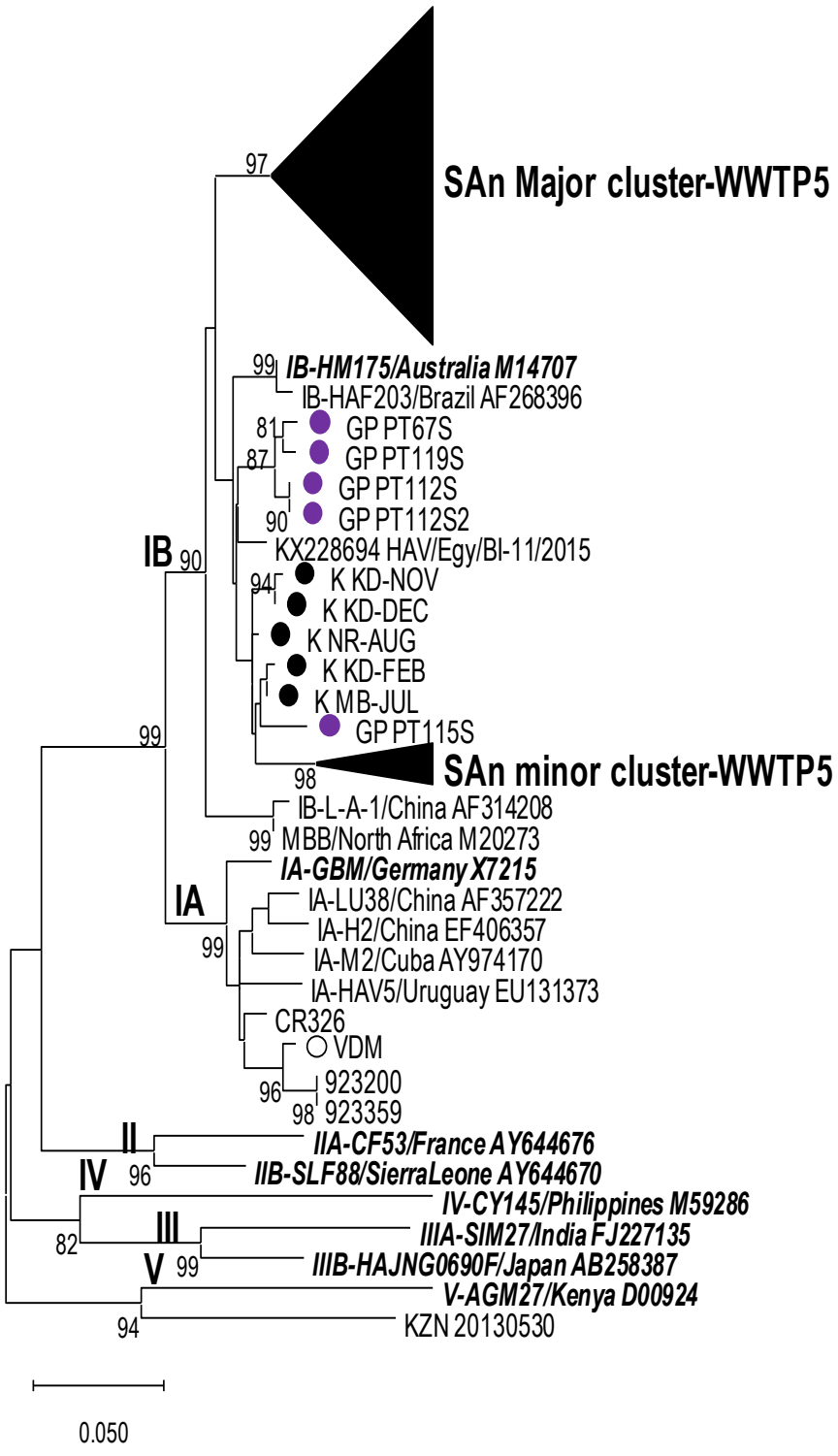


Figure 5.10: Phylogenetic analysis targeting the VP1/P2B junction of HAV strains detected in WWTP5 samples. Previously characterised HAV strains from clinical (●) sources in Gauteng and from other countries (●) are indicated. The VDM (○) strain is shown. Reference strains retrieved from GenBank have accession number next to their name. The name of type strains for each genotype and subgenotype are bolded and italicised. The neighbour-joining tree was constructed using the Kimura two-parameter model. Bootstrap values greater or equal to 75% are shown at the nodes.

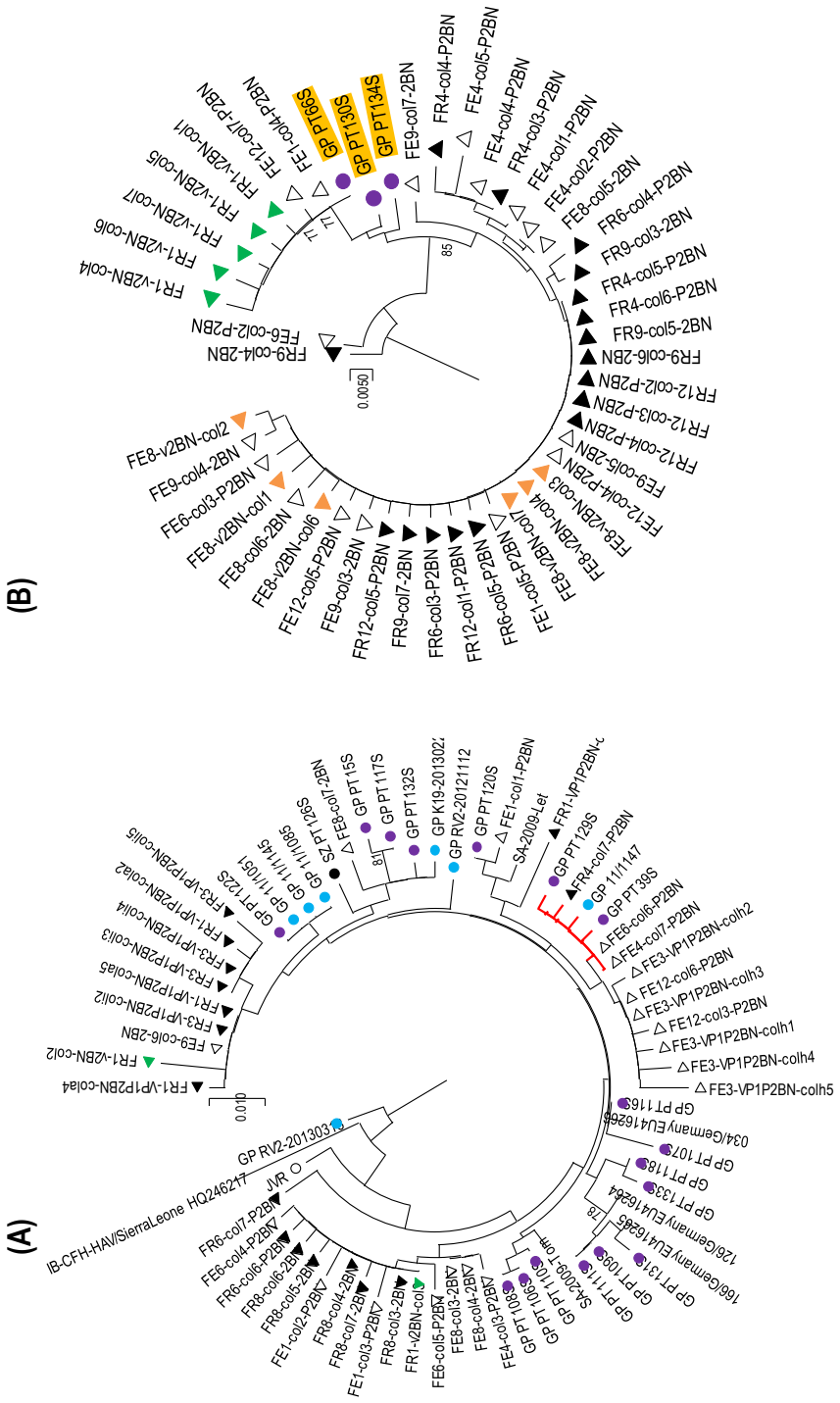


Figure 5.11: Phylogenetic analysis targeting the VP1/P2B junction of HAV strains detected in WWTP4 samples - the tree highlights strains from 'SAn Major cluster-WWTP4' (A) and 'SAn minor cluster-WWTP4' (B). The strains analysed from sewage and treated discharge samples are indicated with ▲ and ∆, respectively. HAV strains detected after viability treatment are indicated with ▲ (sewage) and ▲ (treated discharge). Previously characterised HAV strains from water (●) and clinical (●) sources in Gauteng and from other countries (●) are indicated. The JVR (○) strain is shown. Reference strains retrieved from GenBank have accession number next to their name. The neighbour-joining tree was constructed using the Kimura two-parameter model. Bootstrap values greater or equal to 75% are shown at the nodes.

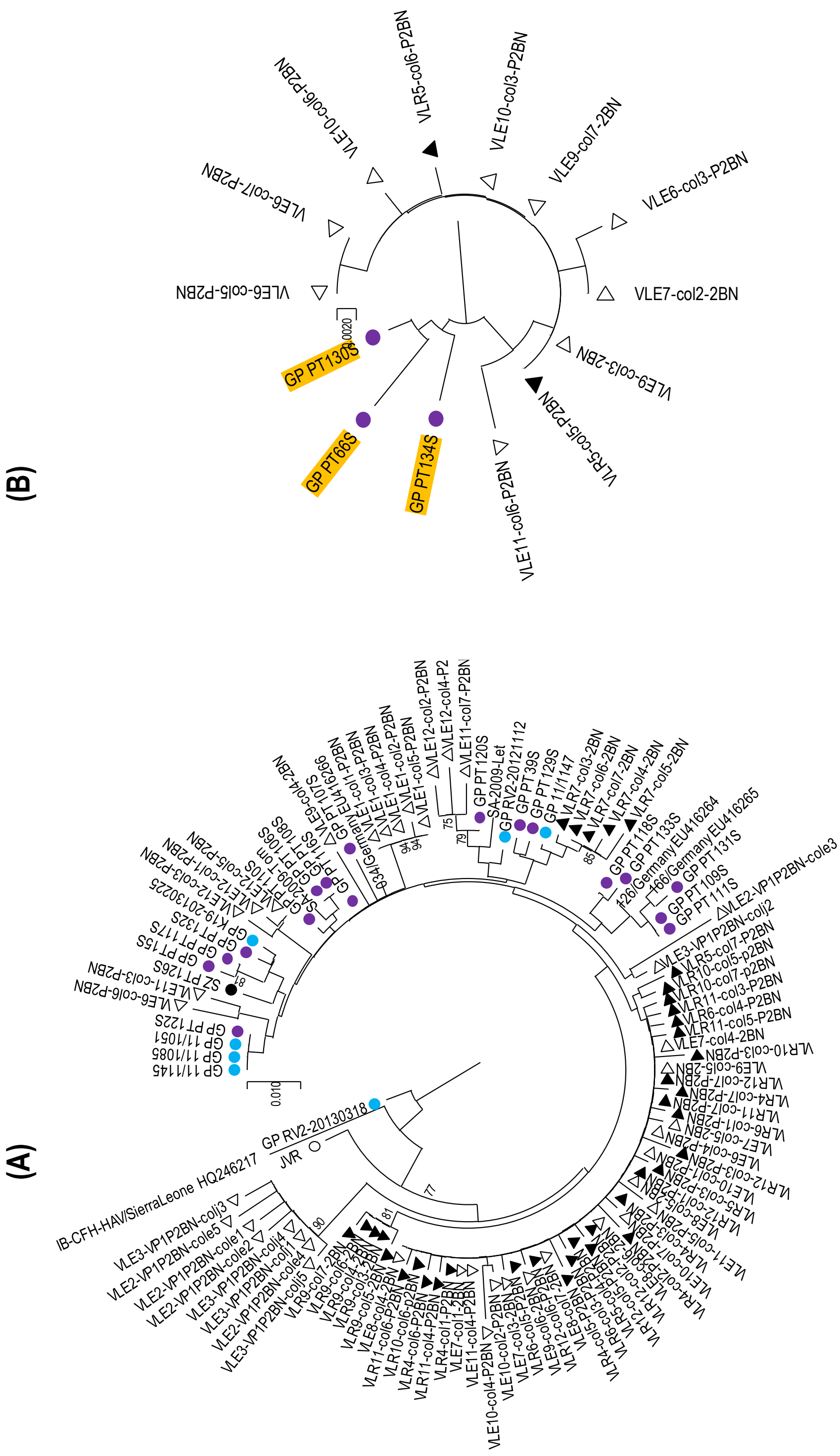


Figure 5.12: Phylogenetic analysis targeting the VP1/P2B junction of HAV strains detected in WWTP5 samples - the tree highlights strains from ‘SAn Major cluster-WWTP5’ (A) and ‘SAn minor cluster-WWTP5’ (B). The strains analysed from sewage and treated discharge samples are indicated with ▲ and ∆, respectively. Previously characterised HAV strains from water (●) and clinical (●) sources in Gauteng and from other countries (●) are indicated. The JVR (○) strain is shown. Reference strains retrieved from GenBank have accession number next to their name. The neighbour-joining tree was constructed using the Kimura two-parameter model. Bootstrap values greater or equal to 75% are shown at the nodes.

5.3.1.3 Mutations detected at the amino acid level

VP1 region: Amino acid changes have been recorded during analysis of the amino acid sequences of the VP1 region of the detected HAV strains. A total of 94% (318/337) of the sequences, obtained from sewage and treated discharge samples, carry the R298K amino acid change as recorded for HAV IIIA (Sim27, FJ227135) and IIIB (HAJNG0690F, AB258387) strains. A total of 11 HAV strains carrying amino acid changes at the immunodominant (S102, V171 and A176) and neutralisation (K221) epitopes were detected in sewage and treated discharge samples (Table 5.8). Amino acid substitutions have also been recorded at the G217, which is known to influence the neutralisation epitope. The majority (6/11) of strains have mutation at position 221 (K221E or K221R) (Table 5.8) (Figure 5.13).

Table 5.8: HAV strains carrying amino acid change at the immunodominant and neutralisation epitopes.

		Name of strain	[a,b]102	[a,b]171	[a,b]176	[c]217	[b]221
WWTP1	[d]R	DR4-col2	-	-	-	G217C	-
	R	DR5-colF	-	-	-	-	K221E
WWTP2	R	OR4-colE	-	V171E	-	-	-
	[e]E	OE7-col302	-	-	-	-	K221R
WWTP4	R	FR6-colB	-	-	A176T	-	-
	R	FR1-col3	S102P	-	-	-	-
	E	FE8-colA	-	-	-	-	K221E
WWTP5	R	VLR4-colA	-	-	-	-	K221E
	R	VLR8-colF	-	-	-	-	K221R
	E	VLE10-colC	-	-	-	-	K221E
	E	VLE11-colD	-	-	-	G217A	-

a: Nainan et al[51]
b: Ping et al[52]
c: Aragonès et al[54]
d: R = sewage
e: E = treated wastewater discharge

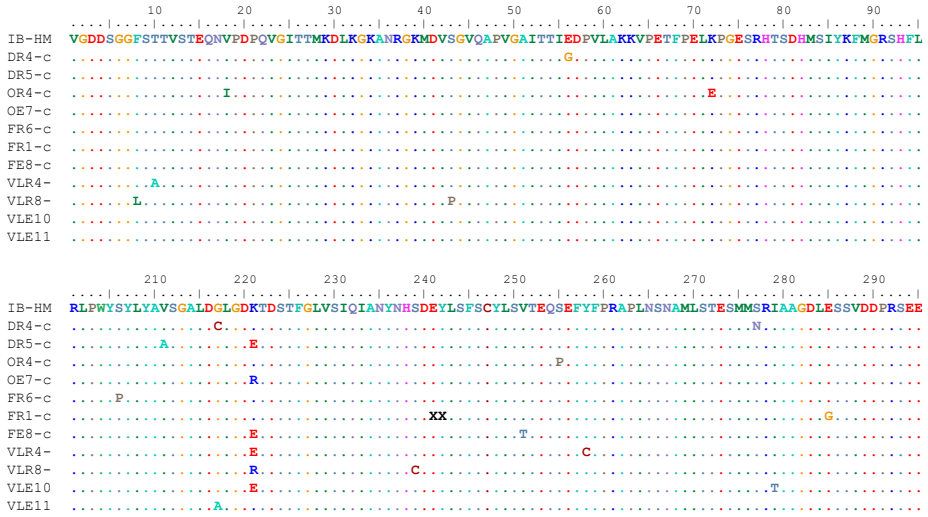


Figure 5.13: Alignment of the deduced amino acid sequences of the VP1 region of the HM175 strain and HAV strains carrying mutations at immunodominant and neutralisation epitopes. Conserved sites and substitutions are represented by dots and single-letter abbreviation, respectively.

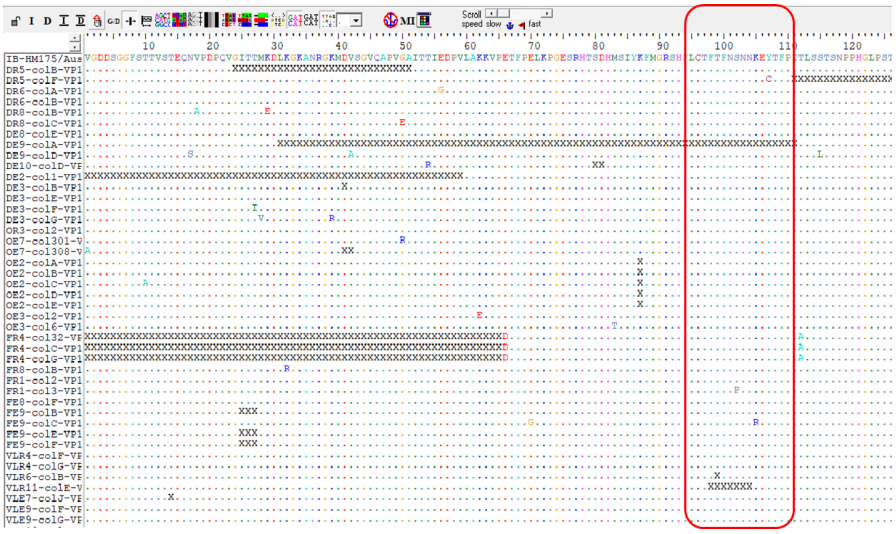
Potentially infectious HAV were quantified from the sewage and treated discharge samples (Chapter 4, Table 4.8) in which Sanger sequencing demonstrated the presence of strains carrying amino acid mutations at immunodominant and neutralisation epitopes (Table 5.9).

Table 5.9: Titres (gc/L) of potentially infectious HAV in sewage and wastewater discharge for WWTPs 1 to 5. Samples in which potential antigenic escape mutants have been characterised are highlighted (orange).

	WWTP1		WWTP2		WWTP3		WWTP4		WWTP5	
	Sewage	Wastewater discharge	Sewage	Wastewater discharge	Sewage	Wastewater discharge	Sewage	Wastewater discharge	Sewage	Wastewater discharge
April	1.35E+05	0.00E+00	7.57E+03	4.27E+02	0.00E+00	ND	6.28E+04	1.74E+03	2.97E+04	1.18E+02
May	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
June	2.04E+04	4.76E+02	6.02E+04	4.12E+03	0.00E+00	0.00E+00	2.16E+05	2.26E+03	3.58E+04	1.15E+04
July	2.13E+05	4.82E+03	1.51E+04	2.35E+04	ND	0.00E+00	ND	ND	7.57E+02	3.35E+04
August	2.06E+04	1.03E+04	2.78E+05	2.36E+04	ND	ND	2.29E+04	1.05E+04	ND	2.68E+04
September	2.10E+04	8.93E+03	2.51E+04	2.41E+04	0.00E+00	ND	Neg	7.96E+02	1.23E+03	1.34E+03
October	3.81E+05	1.55E+03	ND	5.16E+03	ND	ND	ND	ND	4.18E+05	4.33E+02
November	ND	2.93E+03	1.70E+05	2.96E+03	ND	0.00E+00	ND	ND	3.96E+03	2.47E+03
December	ND	ND	ND	2.25E+02	ND	ND	3.28E+04	6.04E+03	1.33E+03	8.01E+01
January	1.45E+04	9.81E+01	ND	5.66E+03	ND	ND	2.62E+05	2.44E+03	0.00E+00	7.83E+01
February	6.51E+03	2.05E+03	9.21E+03	1.44E+02	2.45E+04	ND	ND	ND	ND	2.83E+03
March	0.00E+00	3.25E+02	5.06E+05	1.84E+04	0.00E+00	ND	2.16E+06	1.60E+04	ND	2.90E+03

In addition to amino acid changes, in-frame deletions have also been recorded. The size of these deletions ranges from one amino acid to 105 amino acids and are mostly located within the sequences (Figures 5.14). Some of these in-frame deletions include the epitope and surrounding amino acids (Figures 5.14).

(A)



(B)

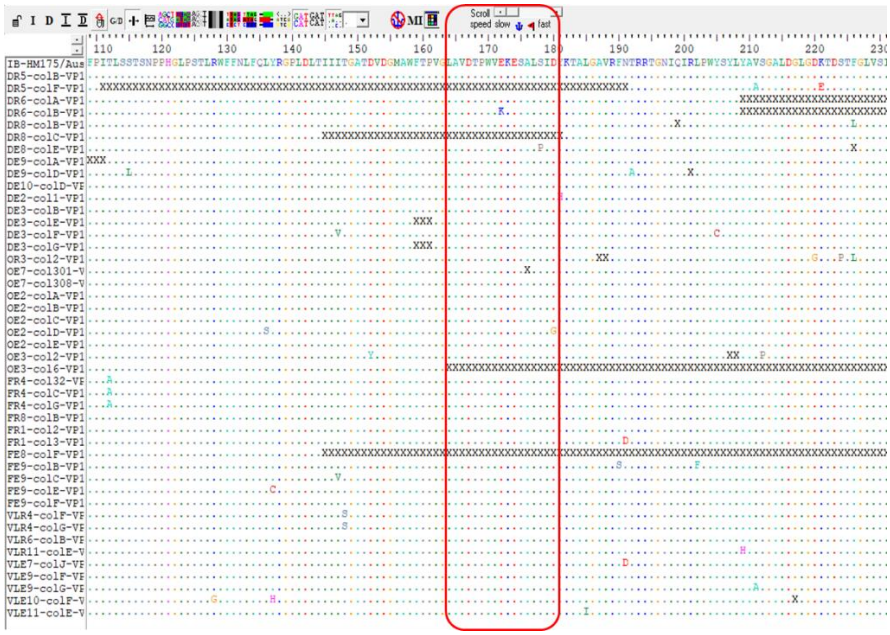


Figure 5.14: Alignment of the deduced amino acid sequences of the VP1 region of the HM175 strain and HAV strains carrying in-frame deletions. Conserved sites, substitutions and deletions are represented by dots, single-letter abbreviation and letter 'X', respectively. The red block highlight regions surrounding epitopes 102 (A), 171 and 176 (B), 217 and 221 (C).

(C)

Figure 5.14: Alignment of the deduced amino acid sequences of the VP1 region of the HM175 strain and HAV strains carrying in-frame deletions. Conserved sites, substitutions and deletions are represented by dots, single-letter abbreviation and letter 'X', respectively. The red block highlight regions surrounding epitopes 102 (A), 171 and 176 (B), 217 and 221 (C).

VP1/P2B junction: Analyses of the amino acid sequences of the VP1/P2B junction of the detected HAV strains revealed that 87% (309/356) of the sequences carry the R63K and R71S amino acid changes. The R63K change corresponds to the R298K change recorded for sequences of the VP1 region. In addition, the amino acid sequences of 47 HAV strains carry the C70S and M104I amino acid change (Figure 5.15). The majority (45) of these strains cluster within 'SAn minor cluster-WWTP4' (34 strains) (Figure 5.11B) and 'SAn minor cluster-WWTP5' (11 strains) (Figure 5.12B). Subgenotypes IIA (CF53, AY644676) and IIB (SLF88, AY644670) carry the C70S change, while the M104I change is present in the sequences of HAV IIIA (Sim27) and IIIB (HAJNG0690F) strains (Figure 5.15). In-frame deletions, ranging from one to four amino acids, were also recorded in 11 sequences.

Figure 5.15: Alignment of the deduced amino acid sequences of the VP1/P2B junction of the HM175 strain and HAV strains carrying the C70S and M104I changes. Conserved sites and substitutions are represented by dots and single-letter abbreviation, respectively.

Analysis of the amino acid sequence of HAV strains detected before and after viability treatment of the FR1 and FE8 wastewater samples from WWTP4 revealed in-frame deletions (Table 5.10).

Table 5.10: In-frame deletion and amino acid changes recorded for HAV strains characterised from FR1 and FE8 samples.

		In-frame deletion		Amino acid changes	
		Before	**After**	**Before**	**After**
VP1	**FR1**	Two [a]aa deletion: position 241 to 242	Two [a]aa deletion: position 241 to 242	S102P, R298K	S102P, G217D, R298K
	FE8	105 aa deletion: position 145 to 249	-	V251T, V251I	V251I
VP1/P2B	**FR1**	-	-	R63K, R71S	R63K, C70S, R71S, M104I
	FE8	-	-	R63K, C70S, R71S, M104I	C70S, M104I

a: aa = amino acid

5.3.2 NGS analyses

The complete CDS (6681 nt) was assembled manually from the genome sequence data of 44S, DE-c and DR. These CDS encode for 2227 amino acid polyproteins. From the sequence data of FE8 and FR partial CDS could be assembled. The 6423 nt CDS of FR was assembled *de novo* and encode for a 2141 amino acid polyprotein. The 5763 nt CDS of FE8 was assembled manually and was translated into a 1921 amino acids polyprotein.

5.3.2.1 Pairwise analyses

Percent identities, between the five partial genomes and reference strains from GenBank, were computed at the nucleotide and amino acid levels (Tables 5.11 to 5.13). Percent identities was lower at the nucleotide level (between 69.9% and 97.5%) but high at the amino acid level (between 87.3% and 99.9%) (Tables 5.11 to 5.13). The highest sequence identities were recorded between DE-c, DR, FR and the CFH-HAV strain over the genomic regions analysed (Tables 5.11 and 5.12). Sequence comparison between FE8 and CFH-HAV also revealed high identities over the genomic regions analysed except the 3CD region which was more similar to the MBB strain (Table 5.12). At the nucleotide level, high percent identities (between 96.1% and 97.1%) were recorded between 44S and the HAV/Egy/BI-11/2015 strain (Table 5.13). However, at the amino acid level, the percentage identity (between 98.3% and 99.7%) recorded between 44S, HM175 and CFH-HAV was similar to the percentage identity recorded between 44S and HAV/Egy/BI-11/2015 (Table 5.13). High percent identity was also recorded at the amino acid level between FE8, FR and HM175 (Table 5.12). The AGM-27 strain was distantly related to DE-c, DR, FE8, FR and 44S as shown by the lowest percent identities (Tables 5.11 to 5.13).

Table 5.11: Percent identities at nucleotide (nt) and amino acid (aa) levels between DE-c, DR and reference HAV strains. Highest (green) and lowest (yellow) identities are highlighted.

DE-c	HM175 (IB)		CFH-HAV (IB)		MBB (IB)		HAV/Egy/BI-11/2015 (IB)		GBM (IA)		CF53/Berne (IIA)		SLF88 (IIB)		SIM27 (IIIA)		HA-JNG06-90F (IIIB)		AGM-27 (V)	
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
CDS	93.5	99.3	97.0	99.5	93.8	98.9	93.4	98.4	89.9	97.9	83.6	96.4	82.8	96.9	79.1	93.8	79.7	94.2	77.8	92.9
P1	93.0	99.6	97.1	99.9	93.7	99.5	93.3	99.6	90.0	99.0	83.7	99.0	82.4	99.0	80.3	97.4	80.8	97.4	79.5	95.9
VP1	93.0	99.7	97.6	99.7	93.0	99.3	93.1	99.7	89.1	98.3	81.9	98.7	79.8	98.3	77.9	95.9	78.1	95.9	77.8	93.5
VP1/P2B	92.3	98.3	96.2	98.3	91.4	97.4	92.7	96.5	88.3	96.5	77.1	95.6	80.0	95.6	71.9	87.3	76.3	89.2	69.9	87.3
3CD	93.9	99.0	96.8	99.6	94.6	99.0	94.4	99.0	90.9	98.0	83.5	94.5	83.2	94.5	80.3	91.7	80.0	92.2	77.7	91.9

DR	HM175 (IB)		CFH-HAV (IB)		MBB (IB)		HAV/Egy/BI-11/2015 (IB)		GBM (IA)		CF53/Berne (IIA)		SLF88 (IIB)		SIM27 (IIIA)		HA-JNG06-90F (IIIB)		AGM-27 (V)	
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
CDS	93.6	99.3	96.9	99.5	93.8	98.9	93.3	98.4	89.9	97.9	83.7	96.3	83.1	96.9	79.2	93.8	79.7	94.2	78.0	93.0
P1	93.1	99.6	97.2	99.9	93.8	99.5	93.3	99.6	90.1	99.0	83.9	99.0	82.6	99.0	80.4	97.4	81.0	97.4	79.4	95.9
VP1	93.1	99.7	97.5	99.7	93.1	99.3	93.3	99.7	89.2	98.3	82.1	98.7	79.9	98.3	77.8	95.9	78.2	95.9	78.0	93.5
VP1/P2B	93.0	98.3	96.2	98.3	92.1	97.4	93.4	96.5	89.0	96.5	77.9	95.6	80.8	95.6	71.9	87.3	77.1	89.2	70.9	87.3
3CD	94.0	99.1	96.7	99.7	94.7	99.1	94.6	99.1	91.1	98.1	83.9	94.3	83.6	94.6	80.5	91.9	80.0	92.4	78.3	92.1

Table 5.12: Percent identities at nucleotide (nt) and amino acid (aa) levels between FE8, FR and reference HAV strains. Highest (green) and lowest (yellow) identities are highlighted.

FE8	HM175 (IB)		CFH-HAV (IB)		MBB (IB)		HAV/Egy/BI-11/2015		GBM (IA)		CF53/Berne (IIA)		SLF88 (IIB)		SIM27 (IIIA)		HA-JNG06-90F (IIIB)		AGM-27 (V)	
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
CDS	94.3	99.5	94.6	99.3	93.8	99.1	94.5	98.6	89.8	98.1	83.2	96.8	83.1	97.6	79.1	94.0	80.2	94.6	77.7	93.3
P1	93.2	99.7	96.3	99.7	93.5	99.6	93.5	99.7	89.8	98.9	83.3	98.7	82.1	98.9	79.9	97.4	81.6	97.6	79.4	95.8
VP1	93.6	99.7	95.8	99.7	93.3	99.3	94.4	99.7	89.4	98.3	82.2	98.7	80.5	98.3	77.8	95.9	80.2	95.9	78.4	93.5
VP1/P2B	92.7	98.3	95.9	98.3	91.7	97.4	93.1	96.5	88.7	96.5	77.6	95.6	80.4	95.6	72.3	87.3	76.7	89.2	70.5	87.3
3CD	96.0	99.0	93.9	99.0	95.6	99.6	96.5	99.6	91.9	98.6	82.8	95.2	84.2	95.8	80.5	91.9	80.6	93.0	78.6	93.0

FR	HM175 (IB)		CFH-HAV (IB)		MBB (IB)		HAV/Egy/BI-11/2015 (IB)		GBM (IA)		CF53/Berne (IIA)		SLF88 (IIB)		SIM27 (IIIA)		HA-JNG06-90F (IIIB)		AGM-27 (V)	
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
CDS	93.3	99.3	96.5	99.4	93.6	98.8	93.2	98.3	89.9	97.8	83.4	96.0	82.6	96.7	78.9	93.7	79.6	94.1	77.7	92.9
P1	92.7	99.9	96.7	99.9	93.4	99.7	93.2	99.7	89.7	99.0	83.3	98.8	81.8	99.0	80.1	97.6	81.8	97.7	79.1	95.9
VP1	92.4	99.7	96.6	99.7	92.6	99.3	93.0	99.7	88.7	98.3	81.9	98.7	79.9	98.3	77.9	95.9	78.1	95.9	78.6	93.5
VP1/P2B	91.3	98.3	95.2	98.3	92.4	97.4	92.4	96.5	88.7	96.5	77.1	95.6	80.8	95.6	72.7	87.3	76.3	89.2	71.3	87.3
3CD	93.7	99.0	96.3	99.6	94.4	99.0	94.2	99.0	91.0	98.0	83.5	94.2	83.0	94.5	80.0	91.6	79.7	92.2	77.9	92.1

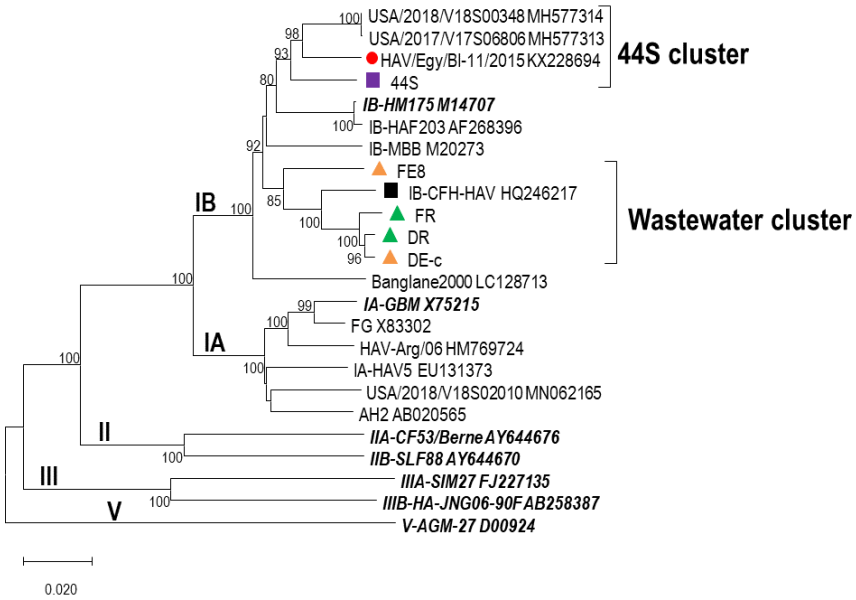
Table 5.13: Percent identities at nucleotide (nt) and amino acid (aa) levels between 44S and reference HAV strains. Highest (green) and lowest (yellow) identities are highlighted.

44S	HM175 (IB)		CFH-HAV (IB)		MBB (IB)		HAV/Egy/BI-11/2015 (IB)		GBM (IA)		CF53/Berne (IIA)		SLF88 (IIB)		SIM27 (IIIA)		HA-JNG06-90F (IIIB)		AGM-27 (V)	
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
CDS	95.1	99.5	93.5	99.3	94.3	99.1	96.1	98.7	90.1	98.0	83.6	96.3	83.5	97.2	79.1	93.8	80.0	94.1	78.2	93.0
P1	95.4	99.9	93.6	99.6	94.7	99.7	96.7	99.9	90.1	98.7	84.2	98.7	83.7	99.0	80.2	97.0	81.6	97.0	78.8	95.7
VP1	94.6	99.7	93.6	99.7	94.4	99.3	96.3	99.7	89.8	98.3	83.1	98.7	81.0	98.3	76.4	95.2	78.7	95.2	76.6	93.5
VP1/P2B	95.9	98.3	93.7	98.3	93.1	97.4	97.1	96.5	89.7	96.5	80.0	95.6	80.8	95.6	71.9	85.3	74.3	87.3	70.0	86.3
3CD	95.8	99.1	94.6	99.4	95.6	99.4	96.8	99.4	92.0	98.1	83.2	94.3	83.9	94.9	80.2	91.9	79.8	92.4	78.9	92.1

5.3.2.2 Phylogenetic analyses

Phylogenetic analyses were performed over the complete CDS at the nucleotide (Figure 5.16A) and amino acid levels (Figure 5.16B) and over the VP1 (Figure 5.17), VP1/P2B (Figure 5.18) and 3CD (Figure 5.19) genomic regions. The partial genome of DE-c, DR, FE8, FR and 44S clustered with sequences representative of genotype IB on all five phylogenetic trees (Figures 5.16 to 5.19). Two distinct clusters were observed, namely the 'Wastewater cluster' and the '44S cluster' (Figures 5.16 to 5.19). The 'Wastewater cluster', supported by high bootstrap values (≥85%) include DE-c, DR, FR, and FE8. However, FE8 grouped outside the cluster, during analyses performed over the CDS at the amino acid level (Figure 5.16B). A close relationship between the CFH-HAV strain (indicated with ■) with DE-c, DR and FR has been observed (Figures 5.16 to 5.19). Hepatitis A virus previously characterised from clinical sources in Gauteng (indicated with ●) also grouped within the 'Wastewater cluster' during analyses performed over the VP1 and VP1/P2B genomic regions (Figures 5.17 and 5.18). Phylogenetic analyses performed over the complete CDS, grouped 44S with the Egyptian strain HAV/Egy/BI-11/2015 (indicated with ●) (Figures 5.16A and B). However, analyses performed over the VP1 and VP1/P2B regions grouped 44S with HAV strains previously characterised from clinical sources in Gauteng (Figures 5.17 and 5.18). For phylogenetic analyses performed over the 3CD region the '44S cluster' includes FE8 and is supported by high bootstrap value (91%) (Figure 5.19).

(A)



(B)

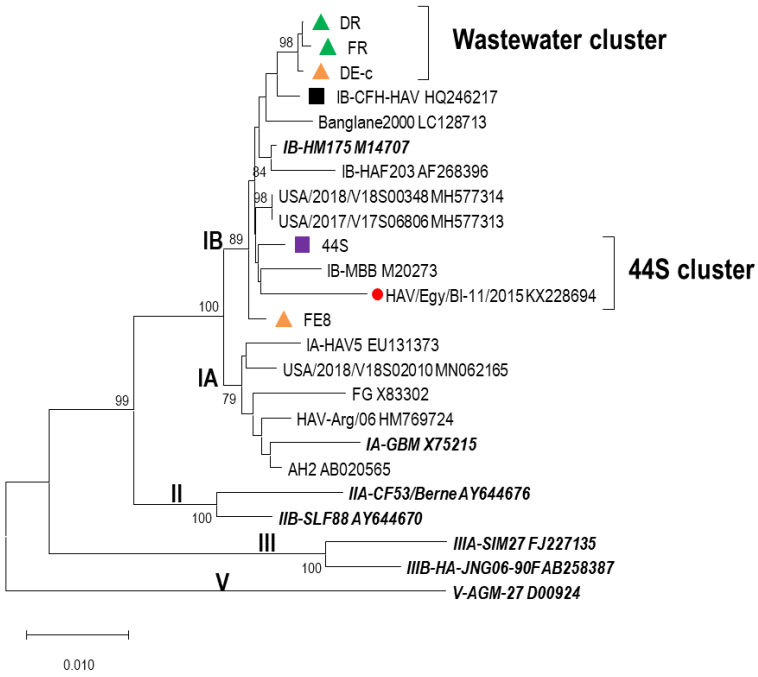


Figure 5.16: Phylogenetic analysis of the CDS of DR, DE-c, 44S, FR and FE8 at the nucleotide (A) and amino acid (B) levels. The sequences from clinical sources (■), sewage (▲) and treated discharge (▲) samples are indicated. Reference strains retrieved from GenBank have accession number next to their name. The name of type strains for each genotype and subgenotype are bolded and italicised. The neighbour-joining tree was constructed using the Kimura two-parameter model. Bootstrap values greater or equal to 75% are shown at the nodes.

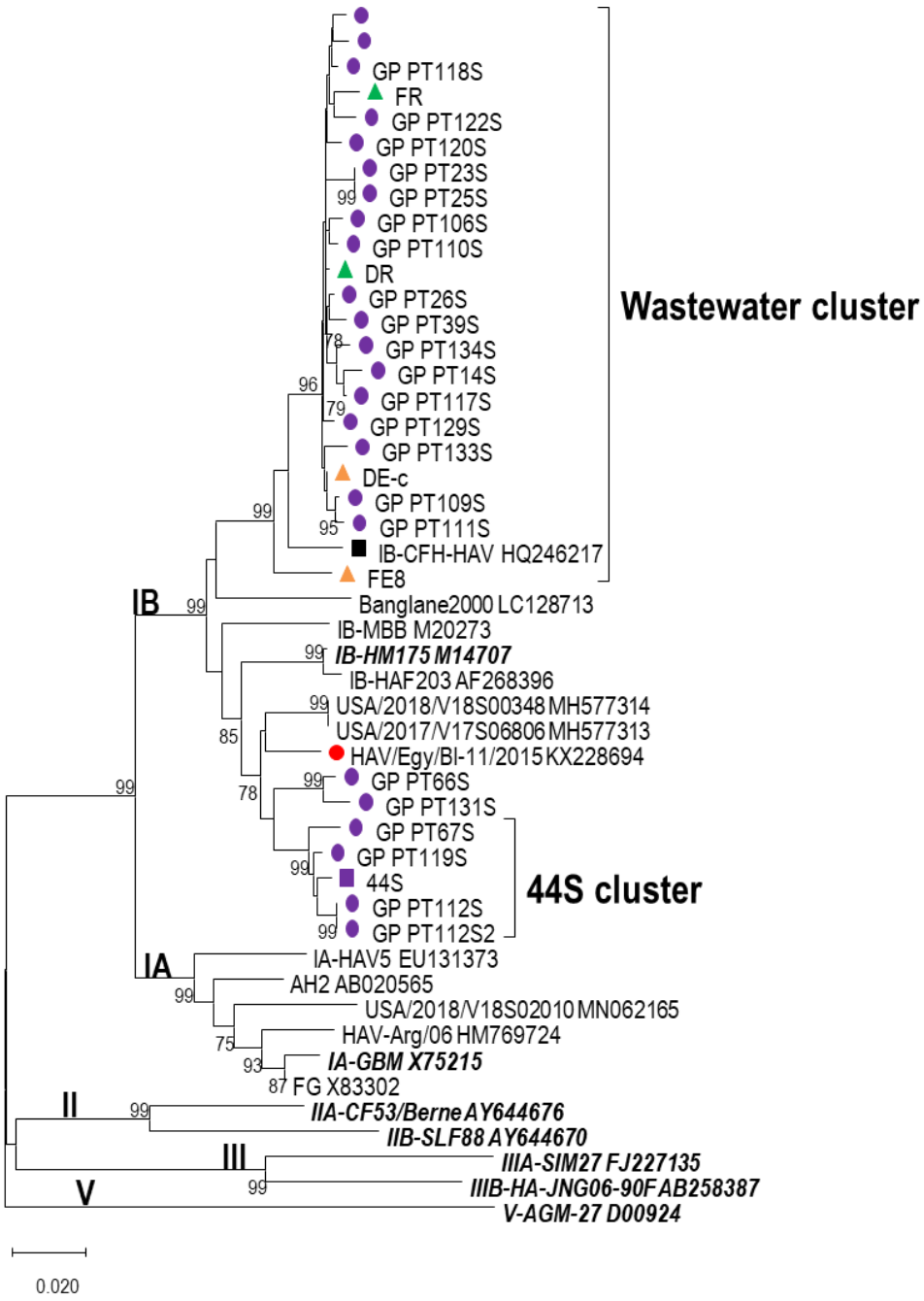


Figure 5.17: Phylogenetic analysis of the VP1 region of DR, DE-c, 44S, FR and FE8. The sequences from clinical sources (■), sewage (▲) and treated discharge (▲) samples are indicated. Reference strains retrieved from GenBank have accession number next to their name. The name of type strains for each genotype and subgenotype are bolded and italicised. The neighbour-joining tree was constructed using the Kimura two-parameter model. Bootstrap values greater or equal to 75% are shown at the nodes.

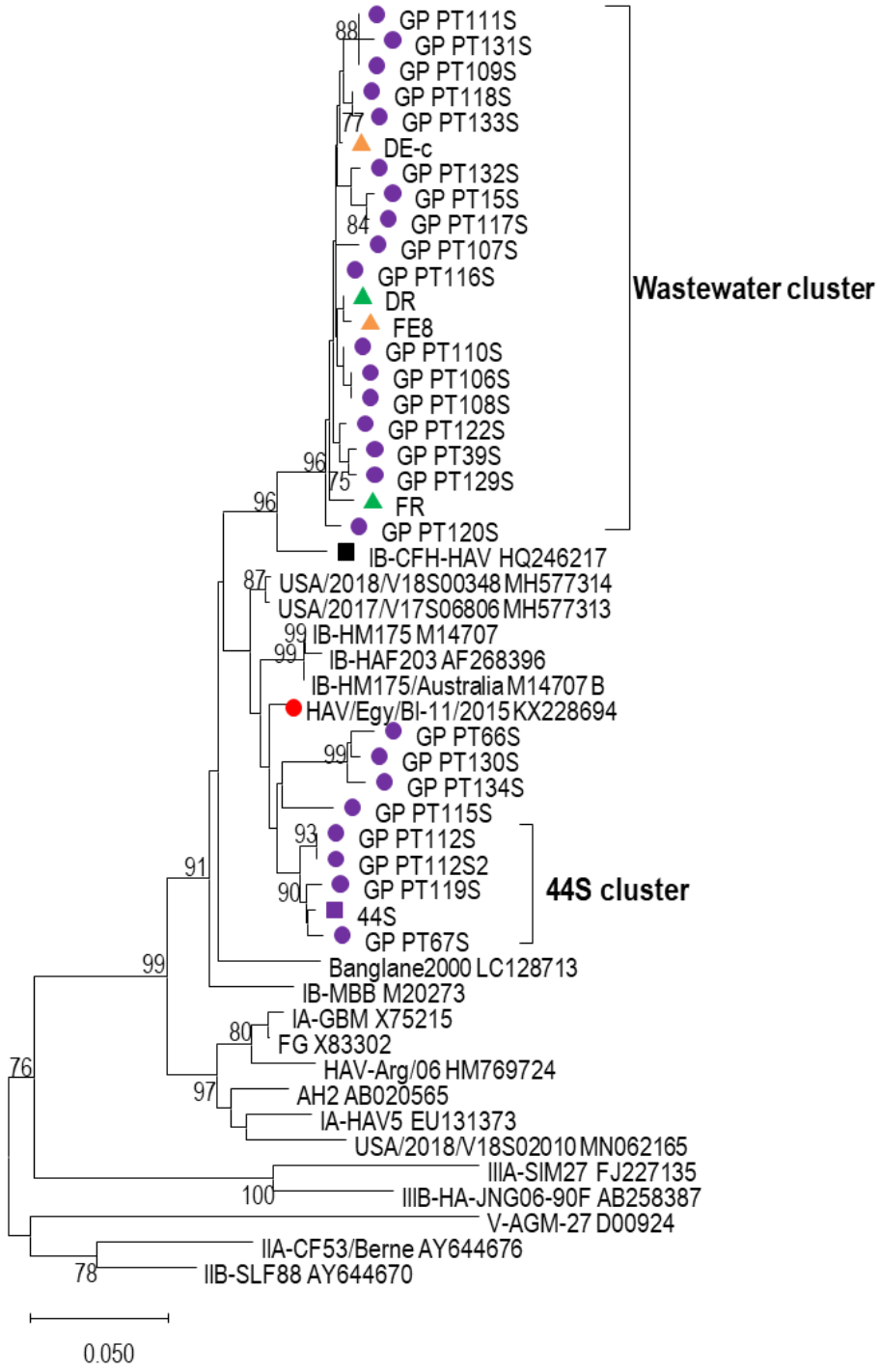


Figure 5.18: Phylogenetic analysis of the VP1/P2B region of DR, DE-c, 44S, FR and FE8. The sequences from clinical source (■), sewage (▲) and treated discharge (▲) samples are indicated. Reference strains retrieved from GenBank have accession number next to their name. The name of type strains for each genotype and subgenotype are bolded and italicised. The neighbour-joining tree was constructed using the Kimura two-parameter model. Bootstrap values greater or equal to 75% are shown at the nodes.

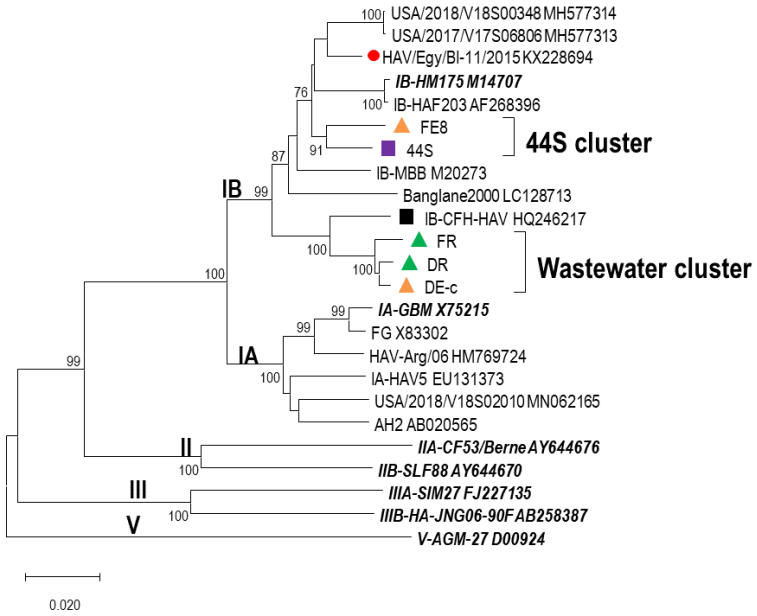


Figure 5.19: Phylogenetic analysis of the 3CD region of DR, DE-c, 44S, FR and FE8. The sequences from clinical sources (■), sewage (▲) and treated discharge (▲) samples are indicated. Reference strains retrieved from GenBank have accession number next to their name. The name of type strains for each genotype and subgenotype are bolded and italicised. The neighbour-joining tree was constructed using the Kimura two-parameter model. Bootstrap values greater or equal to 75% are shown at the nodes.

5.3.2.3 Mutations detected at the amino acid level

Analyses of the polyprotein of all five partial genomes revealed several amino acid changes when compared to the HM175 strain. However, only the R63K and R71S substitutions recorded over the VP1/P2B region could be detected for DE-c, DR, FE8 and FR. The sequence of 44S did not carry the R63K and R71S amino acid changes. None of the amino changes occurred at immunodominant or neutralisation epitopes.

5.3.2.4 Recombination analyses

Recombination analyses were not performed because, none of the characterised sequences clustered with different HAV reference strains during phylogenetic analyses performed over the different genomic regions.

5.4 DISCUSSION

Hepatitis A virus is an antigenically stable virus[46,50] that has geographically distinct genotypes and subgenotypes.[9,58-59] The present study aimed to investigate the genetic identity of potentially infectious HAV strains detected in selected SAn wastewater sources. The results of the study revealed that the surveillance of wastewater samples, using a combination of vPCR, cloning, Sanger sequencing and phylogenies produced from the VP1 and VP1/P2B genomic regions, enabled the characterisation of unique HAV IB variants circulating within a community in Gauteng. In addition, the identity of circulating major variants was confirmed with NGS analysis.

This is the first report of the quasispecies dynamic of HAV in sewage samples. Hepatitis A virus strains with large in-frame deletions (up to 105 amino acids) were detected in sewage samples. In-frame deletions have been previously detected during an *in vitro* study of MARs produced against the H7C27 and K34C8 monoclonal antibodies.[54] The study suggested that the deletions could be an adaptation mechanism by the virus in the presence of new environmental conditions.[54] The in-frame deletions detected in the present study are located around the immunodominant and neutralisation epitopes and suggest the potential emergence of antigenic escape mutants. Structural constraints of the HAV capsid suggests that these deletions arose from immune selection pressure present in the community. South Africa has a large immunocompromised population with approximately 7.97 million people infected with HIV.[196] Previous studies have shown that incomplete vaccination within an immunocompromised population could increase the probability of the emergence of antigenic escape mutants.[50,55] However, evidence for the emergence of new variants of HAV has been provided in both vaccinated and unvaccinated patients.[55] Even though the HAV vaccine is not part of the national expanded program of immunisation, antigenic escape mutants can still emerge in the SAn community as evidenced by the characterisation of HAV strains with amino acid changes at the immunodominant and neutralisation epitopes.

Hepatitis A virus, carrying mutations at the immunodominant and neutralisation epitopes, have been detected in sewage and treated wastewater discharge samples. It has been shown that HAV strains with amino acid changes at the immunodominant site (S102, V171 and A176) have lower fitness compared to wild type strains.[54] That could explain why in the present study HAV strains with amino acid changes at positions 102, 171 and 176 were only detected in sewage samples (Table 5.8). On the other hand, HAV strains carrying amino acid change at position 217 have similar fitness to wild-type HAV and could potentially affect antibody binding at the K221 epitope.[54] This could explain the detection of HAV strains carrying amino acid changes at G217 and K221 in sewage and treated wastewater discharge. The fact that these strains were detected from treated discharge samples from which potentially infectious HAV strains were quantified is a cause for concern.

The results of pairwise and phylogenetic analyses indicated that IB is the only subgenotype present in the sampling region. None of the strains characterised clustered with the JVR or VDM strains, suggesting that there was no cross-contamination during amplification. Hepatitis A virus strains detected in the sampling region showed uniqueness to the population serviced by the treatment work investigated. Even though these strains showed closeness to previously characterised HAV strains from clinical sources in Gauteng, they grouped together during phylogenetic analysis. Of particular importance are HAV strains from WWTP4 and 5 that formed minor clusters with three strains from clinical cases in Gauteng. The strains belonging to both minor clusters were detected before and after viability treatment and carried the same amino acid changes (C70S and M104I over the VP1/P2B genomic junction) suggesting that they might have originated from a group of people with common risk factors or exposure to a common source of HAV. Given that the shedding of HAV peaks during the incubation period, genetic analysis of HAV strains from wastewaters could allow the early detection of an outbreak as shown by previous studies.[144] Unfortunately, clinical data from the community inhabiting the sampling region was not collected. Therefore, it was not possible to confirm if these strains originated from an outbreak or sporadic cases. The diversity of HAV strains observed during Sanger sequencing was not reflected during NGS analyses.

The present study is the first report of three complete and two almost complete CDS of HAV strains from sewage and associated treated discharge samples on the African continent. The results revealed that the methodology, first proposed by Chiapponi et al,[187] can be adapted for the NGS-based characterisation of HAV strains in wastewater samples. The three complete CDS sequences were obtained from a clinical source (44S) in the Western Cape, SA and from pooled sewage (DR) and pooled treated discharge (DE-c) samples from WWTP1. The two nearly complete CDS were obtained from a treated discharge (FE8) and from pooled sewage (FR) samples from WWTP4. Except for 44S the remaining four sequences carry the R63K and R71S observed over the VP1/P2B junction, suggesting that these are the major variants of HAV circulating within the sampling region. The low genetic diversity observed during NGS analysis cannot be attributed to the pooling of samples, because the sequence of FE8 originated from a single sample. However, the CDS obtained from wastewater samples represent consensus sequences that were assembled from the sequences of pooled amplified subgenomic regions. In order to detect minor variants, NGS should have been performed on each amplified subgenomic region separately. A recent study, used NGS and Sanger sequencing to characterise human adenoviruses from urban wastewaters in Italy.[197] A single fragment of the hexon coding region was amplified using nested PCR and sequenced. In addition, pools of amplicons from the same fragment were analysed by NGS. While Sanger sequencing identified four species of human adenovirus and four types, NGS could identify 16 additional types.[197]

The main limitations of the study were that the methodology used to characterise the circulating strains was lengthy and included many steps, making it difficult to apply for routine analysis. The amplification of subgenomic regions by PCR is fast, but the cloning step required to identify single HAV strain by Sanger sequencing increases the processing time. The cloning step could be replaced by the characterisation of individual amplicon using NGS. In addition, the methodology used here produced partial CDS. The partial CDS obtained could be due to performing the reverse transcription step at 60°C instead of 50°C during ds-cDNA synthesis. According to the manufacturer's instructions, performing the reverse transcription step at 60°C removes

secondary structures in the RNA genome (Thermo Fisher Scientific). In the present study, incubation at 60°C might have cause degradation at the extreme ends of the RNA genome leading to the production of partial CDS. The reverse transcription step for DR, DE-c and 44S was performed at 50°C and complete CDS were sequenced for these samples. Despite these limitations, the results of the present study have implications for the WWTPs investigated.

Potentially infectious antigenic escape variants of HAV, with similar fitness to wild-type HAV, are circulating in the population serviced by WWTPs 1, 2, 4 and 5. Universal childhood vaccination is recommended for a region experiencing epidemiological shift.[101] However, in the community serviced by the four WWTPs, it should be delayed until additional wastewater-based surveillance studies coupled with clinical studies can confirm or refute the circulation of these variants. Routine vaccination in the presence of environmentally stable antigenic escape variants could be ineffective and lead to their emergence and transmission within and between communities.[15,55]

Unique HAV IB strains were characterised by Sanger and NGS sequencing, from the sewage and treated discharge samples collected from WWTPs 1, 2, 4 and 5. These potentially infectious strains are closely related to HAV strains previously detected in clinical specimens from Gauteng and could escape the currently available HAV vaccine. There is a potential risk of HAV exposure to communities using water sources downstream of WWTPs 1, 2, 4 and 5.

CHAPTER 6

GENERAL DISCUSSION

Hepatitis A virus, which is the leading cause of acute hepatitis, is an environmentally stable picornavirus.[2,11] The virus is shed in faeces by infected individuals and ends up in the sewage system.[79,150,151] The discharge of HAV contaminated wastewaters into surface waters, such as dams or rivers, could potentially expose users to hepatitis A. The detection and characterisation of HAV strains from sewage provides information on the type of strains circulating within a specific geographical area. In SA, unique HAV strains have been detected in raw and treated wastewaters, in surface waters and on fresh produce at the point of retail.[16-19] However, until the recent developments in PCR-based methods, the quantity and infectivity of the detected strains were unknown. Given that the propagation or isolation of wild-type HAV in cell culture is limited, pretreatment of water samples with an intercalating dye, followed by RT-qPCR, offers a cost-effective alternative for the quantification of infectious HAV. These data could help refine risk assessment studies and downstream Sanger sequencing and NGS could help identify the circulation of potentially infectious new variants of HAV. For these reasons, the present study aimed to detect and characterise infectious HAV strains from selected SAn water sources.

The first objective of the study was to detect and quantify HAV strains from selected SAn water sources. From April 2015 to March 2016, sewage (1 L), treated wastewater discharge (10 L) and dam water (10 L) samples were collected monthly from five WWTPs (WWTP 1, 2, 3, 4, 5). Viruses were recovered from the treated discharge and dam water samples using the glass wool adsorption-elution method[19,114,168] and further concentrated by PEG_{8000}/NaCl precipitation.[117] From the sewage samples, viruses were concentrated using the PEG_{8000}/NaCl method. A RT-qPCR assay was used to quantify HAV from the virus concentrates of the samples. Hepatitis A virus was

detected in the samples collected from all five WWTPs. A total of 80% and 83% of the sewage and treated discharge samples, respectively, tested positive. Given that the detection assay was not inhibited, the high detection rate in treated discharge could be attributed to the dissociation of HAV particles from organic matter in the sewage by wastewater treatment processes. The highest and lowest detection rates of HAV were recorded from WWTPs 4 (100% in both sewage and treated discharge samples) and 3 (42% in sewage and 27% in treated discharge samples), respectively. The titres of HAV ranged from 1.34 x 10^5 to 3.70 x 10^{10} gc/L of sewage and from 4.74 x 10^3 to 3.39 x 10^7 gc/L of treated wastewater discharge. The concentration of HAV in SAn sewage samples is unprecedented and high, but similar to other highly endemic regions like Tunisia. The presence of high titres of HAV in treated discharge samples suggest that wastewater treatment is ineffective at removing and/or inactivating the virus from sewage.

The second objective of the study was to determine the infectivity of detected HAV strains using molecular-based assays. Firstly, a viability RT-qPCR assay for the detection of potentially infectious HAV in water samples was optimised. Different methods of pretreatment were used on a cell culture suspension of HAV and HAV diluted in river water. The vdyes PMA-DMSO (50 µM), PMA-water (50 µM) and EMA-DMSO (20 µM) with or without the surfactants Triton® X100 (0.5%), Tween®20 (0.5%) and Tween®20 (0.1%), were tested. The results indicated that pretreatment with a combination of EMA-DMSO (20 µM) and Triton® X100 (0.5%) was best at quantifying intact HAV particles from cell culture suspension following thermal inactivation. However, the pretreatment of river water samples with a combination of PMA-water (50 µM) and Tween®20 (0.5%) was more efficient. The difference could be attributed to the matrices of the river water samples used during optimisation which favours pretreatment with the PMA-water (50 µM) and Tween®20 (0.5%) combination.

Following pretreatment with PMA-water (50 µM) and Tween®20 (0.5%), high titres of potentially infectious HAV was detected from the virus concentrates of sewage (0 to 2.16 x 10^6 gc/L) and treated wastewater discharge (0 to 3.35 x 10^4 gc/L)

samples collected from WWTPs 1, 2, 4 and 5. None of the virus concentrates corresponding to treated discharge samples collected from WWTP3 tested positive. The high titres might have been an overestimation, since in wastewater samples viruses could be exposed to multiple mechanisms of inactivation that do not necessarily involve the loss of capsid integrity. However, the reduction in PCR signal indicates that quantification by RT-qvPCR is more accurate than by RT-qPCR.

The third objective was to characterise the HAV strains detected in the sewage and treated wastewater discharge by nucleotide sequence and phylogenetic analysis. Hepatitis A virus strains detected in wastewater samples were characterised by PCR amplification of the VP1 and VP1/P2B genomic regions, followed by cloning and Sanger sequencing. Pairwise and phylogenetic analyses showed that the 337 and 356 HAV strains characterised by Sanger sequencing of the VP1 and VP1/P2B regions, respectively, belonged to subgenotype IB. The majority of these strains carried the R298K amino acid change over the VP1 region or the R63K and R71S change over the VP1/P2B junction. These amino acid changes are specific to SA HAV strains and phylogenetic analysis showed that the strains detected are unique to the Vaal catchment region. A subset of the HAV strains from the samples collected from WWTPs 4 and 5 carried the C70S and M104I changes over the VP1/P2B junction. Sanger sequencing, performed after viability treatment, confirmed the circulation of HAV strains carrying the C70S and M104I changes over the VP1/P2B junction. Further analysis of the amino acid sequences provided the first evidence of quasispecies dynamics of HAV in sewage samples, as evidenced by the detection of strains with in-frame deletions. This suggests that circulating HAV strains are subjected to immune selection pressure in the community serviced by the WWTPs investigated. In addition, HAV strains, carrying mutations at the immunodominant antigenic and neutralisation epitopes, were detected in the sewage and treated discharge samples of WWTPs, 1, 2, 4 and 5. Some of these potential vaccine escape mutants have similar fitness to wild-type HAV and could survive wastewater treatment. However, clinical data of the surrounding community is needed to confirm if these strains can cause disease.

The fourth objective was to characterise novel and/or emerging strains using full genome analysis. Following viability treatment of virus concentrates, sewage and treated discharge samples from WWTPs 1 (DR and DE-c, respectively) and 4 (FE8 and FR, respectively) which were found to harbour potential new variants of HAV were subjected to NGS analysis. Four overlapping fragments were amplified from pools of RNA and from a single RNA extract. The amplicons were pooled and submitted for NGS. A total of two complete CDS and two partial CDS were obtained. Pairwise and phylogenetic analyses performed at the nucleotide and amino acid levels revealed that these CDS could be characterised as subgenotype IB. NGS analysis recorded lower diversity compared to Sanger sequencing, probably due to the pooling of amplicons prior to NGS. However, NGS analysis did confirm the circulation of HAV strains carrying the R63K and R71S amino acid changes over the VP1/P2B junction.

The fifth and final objective was to determine the clinical relevance of the potentially infectious environmental HAV strains by comparison to those detected in clinical specimens. The complete CDS, of HAV strain detected in a clinical specimen (44S) collected in the Western Cape, was sequenced. The CDS of 44S did not group with the CDS obtained from wastewater samples, except during analysis performed over the 3CD region. This is probably due to a difference in geographical location. Phylogenies produced from the nucleotide sequence analysis of the VP1 and VP1/P2B junction, did group DE-c, DR, FE8 and FR with HAV strains previously characterised from clinical cases in Gauteng.[21]

The analysis of sewage and treated wastewater discharge samples collected from five WWTPs revealed treatment failure at four plants and the circulation of potential new variants of HAV sugbenotype IB, some of which could escape the currently available vaccine. The methodology used in the present study allowed for the quantification and genetic characterisation of potentially infectious HAV strains from wastewater sample using a molecular assay. Despite some technical limitations, the methodology can be adapted for surveillance studies. As SA could be experiencing an epidemiological shift, data from wastewater-

based surveillance studies coupled to clinical surveillance will help public health officials on the formulation of appropriate policies especially with regard to the use of vaccine.

CHAPTER 7

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APPENDIX A

ABSTRACTS OF PRESENTATIONS

A.1: Rachida S, Taylor MB. Hepatitis A virus in wastewater discharge: the great escape [Presentation]. Faculty Day, Faculty of Health Sciences, University of Pretoria 21-22 August 2018: Tswelopele Building, Prinshof Campus, Pretoria, South Africa.

Background:
Hepatitis A virus (HAV) infection is a significant cause of morbidity worldwide with unique strains of HAV circulating in South Africa (SA). The virus, which has a low (10-100 virions) infectious dose, is stable in the environment. HAV has been detected, but not quantified, in water sources in the Vaal catchment area. This study aimed to quantify and characterise HAV in treated and untreated wastewater to estimate the efficacy of wastewater treatment processes for HAV.
Method:
Over a period of one year (April 2015 to March 2016) water samples (*n=120;* sewage, wastewater discharge, dam) were collected from five wastewater treatment plants (WWTPs) and downstream dam in the Vaal catchment area. Viruses were recovered and concentrated from the water samples. After nucleic acid extraction, HAV was quantified in the virus concentrates by real-time qRT-PCR using the hepatitisA@ceeramTools™ kit. Detected HAV strains were characterised by nucleotide sequence and phylogenetic analyses of the VP1 genomic region.
Results:
A total of 72% (86/120) of the water samples tested positive for HAV. The HAV concentration ranged from 1.34 x 10^7 to 3.94 x 10^9 genome copies (gc) per 1 L of sewage sample and 3.40 x 10^6 to 1.65 x 10^9 gc/L of treated wastewater discharge. None of the dam water samples tested positive for HAV. The concentrations of HAV remain constant throughout the sampling period and no seasonal pattern was observed. The highest concentrations of HAV in sewage samples and corresponding discharge were recorded from a single WWTP in August and September, respectively. Phylogenetic analyses revealed that HAV genotype IB strains unique to SA circulated in the region, with the presence of three potential antigenic escape variants, one of which was detected in a treated discharge.
Discussion and Conclusion:
High concentrations of HAV are being released into downstream water sources by the five WWTPs sampled in this study, one of which has discharged potential antigenic or vaccine escape variants of HAV. Although the potential infectiousness of the detected HAV has yet to be determined, the use of water sources downstream of the WWTPs for domestic, recreational and irrigation purposes is a cause of concern.

A.2: Rachida S, Taylor MB. Hepatitis A virus in wastewater discharge: a potential public health concern? [Presentation]. 2nd International Conference for Food Safety and Security 15-17 October 2018: Saint George's Hotel and Conference Centre, Pretoria, South Africa.

Hepatitis A virus (HAV) infection is a significant cause of morbidity worldwide with unique strains of HAV circulating in South Africa (SA). The virus, which has a low (1-10 virions) infectious dose, is stable in the environment. As a result, HAV can survive in sewage and food, causing multistate foodborne outbreaks. HAV has been detected, but not quantified, in water sources in the Vaal catchment area. This study aimed to quantify and characterise HAV in treated and untreated wastewater to estimate the efficacy of wastewater treatment processes for HAV. Over a period of one-year, water samples (*n=120;* sewage, wastewater discharge, dam) were collected from five wastewater treatment plants (WWTPs) and downstream dam in the Vaal catchment area. Viruses were recovered and concentrated from the water samples. HAV was quantified in the virus concentrates by real-time qRT-PCR. Detected HAV strains were characterised by nucleotide sequence and phylogenetic analyses of the VP1 genomic region. A total of 72% (86/120) of the water samples tested positive for HAV. The HAV concentration ranged from 1.34 x 10^7 to 3.94 x 10^9 genome copies (gc) per 1 L of sewage sample and 3.40 x 10^6 to 1.65 x 10^9 gc/L of treated wastewater discharge. None of the dam water samples tested positive for HAV. The concentrations of HAV remain constant throughout the sampling period and no seasonal pattern was observed. The highest concentrations of HAV in sewage samples and corresponding discharge were recorded from a single WWTP in August and September, respectively. Phylogenetic analyses revealed that HAV genotype IB strains unique to SA circulated in the region, with the presence of three potential antigenic escape variants, one of which was detected in a treated discharge. High concentrations of HAV are being released into downstream water sources by the five WWTPs sampled in this study, one of which has discharged potential antigenic or vaccine escape variants of HAV. Although the potential infectiousness of the detected HAV has yet to be determined, the use of water sources downstream of the WWTPs for domestic, recreational and irrigation purposes is a cause of concern.

A.3: Rachida S, Taylor MB. Viability PCR for the detection of potentially infectious hepatitis A virus in wastewater sources, Gauteng, South Africa [Presentation]. Virology Africa 10-14 February 2020: Radisson Blu Hotel, Cape Town, South Africa.

Hepatitis A virus (HAV) is a re-emerging pathogen, with waterborne transmission recognised as a major public health concern. The virus, which has a low (1-10 virions) infectious dose, is stable in the environment and can survive in water and food sources, causing multistate foodborne outbreaks. HAV has been detected in water sources in the Vaal catchment area. However, in the absence of cell culture pre-amplification, the potential infectivity of strains cannot be determined by molecular-based detection methods like RT-PCR. This study aimed to quantify and characterise potentially infectious HAV in effluent water using viability PCR. For one-year, samples of wastewater discharge from five wastewater treatment plants (WWTPs) and downstream dam in the Vaal catchment area were monitored for the presence of HAV by RT-qPCR. Positive samples were retested using viability RT-qPCR, to determine the concentration and infectivity of detected virus. Phylogenetic analysis based on the VP1 genomic region was used to characterise the detected strains. Of the 83% (43/52) HAV-positive effluent samples, a total of 81% (34/42) contained potentially infectious viruses with concentrations recorded as high as 2.66 x 107 genome copies/litre of effluent. None of the dam water samples tested positive. Genetic analyses revealed that clinically relevant HAV genotype IB strains unique to SA circulated in the region. High concentrations of potentially infectious HAV are being released into downstream water sources by the WWTPs sampled in this study. Therefore, the use of water sources downstream of the WWTPs for domestic, recreational and irrigation purposes is a public health concern.

APPENDIX B

ETHICAL APPROVAL

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 22/04/2017.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

18/08/2016

Approval Certificate
New Application

Ethics Reference No.: 327/2016

Title: Genetic assessment of infectious hepatitis A virus strains detected in selected water sources in Gauteng, South Africa

Dear Ms Said Rachida

The **New Application** as supported by documents specified in your cover letter dated 22/07/2016 for your research received on the 22/07/2016, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 17/08/2016.

Please note the following about your ethics approval:
- Ethics Approval is valid for 3 years
- Please remember to use your protocol number (**327/2016**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of **6 monthly written Progress Reports**, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

*** Kindly collect your original signed approval certificate from our offices, Faculty of Health Sciences, Research Ethics Committee, Tswelopele Building, Level 4-60*

Dr R Sommers; MBChB; MMed (Int); MPharMed,PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

14 February 2019

Approval Certificate
Annual Renewal

Ethics Reference No.: 327/2016
Title: Genetic assessment of infectious hepatitis A virus strains detected in selected water sources in Gauteng, South Africa

Dear Ms S Rachida

The **Annual Renewal** as supported by documents received between 2019-01-23 and 2019-02-13 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 2019-02-13.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2020-02-14.
- Please remember to use your protocol number (327/2016) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers
MBChB MMed (Int) MPharmMed PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

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