THE INCIDENCE OF HEPATITIS A VIRUS IN SELECTED WATER SOURCES AND ASSOCIATED RISK OF INFECTION IN SOUTH AFRICA

by

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SUMMARY

Hepatitis A virus (HAV) is a non-enveloped, positively charged single stranded RNA hepatotropic agent from the family *Picornaviridae*, and the sole member of the genus *Hepatovirus*. There is only one HAV serotype but there are seven genotypes. Hepatitis A (HA) infection is usually self-limiting and the severity of the illness is age dependant. In children, infection with HAV is usually asymptomatic, while most adults and immunocompromised patients develop moderate to severe clinical disease. HA is endemic in South Africa (SA) with 100% of children from the lower socio-economic population acquiring immunity before the age of 10. With the current trends in urbanisation, a change in the epidemic vulnerability of the SA population can be expected. HAV is predominantly transmitted by the faecal-oral route and contaminated food and water are important sources of infection. However, the contribution of waterborne HAV to the burden of HA disease in SA is unknown.

The aims of this investigation were to assess techniques for the recovery, isolation and detection of HAV from water sources. Thereafter these techniques were applied to estimate the potential risk of infection posed to communities using the water sources for recreational and domestic purposes. This would elucidate whether or not water plays a role in the spread of HAV infection in SA.

An effective and sensitive concentration method is fundamental to the successful detection of HAV in water sources. Three primary recovery and two secondary concentration techniques were investigated in this study. An in-house modified glass wool technique, using 15 g of glass wool and with the addition of three metal gauze grids at 5 g intervals, proved to be the most sufficient and cost effective technique for the primary recovery of HAV from water sources. The packing density of the glass wool and positioning of the grids proved to be essential for efficient HAV recovery. A polyethylene glycol/sodium chloride secondary concentration technique proved to be more cost effective than commercial centrifugal devices. Combinations of cell cultures, propagation conditions, RNA extraction protocols and detection techniques were assessed for the isolation and detection of HAV. A combination of FRhK-4R cell culture propagation and reverse transcription-polymerase chain reaction-oligonucleotide probe assay was demonstrated to be the simple, most efficient technique for the detection of HAV. The nucleotide sequences of HAV strains from water sources and clinical specimens were compared to ascertain whether the strains from water were a potential source of infection. Although the majority of clinical strains clustered seperately from the water strains, one strain from an asymptomatic patient was identical to a number of strains from water. This suggests that HAV in the environment is a potential source of infection in SA. To assess the potential risk of infection constituted by HAV to persons using surface dam and river water for domestic and recreational purposes, a deterministic exponential risk assessment model which works with mean values and conservative assumptions was applied. Results indicated a minimal risk of infection to the higher socio-economic, non-immune population using the water for recreational purposes, if 100 ml of water was ingested per day. No risk was identified for the lower socio-economic, predominantly immune, population who uses the same water sources for domestic and drinking purposes. This study represents the first comprehensive data on risk of infection constituted by waterborne HAV in SA.

Keywords: Hepatitis A virus, water, detection techniques, recovery, molecular characterisation, risk assessment

DIE INSIDENSIE VAN HEPATITIS A VIRUS IN GESELEKTEERDE WATERBRONNE EN DIE GEASSOSIEERDE RISIKO VAN INFEKSIE IN SUID AFRIKA

deur

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OPSOMMING

Hepatitis A virus (HAV) is ‘n positief-gelaaide, enkelband RNA, hepatotropiese virus sonder ‘n membraan uit die familie *Picornaviridae*, en is die enigste lid van die genus *Hepatovirus*. Daar is slegs een serotipe maar sewe genotipes. Hepatitis A (HA) infeksie is gewoonlik selfbeperkend en die ernstigheidgraad van die siekte is ouderdoms gebonde. Die infeksie is gewoonlik asimptomaties in kinders, terwyl meeste volwassenes en immuun onderdrukte pasiënte met matige tot ernstige kliniese simptome presenteer. HA is endemies in Suid Afrika (SA) en 100% van die kinders in die laer socio-ekonomiese gemeenskappe ontwikkel immuniteit voor die ouderdom van 10 jaar. Soos wat verstedeliking egter toeneem word ‘n verandering in die epidemiologiese vatbaarheid van die SA populasie verwag. HAV word hoofsaaklik deur die fekaal-orale roete versprei en gekontamineerde voedsel en water is belangrike bronne van infeksie. Die bydrae van watergedraagde HAV tot die siektelas in SA is egter onbekend.

Die doel van hierdie studie was om tegnieke vir die herwinning, isolasie en opsporing van HAV in waterbronne te ondersoek. Daarna is hierdie tegnieke toegepas om die potensiële risiko van infeksie aan die SA gemeenskappe wat hierdie waterbronne vir ontspannings en huishoudelike doeleindes gebruik, te

bepaal. Die rol wat water in die verspreiding van HAV speel, sal sodoende verklaar kan word.

'n Effektiewe en sensitiewe konsentrasie metode is noodsaaklik om HAV suksesvol in water op te spoor. Drie primêre herwinnings- en twee sekondêre konsentrasie tegnieke was ondersoek gedurende hierdie studie. 'n Gemodifiseerde glaswol tegniek, wat 15 g glaswol benut met metaalgaas roosters wat op 5 g intervalle tussen die glaswol geplaas is, is bewys as die doeltreffendste en mees koste effektiewe tegniek vir die primêre herwinning van HAV vanuit water. Die verpakkingsdigtheid van die glaswol en posisionering van die metaalgaas roosters is belangrik vir die doetreffende herwinning van HAV. 'n Polietileenglikol/natrium chloried sekondêre konsentrasie metode is bewys as meer koste effektief as die kommersiële sentrifugale toestelle. Kombinasies van selkulture en amplifiserings toestande, RNA ekstraksie protokolle en opsporings tegnieke was ondersoek vir die isolasie en opsporing van HAV. Die eenvoudigste en doeltreffendste metode vir die opsporing van HAV was 'n kombinasie van FRhK-4R selkultuur amplifisering en 'n tru-transkripsie polimerase ketting reaksie-oligonukleotied peiler toets. Die nukleotied basis volgordes van HAV stamme wat uit water en kliniese monsters geïsoleer is, was met mekaar vergelyk om te bepaal of die stamme vanuit water 'n potensiële bron van infeksie was. Al het die meerderheid van die kliniese stamme apart van die water stamme gegroepeer, was een van die kliniese stamme identies aan die stamme wat uit water geïsoleer was. Hierdie bevinding suggereer dat HAV in die omgewing 'n moontlike bron van infeksie kan wees. 'n Deterministiese eksponensiële risiko-bepalingsmodel, gebaseer op die gebruik van gemiddelde waardes en konserwatiewe aannames, is gebruik om die potensiële risiko van infeksie wat deur HAV aan individue, wat dam en rivier water vir huishoudelike en ontspannings doeleindes gebruik, te bepaal. Die resultate impliseer dat daar slegs 'n minimale risiko van infeksie is vir die hoër sosio-ekonomiese, non-immuun, populasiegroep wat die waterbronne vir ontspannings doeleindes gebruik, indien 100 ml water per dag ingeneem sou word. Daar is geen risiko van infeksie vir die laer sosio-ekonomiese, hoofsaaklik immuun, populasie wat dieselfde waterbronne vir huishoudelike en drink doeleindes gebruik nie. Hierdie is die eerste omvattende studie in verband met die risiko van water gedraagde HAV infeksie vir die SA bevolking.

Sleutelwoorde: Hepatitis A virus, water, opsporingstegnieke, herwinning, molekulêre karakterisering, risiko bepaling

PRESENTATIONS AND PUBLICATIONS

NATIONAL PRESENTATIONS

JME Venter, WOK Grabow, MB Taylor. Comparison of methods for the isolation and detection of hepatitis A virus from water samples [Poster]. Faculty Day, Faculty of Health Sciences, University of Pretoria. 21-22 August 2001: Pretoria, South Africa.

JME Venter, WOK Grabow, MB Taylor. Comparison of methods for the isolation and detection of hepatitis A virus from water samples [Presentation]. "Microbial Diversity". 12th Biennial Congress of the South African Society of Microbiology, University of the Free State. 2-5 April 2002: Bloemfontein, South Africa.

WB van Zyl, S Nadan, JC Vivier, **JME Venter**, K Riley, EMK Tlale, LR Seautlueng, WOK Grabow, MB Taylor. The prevalence of enteric viruses in patients with gastroenteritis in the Pretoria and Kalafong Academic Hospitals, South Africa [Poster]. Microbial Diversity" 12th Biennial Congress of the South African Society for Microbiology, Faculty of Health Sciences, University of the Free State. 2-5 April 2002: Bloemfontein, South Africa.

JME Venter, JC Vivier, WOK Grabow, MB Taylor. Is hepatitis A virus in surface recreational water a cause for concern? [Poster Presentation]. Faculty Day, Faculty of Health Sciences, University of Pretoria. 20-21 August 2002: Pretoria, South Africa.

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INTERNATIONAL PRESENTATIONS

JC Vivier, MM Ehlers, J van Heerden, **JME Venter**, MB Taylor, WOK Grabow. An assessment of candidate viruses for harmonised risk assessment of water related microbiological hazards [Oral Presentation]. 11th International Water Association (IWA) Health-Related Water Microbiology Symposium, Melbourne 7-12 April 2002: Melbourne, Australia.

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MA Vrey, MM Ehlers, WB van Zyl, MB Taylor, DN Pavlov, J van Heerden, **JME Venter**, JC Vivier, JC de Villiers, E van Zyl, WOK Grabow. Microbiological quality of ground and surface water used for domestic purposes in rural areas of South Africa [Poster]. IWA International Symposium on Health-Related Water Microbiology, Cape Town 14-19 September 2003: Cape Town, South Africa.

MB Taylor, S Nadan, WB van Zyl, **JME Venter**, D Pavlov, N Potgieter, JM Barnes. Application of integrated-cell culture RT-PCRs to determine the occurrence of enteric viruses in irrigation water and associated minimally processed foods [Poster]. IWA International Symposium on Health-Related Water Microbiology, Cape Town 14-19 September 2003: Cape Town, South Africa.

PUBLICATIONS

JME Venter, J van Heerden, JC Vivier, WOK Grabow, MB Taylor.

Hepatitis A virus in surface water in South Africa: What are the risks?

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ABBREVIATIONS

3AEC	=	3-amino-9-ethylcarbazole
AC-PCR	=	antigen-capture polymerase chain reaction
Ag	=	antigen
AGMK	=	African green monkey kidney cell line
ALT	=	alanine aminotransferase
AMV	=	avian myeloblastosis virus
anti-HAV	=	antibodies to hepatitis A virus
anti-PPLO	=	tylocine
APHA	=	American Public Health Association
ATCC	=	American Type Culture Collection
BGM	=	Buffalo green monkey kidney cell line
bp	=	base pair(s)
BSA	=	bovine serum albumin
BS-C-1	=	African green monkey kidney cell line
4CN	=	4-chloro-1-napthol
C.	=	*Cercopithecus* species of monkeys
CDC	=	Centers for Disease Control and Prevention
cDNA	=	complimentary DNA
cfu	=	colony forming units
cm	=	centimetre(s)
cm^2	=	square centimetre
cm^3	=	cubic centimetre
CO_2	=	carbon dioxide
CPE	=	cytopathic effect
CsCl	=	caesium chloride
D	=	Dalton
DAB	=	3,3'-Diaminobenzidine tetrahydrochloride
DIF	=	direct immunofluorescence assay
DIG	=	digoxigenin

DNA	=	deoxyribonucleic acid
DNase	=	an enzyme that specifically destroys DNA
dNTP	=	deoxy nucleotide triphosphates (dGTP, dATP, dTTP and dCTP)
DWAF	=	Department of Water Affairs and Forestry
ºC	=	degree Celsius
ECACC	=	European Collection of Cell Cultures
EDTA	=	ethylenediaminetetraacetic acid
e.g.	=	for example
EIA	=	enzyme immunoassay
ELISA	=	enzyme-linked immunosorbent assay
EM	=	electron microscopy
E-MEM	=	Eagle's minimal essential media with Earle's salts
EMEM-HMEM	=	Eagle's minimal essential media with Earle's salts and Eagle's minimal essential media with Hank's salts
EOR	=	efficiency of recovery
EtBr	=	ethidium bromide
EU	=	endotoxin unit(s)
FCS	=	foetal calf serum
FDA/CFSAN	=	US Food and Drug Administration/Center for Food Safety and Applied Nutrition
FITC	=	fluorescein-isothiocyanate
FRhK-4	=	cloned cell line of foetal rhesus monkey kidney
FRhK-4R	=	rapidly growing variant of FRhK-4 cell line
FRhK-6	=	foetal rhesus monkey kidney cell line
Frp/3	=	cell line derived from the FRhK-4R cell line
g	=	gram(s)
g	=	gravitational force
GBEB	=	glycine-beef extract buffer

GBG, GJA & GMB	=	HAV strains isolated from human faeces in Gormaringer
GPE	=	non-primate cell line from guinea pig origin
h	=	hour(s)
HA	=	hepatitis A
HAAg	=	HAV antigen
HAV	=	hepatitis A virus
HB	=	hepatitis B
HBV	=	hepatitis B virus
HBsAg	=	hepatitis B surface antigen
HCl	=	hydrochloric acid
HIV	=	human immunodeficiency virus
HM-175	=	Australian strain of HAV
HRP	=	horse radish peroxidase
IB-RS-2 D10	=	non-primate cell line from pig origin
IC-PCR	=	immunomagnetic capture PCR
IEM	=	immune electron microscopy
IF	=	immunofluorescence assay
Ig	=	immunoglobulin
IgM	=	immunoglobulin of the M-class
IgG	=	immunoglobulin of the G-class
IIF	=	indirect immunofluorescence assay
IP	=	immunoperoxidase
IPFA	=	immunoperoxidase focus assay
IV	=	intravenous
kb	=	kilobase(s)
KCl	=	potassium chloride
kD	=	kilodalton
kPa	=	kilo Pascal
l	=	litre
LIFA	=	luminescent immunofocus assay
M	=	molar

Mabs	=	monoclonal antibodies
MEM	=	minimal essential media
µg	=	microgram(s)
mg	=	milligram(s)
$MgCl_2$	=	magnesium chloride
min	=	minute(s)
µl	=	microlitre
ml	=	millilitre
µm	=	micrometer(s)
mm	=	millimetre
mm^2	=	square millimetre
mM	=	millimolar
MPF	=	minimally processed food
MPN	=	most probable number
MRC-5	=	continuous cell culture derived from human fibroblasts
mRNA	=	messenger RNA
MS-1	=	HAV strain isolated in Willowbrook State hospital for the mentally handicapped during an outbreak
µW	=	microwatt
MW	=	molecular weight
NaCl	=	sodium chloride
NaOH	=	sodium hydroxide
$Na_2S_2O_3$	=	sodium thiosulfate
NCR	=	non-coding region
NICD	=	National Institute for Communicable Diseases
NIV	=	National Institute for Virology
ng	=	nanogram
nm	=	nanometer
nt	=	nucleotide(s)
ORF	=	open reading frame

P1, 2, 3	=	cleavage sites on picornavirus polyprotein
PBS	=	phosphate buffered saline
PCR	=	polymerase chain reaction
PEG	=	polyethylene glycol
pfu	=	plaque forming units
pHM-175	=	cell culture adapted HAV strain (HM-175 43c) derived from HM-175 which causes CPE
p.i.	=	post infection
PLC/PRF/5	=	continuous line of human hepatoma cells
RH	=	relative humidity
RIA	=	radio-immunoassay
RIFA	=	radio-immunofocus assay
RNA	=	ribonucleic acid
rRNA	=	ribosomal RNA
RNase	=	an enzyme that specifically destroys RNA
RT	=	reverse transcriptase
RT-PCR	=	reverse transcriptase polymerase chain reaction
s	=	second(s)
ss	=	single stranded
S	=	sedimentation coefficient (Svedburg units)
S.	=	*Saguinus* species of marmosets
SA	=	South Africa
SD-11	=	American strain of HAV isolated from a naval recruit in San Diego
SP 1K	=	non-primate cell line of dolphin origin
ssRNA	=	single stranded ribonucleic acid
$TCID_{50}$	=	50% tissue culture infectious dose
TMB	=	3,3′,5,5′-tetramethylbenzidine
tRNA	=	transfer RNA
trypsin-EDTA	=	activated trypsin-versene
U	=	unit(s)
UK	=	United Kingdom

USA	=	United States of America
US EPA	=	United States Environmental Protection Agency
UV	=	ultraviolet
Vero	=	continuous cell line derived from African green monkey kidney cells
VK	=	Vervet monkey kidney cells
VP	=	Virion protein
VP1-4	=	the major structural proteins of HAV
VPg	=	virion protein, genome
WHO	=	World Health Organization
WT HAV	=	wild type HAV
w/v	=	weight per volume

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CHAPTER 1

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Acute viral hepatitis is a public health problem worldwide (World Health Organization [WHO], 2000). The past two decades have witnessed an explosion in the knowledge of viral hepatitis. Five viruses that differ immunologically and epidemiologically from each other, hepatitis A, B, C, D and E, are recognized as hepatotropic viruses (Grabow, 1997; Ryder, 1999). Additional potential hepatotropic viruses, namely GB virus C, now classified as hepatitis G virus, Sentinel, SEN, SANBAN, and TT viruses, have also been identified (Bowden et al., 1996; Mushahwar, 2000). Of the five recognised hepatotropic viruses, hepatitis A virus (HAV) is the major cause of acute viral hepatitis in the world (WHO, 2000).

HAV, previously classified as *Enterovirus 72* in the family *Picornaviridae* (Gust and Feinstone, 1988; Weitz and Siegl, 1993; Hollinger and Ticehurst, 1996), has been a health problem plaguing human society for centuries (Hollinger and Ticehurst, 1996; Beard and Lemon, 1999). The unique biochemical and biophysical characteristics of HAV, namely: i) thermostability, ii) liver tropism, iii) relatively high degree of secondary genome structure, iv) small or possibly absence of a virion protein 4 (VP4), and v) ability to induce persistent nonlytic infections in some cell lines, forced taxonomists to re-evaluate their decision (Anderson, 1987; Gust and Feinstone, 1988; Hollinger and Ticehurst, 1996; Beard and Lemon, 1999). HAV was therefore reclassified within a unique genus, genus *Hepatovirus*, in the family *Picornaviridae* (Gust and Feinstone, 1988; Minor, 1991; Feinstone

and Gust, 1997). HAV has only one neutralization site that is immunodominant, and enteroviral-specific monoclonal antibodies (Mabs) do not react with HAV, therefore providing further evidence that this virus cannot be classified within the genus *Enterovirus* (Hollinger and Ticehurst, 1996). Two biotypes of HAV, namely human HAV and simian HAV, are included in the genus *Hepatovirus* (Gust and Feinstone, 1988; Hollinger and Ticehurst, 1996; Feinstone and Gust, 1997; Grabow, 1997; King et al., 2000). Initially, these two biotypes were classified into a single serotype, but in a recent publication the human and simian HAVs were grouped into two serotypes (Racaniello, 2001). On the basis of primary sequence variability there are four human genotypes (Robertson et al., 1991; Hollinger and Ticehurst, 1996; Feinstone and Gust, 1997; Taylor, 1997; King et al., 2000) and an additional three genotypes associated exclusively with Old World monkeys (Robertson et al., 1992; Taylor, 1997; Robertson, 2001). Individual strains of HAV have differences at molecular level, which facilitate molecular epidemiological studies (Feinstone and Gust, 1997).

1.2 HISTORY

During the 17th to 19th centuries a disease called "*campaign jaundice*" appeared to follow military camps (Gust and Feinstone, 1988; Zachoval and Deinhardt, 1993; Hollinger and Ticehurst, 1996). Clinical features of "*campaign jaundice*" included thick dark urine, high fever, weight loss, lassitude, nausea and abdominal discomfort and later jaundice. Some clinicians suspected that the agent causing this infection was not the same aetiological agent that caused the more severe type of jaundice later known as hepatitis B (HB) (Hollinger and Ticehurst, 1996; Feinstone and Gust, 2002). In 1923 a study of the pattern of illness in 63 hepatitis outbreaks in the United States of America (USA), between 1812 and 1922, was done (Hollinger and Ticehurst, 1996). The observed incubation period ranged between seven and ten days. The disease peaked in autumn and winter and appeared to be

transmitted by person-to-person contact. These early observations established the existence of one of the most common forms of hepatitis now known as hepatitis A (HA) (Hollinger and Ticehurst, 1996; Beard and Lemon, 1999). During World War II a Jaundice Committee was created in Great Britain, and various laboratories were established in the USA, to determine how the disease was transmitted, what specimens contained the aetiological agent and how it could be managed. Based on previous work done by scientists in Japan and Germany, their focus turned to blood and faeces as source of the agent. They succeeded in transmitting the agent to human volunteers in 1944. The importance of the faecal-oral route of infection was demonstrated by infecting volunteers with faecally contaminated water collected from a well in Pennsylvania during the height of a HA epidemic. The volunteers all developed acute hepatitis (Gust and Feinstone, 1988).

Various human volunteer and epidemiological studies performed during World War II confirmed the virological aetiology of the disease known as "*epidemic catarrhal jaundice"* (Hollinger and Ticehurst, 1996; Beard and Lemon, 1999). This disease could be distinguished from the more severe jaundice with a much longer incubation period and the terms, HA and HB, were introduced (Hollinger and Ticehurst, 1996; Feinstone and Gust, 2002). Thereafter various human volunteer trials were done to determine the exact route of infection, precise incubation period and whether or not infection leads to permanent immunity. One of these trials was done at the Willowbrook State School for the mentally handicapped (Ward et al., 1958). The now well-known MS-1 strain of HAV was isolated during this investigation (Gust and Feinstone, 1988; Hollinger and Ticehurst, 1996). HA could be induced with as little as 25 µl of the serum containing the MS-1 agent administered via the oral or parenteral route (Gust and Feinstone, 1988; Hollinger and Ticehurst, 1996). In 1973 HAV virions were observed for the first time by immune electron microscopy (IEM) (Feinstone et al., 1973; Hollinger and Ticehurst, 1996).

1.3 COMPOSITION AND MORPHOLOGY

1.3.1 Physical properties

The HAV virion is a spherical particle with icosahedral symmetry, a diameter of 27 nm and without a lipid envelope (Figure 1), which is slightly (± 2 nm) smaller than that of the other members in the family *Picornaviridae* (Hollinger, 1985; Robertson et al., 1991; Hollinger and Ticehurst, 1996).

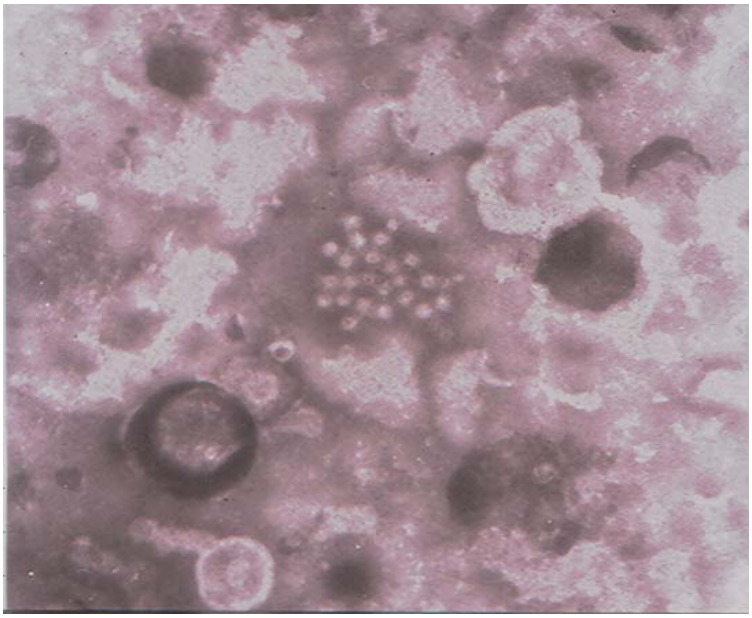


Figure 1: Negatively stained HAV particles in a human faecal specimen. (Kindly provided by Prof MB Taylor)

There are three distinct populations of HAV particles in purified HAV preparations (Hollinger, 1985; Gust and Feinstone, 1988; Hollinger and Ticehurst, 1996; Feinstone and Gust, 1997). Mature HAV virions have a buoyant density of approximately 1.32–1.34 g/ml in caesium chloride (CsCl) and sedimentation value of 160 S. Another HAV particle population bands at 1.20–1.32 g/ml in CsCl with a sedimentation value of 70–80 S. This population represents empty antigenic particles, known as procapsids, in the preparations and are therefore not infectious. In general they are detected in

abundance during the early stages of infection (Hollinger, 1985; Gust and Feinstone, 1988; Bishop and Anderson, 1997; Feinstone and Gust, 1997). The third population of particles was found to band at 1.39–1.50 g/ml in CsCl and it was suggested that this was due to a more open virion structure, allowing the CsCl to penetrate deeper into the virion. These particles contain RNA but were less stable than mature virions, and when sedimented, the virions dissociated into components that sediment at 50, 90, 160 and 230 S (Gust and Feinstone, 1988; Bishop and Anderson; 1997). Morphologically these virions are indistinguishable from the mature particles and express the same major surface antigens (Gust and Feinstone, 1988; Feinstone and Gust, 1997).

1.3.2 Structural proteins

The icosahedral protein shell of the HAV virion is very complex and consists of four different proteins or polypeptides (Beard and Lemon, 1999). These polypeptides, previously termed VP 1–4, are now officially known as 1D, 1B, 1C and 1A respectively (Levy et al., 1994). The molecular weight (MW) values of these structural proteins recognized at present are as follows: 1D (VP1) = 30-33 kD, 1B (VP2) = 30 kD, 1C (VP3) = 28 kD and 1A (VP4) = 14 kD (Hollinger and Ticehurst, 1996). A capsomer contains one copy of each of these four polypeptides and the protein shell of the virus consists of 60 capsomers. Protein 1A (VP4) is embedded deep inside the particle in contact with the RNA molecule and is thus not an integral part of the capsid. The other three polypeptides are exposed on the surface of the virion (Levy et al., 1994; Beard and Lemon, 1999).

1.3.3 Nucleic acid

Early studies provided evidence that HAV has a positive sense, linear, single stranded (ss) RNA genome (Hollinger and Ticehurst, 1996) implying that the RNA can be utilised as a messenger RNA (mRNA) during the replication cycle (Levy et al., 1994; Feinstone and Gust, 1997). The genome sediments at

a value of 33 S, has a buoyant density of 1.64 g/ml and a MW of 2.25 X 10^6 D (Gust and Feinstone, 1988; Hollinger and Ticehurst, 1996).

1.3.4 Replication cycle

RNA replication starts as soon as the RNA has been uncoated. During the initial stages of infection the rate of RNA synthesis doubles every 15 min and many of the progeny positive sense RNA molecules are used as viral mRNA. In the first two to four hours, there are more replicative intermediates than double stranded RNA molecules. Replicative intermediates are partially double stranded molecules formed by the simultaneous synthesis of one or more complementary strands from a single template strand. Replicative forms only accumulate in later stages of the cycle. Later, when RNA synthesis reaches a constant tempo, synthesis of positive sense RNA strands predominate, approximately 50% of these positive sense RNA strands are being packaged into virions. At this stage, 5-10% of the total cellular RNA consists of negative sense RNA strands. Virion assembly occurs on the smooth endoplasmic reticulum in association with the virus replication complex. The complete and infective virions are then released from the host cell to infect and replicate another cell (Levy et al., 1994). Because of rapid encapsidation, the RNA pool shrinks and replication slows down as a result. This could explain the lengthy logarithmic phase in HAV replication (Cromeans et al., 1989).

1.3.5 Genomic organisation

The linear 7.5 kb RNA genome of HAV can be divided into three regions (Figure 2), each with its own genomic characteristics (Table 1):

i) 5′ noncoding region (NCR),

ii) an open reading frame (ORF), and

iii) a short NCR at the 3′ end.

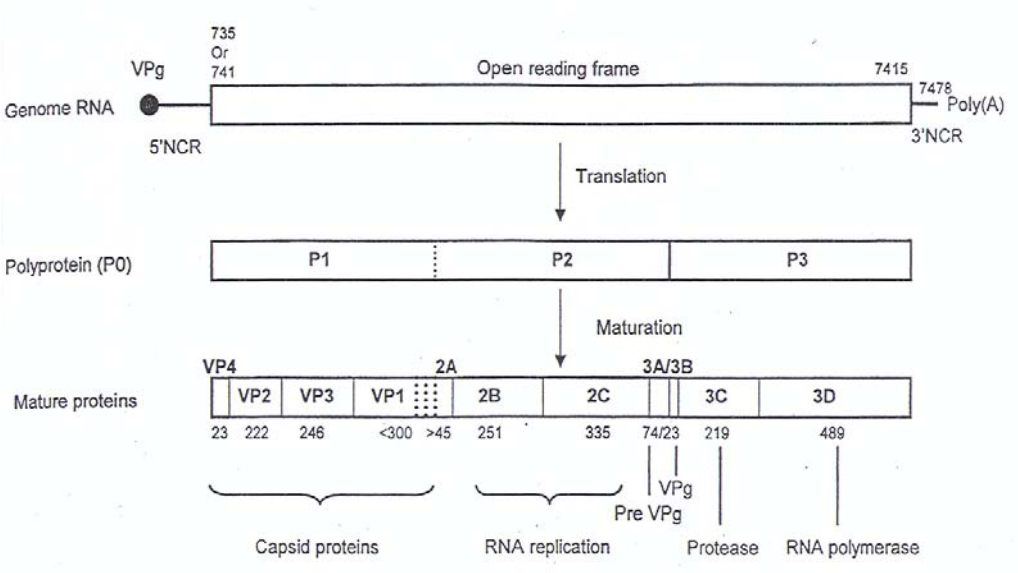


Figure 2: Organisation of positive sense HAV genome, polyprotein translation product and mature proteins. Mature protein sizes given as the number of amino acid residues.
(Feinstone and Gust, 2002)

The 5′ NCR comprises approximately 10% of the total viral genome. It is uncapped and covalently linked at the 5′ terminus to the VPg protein (Hollinger and Ticehurst, 1996; Feinstone and Gust, 2002). This region stretches from nucleotide (nt) 1-734 and is the most conserved part of the genome (Hollinger and Ticehurst, 1996; Beard and Lemon, 1999). There is a high ratio of uracil and cytosine in this region, which is unique to HAV. Deletion of nt 140-144 leads to temperature sensitive mutant strains, but the virus will not survive deletion of nt 93-134. Deletions of nt 96-139 however, do not influence the replication of infectious HM-175 in cell culture. The region from nt 99-207 is susceptible to natural occurring mutations. Mutations in this region enhance adaptation of the virus to grow in cell culture, but are not necessary to accomplish this goal (Hollinger and Ticehurst, 1996). A stem-and-loop structure is found near the 5′ terminus. Should nt 1 and 2, or 2 and 3 be deleted from this loop, the resulting mutant RNA will not be infectious, therefore it can be concluded that this secondary structure is

important in the replication cycle of the virus (Hollinger and Ticehurst, 1996; Beard and Lemon, 1999).

Table 1: Genomic characteristics of HAV

(Adapted from Hollinger and Ticehurst, 1996)

Encoding Region	Nucleotides (nt)	Length in amino acids	Proposed or known function
Polyprotein	741-7415 or 735-7415	2225 or 2227	AUG codon downstream
P1 (Capsid)			
1A	741-803 or 725-803	21 or 23	VP 4? (not detected in virions)
1B	804-1469	222	VP 2
1C	1470-2207	246	VP 3
1D	2208-≤3107	≤300	VP 1
P2 (Non-structural)			
2A	≤3108-≈3230	≥40	Unknown
2B	≈3231-3995	≈260	Unknown
2C	3996-5000	335	RNA synthesis
P3 (Non-structural and VPg)			
3A	5001-5222	74	Pre-VPg
3B	5223-5291	23	VPg
3C	5295-5948	219	Protease
3D	5949-7415	489	RNA polymerase

The ORF encodes for all the viral proteins. The region coding for the capsid proteins (1A-1D) is designated P1, and is 2 373 nt in length. P2 and P3 code for the non-structural proteins, 2A-2C and 3A-3D respectively, and are found

downstream from P1 (Levy et al., 1994; Beard and Lemon, 1999). These regions therefore code for proteins needed for RNA synthesis and the three proteases required for polyprotein cleavage (Table 1) (Levy et al., 1994; Hollinger and Ticehurst, 1996; Cuthbert, 2001; Feinstone and Gust, 2002). The 3′ NCR, is a short virus coded poly-(A) tail of 40-80 nt (Hollinger and Ticehurst, 1996; Feinstone and Gust, 1997).

1.4 RESISTANCE TO CHEMICAL AND PHYSICAL AGENTS

A major feature of HAV is its stability that is directly related to the virions structure. HAV is resistant to various physicochemical agents and may therefore survive for days and even weeks in water, shellfish, marine sediments and soil and so pose a potential threat to public health (Feinstone and Gust, 1997; Cuthbert, 2001).

1.4.1 Temperature

HAV have been found to survive elevated temperatures of up to 60°C for one hour, 25°C for several weeks and years at -20°C (Anderson, 1987; Feinstone and Gust, 1997; Koopmans et al., 2002). At temperatures greater than 60°C, HAV rapidly loses its infectivity if present in an isotonic solution and inactivation is virtually instant at temperatures of 85°C and higher. The virus will however survive temperatures of up to 80°C in the presence of 1 M magnesium chloride ($MgCl_2$) (Gust and Feinstone, 1988; Zachoval and Deinhardt, 1993).

Faecal suspensions of HAV are more resistant to heat than cell culture derived viruses. This may be due to small particles of faecal material clustering around the virus, shielding it from direct heat (Gust and Feinstone, 1988). In faecal material, HAV does not loose any infectivity after treatment for two hours at 60°C. Only a 100-fold loss in titre was recorded when incubated for

seven days at 37°C or four weeks at 4°C (Gust and Feinstone, 1988).

HAV has also remained viable in pasteurised milk products (Gust and Feinstone, 1988). Dairy foods with high fat content, such as cream and milk, need an exposure time of at least 30 s at 85°C to cause a 5-log reduction in the HAV titre (Bidawid et al., 2000a). Foodborne outbreaks of HA have been linked to the consumption of both raw and cooked shellfish harvested from water contaminated with human sewage (Bidawid et al., 2000a; Mullendore et al., 2001). For the internal temperature of shellfish to reach 100°C, four to six minutes steaming is required (Bidawid et al., 2000a). Steaming or just heating the shellfish to open the shell is insufficient to inactivate any HAV present (Gust and Feinstone, 1988; Abad et al., 1997).

1.4.2 Relative Humidity

HAV infectivity can be preserved for up to one month when dried and stored at 25°C at relative humidity (RH) of 42% (Hollinger, 1985). Faecally suspended HAV survival is inversely proportional to the level of RH and temperature, and the half-life of the virus ranges from more than seven days at low RH (25% ± 5%) and 5°C to two hours at ultrahigh RH (95% ± 5%) and 35°C. In comparison to faecally suspended poliovirus, HAV survives longer on nonporous environmental surfaces (Mbithi et al., 1991). This emphasizes the need for careful handling of any potentially infectious specimens or samples (Gust and Feinstone, 1988; Feinstone and Gust, 1997).

1.4.3 pH

HAV is stable at pH values as low as pH 3, a property the virus shares with other picornaviruses such as enteroviruses (Hollinger, 1985; Gust and Feinstone, 1988). Survival of HAV at pH values as low as pH 1 has been reported (King et al., 2000; Koopmans et al., 2002). HAV can survive in pH 3

for at least three hours at room temperature, but loses its characteristic morphology when left at pH 3 for more than three hours at 25°C or for an hour at 60°C (Hollinger, 1985; Feinstone and Gust, 2002).

1.4.4 Chemical agents

The susceptibility of HAV to chemical agents is very similar to that of poliovirus (Gust and Feinstone, 1988). Since HAV does not have a lipid soluble outer envelope, it is resistant to treatment with 20% diethyl ether, chloroform and 50% trichlorotrifluoroethane (Gust and Feinstone, 1988). Chloroform treatment of faecal suspensions is therefore an important step for virus purification and preparation of bacteria free inocula for cell culture (Gust and Feinstone, 1988). The titre of HAV can be reduced by 5.5 $\log_{10}$ when treated for at least one hour at room temperature with 70% ethanol (Gust and Feinstone, 1988). HAV can be inactivated by treatment with formalin (at a dilution of 1:4 000 for three days at 37°C, 3% for five minutes or 8% for one minute at 25°C) (Hollinger and Emerson, 2001; Feinstone and Gust, 2002) and chlorine (10-15 mg/l residual chlorine concentration for 30 min) (Hollinger, 1985) or chlorine-containing compounds such as sodium hypochlorite at concentrations of 10 mg/l for 15 min at 20°C (Grabow et al., 1983, 1984; Hollinger, 1985). Treating HAV preparations with 0.5-1.5 mg free residual chlorine/l for one hour at pH 7 reduces HAV infectivity (Gust and Feinstone, 1988). To inactivate any infectious HAV in water, treatment with 2.0-2.5 mg of free-residual chlorine/l of water for 15 min should be considered (Hollinger and Ticehurst, 1996; Feinstone and Gust, 2002).

1.4.5 Antiviral agents

Antiviral agents, such as arildone, disoxaril, 3′-methylquercetin, 2,4-dichloropyrimidine and 2-benzimidazole, that usually inhibit the growth of poliovirus and other picornaviruses, do not affect the replication of HAV *in*

vitro. Ribavirin, amantidine, 2-deoxy-D-glucose, dinitrophenol and sodium azide however inhibit HAV replication (Hollinger and Ticehurst, 1996).

1.4.6 Radiation

HAV can readily be inactivated in water by ultraviolet (UV) irradiation at 1-1 W for one minute at a depth of 0.9 cm, or 197 $\mu W/cm^2$ for four minutes. At 60°C HAV becomes sensitive to UV light (Gust and Feinstone, 1988; Hollinger and Ticehurst, 1996).

1.5 ANIMAL MODELS

In early attempts to understand the pathogenesis and epidemiology of viral hepatitis, a number of animals, including nonhuman primates were assessed for susceptibility to HAV infection (Gust and Feinstone, 1988; Hollinger and Emerson, 2001). Researchers attempted to transmit HAV to Old World monkeys such as baboons (Taylor, 1985; Taylor and Prozesky, 1985), chimpanzees (*Pan troglodytes*) (Dienstag et al., 1975, 1976), gorillas, Celebes apes, gibbons, Wooley monkeys, Patas monkeys, Mangabeys and various species of guenons (Bearcroft, 1968; Gust and Feinstone, 1988). New World monkeys such as marmosets (*Saguinus nigricolis*, *S. oedipus*, *S. mystax*) (Hollinger, 1985; Gust and Feinstone, 1988; Hollinger and Ticehurst, 1996; Hollinger and Emerson, 2001) and lesser bushbabies (*Galago senegalensis*) (Grabow and Prozesky, 1975; Grabow et al., 1981) were also investigated as potential animal models for *in vivo* studies. The animal models used most commonly for *in vivo* studies are chimpanzees (Tassopoulos et al., 1986; Bower et al, 2000), marmosets (*S. mystax*), Panamanian owl monkeys (*Aotus trivirgatus*) (Asher et al., 1995) and stump-tailed monkeys, and although the clinical course of HAV infection is similar to that in man, the disease is usually milder than human infection (Vitral et al. 2000; Hollinger and Emerson, 2001; Hornei et al., 2001).

1.6 CLINICAL FEATURES

1.6.1 Clinical course

The course of acute viral hepatitis can be divided into four clinical phases: i) the incubation or preclinical period, ii) the prodromal or preicteric phase, iii) the icteric phase and iv) the convalescent period (Hollinger and Emerson, 2001). The incubation period is the time between exposure and the development of the first symptoms or jaundice and ranges from 10-50 days (average 28-30 days), but depends greatly on the infectious dose at exposure (Beard and Lemon, 1999; Ryder, 1999; Cuthbert, 2001). The ultimate target site of replication for HAV is the hepatocyte where the virus induces disease through an immunopathologic mechanism (Hollinger and Ticehurst, 1996; Beard and Lemon, 1999), but recent reports identified the gastrointestinal tract as the initial site of replication before viruses are transported to the liver via the blood (Asher et al., 1995; Beard and Lemon, 1999; Cuthbert, 2001). HAV infection does not result in permanent liver damage (Beard and Lemon, 1999; Zuckerman and Zuckerman, 1999).

The short prodromal or pre-icteric phase is typically characterised by non-specific symptoms such as fever, fatigue, chills, malaise, myalgia, anorexia, nausea, and vomiting (Zuckerman and Zuckerman, 1999) which are usually as a result of interferon induction (Feinstone and Gust, 1997; Cuthbert, 2001). Diarrhoea, nausea and vomiting are more frequently associated with infections of children (Hollinger and Ticehurst, 1996; Beard and Lemon, 1999). The icteric phase includes the appearance of dark, golden brown urine due to bilirubin being passed into the urine from the liver. The passing of yellow stools and yellow discoloration of skin, mucous membranes, conjunctivae and sclera follows within one week (Hollinger and Ticehurst, 1996; Zuckerman and Zuckerman, 1999; Cuthbert, 2001). Splenomegaly occurs in 5-15% of cases (Hollinger, 1985; Ryder, 1999). Relapsing HA, on one or more occasions, occurs in 3-20% of cases (Hollinger and Ticehurst, 1996; Cuthbert,

2001), but chronic HA has never been reported (Hollinger and Ticehurst, 1996). Mortality due to HA is <1.5% (Cuthbert, 2001; Hollinger and Emerson, 2001) and mostly associated with old age (>70% of deaths in patients older than 49 years old) (Hollinger and Ticehurst, 1996; Feinstone and Gust; 1997; Cuthbert, 2001), liver transplantations, and underlying conditions such as immunodeficiencies, malnutrition, and chronic liver disease (Grabow, 1997; Cuthbert, 2001).

1.6.2 Viremia and viral excretion

HAV RNA can be detected in serum for an average of 17 days before the alanine aminotransferase (ALT) peak and viremia persists for an average of 79 days thereafter (Bower et al., 2000). The average duration of viremia is 95 days, but ranges from 36 to 391 days (Bower et al, 2000), with peak levels in the blood reached prior to maximum ALT levels (Hollinger and Ticehurst, 1996; Bower et al., 2000). Faeces are infectious as early as two to three weeks prior to the onset of jaundice (Gust and Feinstone 1988; Hollinger and Ticehurst, 1996; Zuckerman and Zuckerman, 1999; Cuthbert, 2001), and remain infectious for three to four weeks after ALT levels peak (Polish et al., 1999). HAV RNA can be detected, by reverse transcriptase (RT)-polymerase chain reaction (PCR), in stools for up to 10 weeks after the onset of symptoms (Yotsuyanagi et al., 1996; Robertson et al., 2000). Infectivity studies in tamarins indicate that the RT-PCR positive stools were less infectious than stool specimens where HAV was detected by enzyme immunoassay (EIA) (Polish et al., 1999; Robertson et al., 2000). In the case of relapsing HA, a second phase of HAV excretion can be detected which could prove to be infectious (Cuthbert, 2001). HAV detection in stool, by RT-PCR, detectable anti-HAV IgM and elevated ALT levels have been reported for a patient for up to 11 months after the onset of illness (Inoue et al., 1996; Cuthbert, 2001). In patients infected with human immunodeficiency virus (HIV), the HAV load is higher and the duration of HAV viremia is longer than in non-HIV infected individuals at the onset of symptoms, but the ALT elevation level is less. This

could be due to the compromised immune system of HIV positive patients (Ida et al., 2002).

1.6.3 Treatment

Treatment of HA is mostly supportive and must strive to support, comfort and provide adequate balanced nutrition to the patient. Intravenous (IV) infusion of carbohydrates and protein restriction are usually prescribed to hospitalised patients (Hollinger and Ticehurst, 1996).

1.7 LABORATORY DIAGNOSIS

The clinical, serological and virological events during HAV infection are demonstrated in Figure 3.

1.7.1 Serology

As the faecal viral excretion peaks prior to the onset of symptoms, direct detection or isolation of the virus in faeces during the icteric phase is usually impractical. Diagnosis depends on serological techniques for the detection of anti-HAV IgM during the acute phase of the infection (Koff, 1998; Ryder, 1999; Cuthbert, 2001). Anti-HAV IgM persists for three to six months after infection and is, as a rule, not present after vaccination (Koff, 1998). A variety of sensitive and specific immunoassays, which distinguish between anti-HAV IgM (current or recent infection) and anti-HAV IgG (previous infection) are available (Feinstone and Gust, 1997; Koff, 1998; Ryder 1999; Cuthbert, 2001). Specific HAV IgM and IgG antibodies can be detected by radio-immunoassay (RIA) (Flehmig, 1981; Stapleton et al., 1991; Feinstone and Gust, 1997), enzyme-linked immunoabsorbent assay (ELISA) (Feinstone and Gust, 1997; Mims et al., 1998), EIA (Bower et al., 2000; Feinstone and Gust, 1997), and immunofluorescence (IF) assays (Gust and Feinstone, 1988;

Nadala and Loh; 1990).

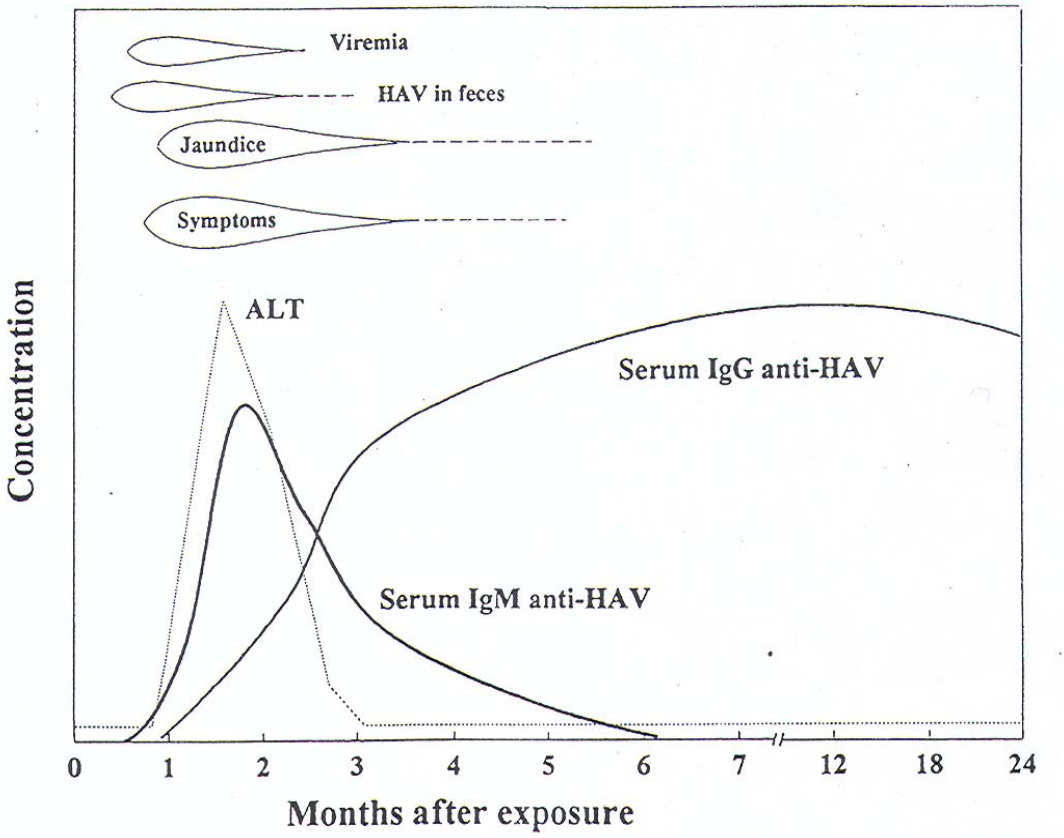


Figure 3: Clinical, virological and serological events associated with HA (Feinstone and Gust, 2002)

1.7.2 Viral detection

HAV can be detected in faecal specimens by electron microscopy (EM) if present in concentrations of at least 10^5-10^6 particles/ml (Taylor et al., 2001), but is indistinguishable from other picornaviruses. Direct demonstration of HAV is therefore impractical for routine diagnostic purposes. Several sensitive antigen (Ag) detection techniques have been developed for the

immunological detection of hepatitis A Ag (HAAg). These include RIA (Stapleton et al., 1991; Feinstone and Gust, 1997), ELISA (Samuel et al., 2000), or EIA (Gust and Feinstone, 1988). Molecular techniques such as RT-PCR (Robertson et al., 1991, 1992; Tsai et al., 1993; Taylor, 1997), Ag-capture PCR (AC-PCR) (Jansen et al., 1990; Robertson et al., 1992), and oligonucleotide probe hybridisation (Divizia et al., 1989a; Legeay et al., 2000) can be used for the accurate detection of HAV in a variety of specimens.

1.7.3 Viral isolation

Wild type (WT) HAV is difficult to propagate in conventional cell culture (Grabow, 1997; Hollinger and Emerson, 2001; Taylor et al., 2001), and even if successful, WT HAV can take weeks, even months, to grow (Hollinger and Ticehurst, 1996). WT HAV does not cause a cytopathogenic effect (CPE) in cell culture and propagation must be monitored by immunological-based Ag detection techniques such as IF, EIA, RIA, radio-immunofocus assay (RIFA) or molecular-based techniques such as RT-PCR-oligonucleotide probe hybridisation and AC-PCR (Taylor, 1985; Stapleton et al., 1991; Robertson et al., 1992; Yap and Lam, 1994; Bishop and Anderson, 1997; Samuel et al., 2000; Taylor et al., 2001). Thus for diagnostic purposes the isolation of HAV is impractical.

1.8 EPIDEMIOLOGY

HAV is highly infectious with an estimated 1.5 million cases reported annually worldwide (WHO, 2000). Epidemics are a prominent feature of the disease (Beard and Lemon, 1999; Taylor et al., 2001), but the true incidence of HA is uncertain because of gross underreporting of cases (Zuckerman and Zuckerman, 1999; Aszkenasy, 2000). Susceptible individuals housed together or in close contact, such as families, young children attending daycare centres as well as the staff in these centres, and individuals held at penal, mental or

other institutions are at increased risk. High-risk groups also include the elderly, homosexual men, drug abusers, sewage workers, foreign travellers, military personnel and immunocompromised people (Lemon and Stapleton, 1993; Hollinger and Ticehurst, 1996; Feinstone and Gust, 2002; Promed, 2004). In addition the disease is predominantly asymptomatic in approximately 90% of small children (Zuckerman and Zuckerman, 1999) who then pose as a reservoir of infection to the adult population who become clinically ill (Aszkenasy, 2000; Termorshuizen et al., 2000). The infection rate in men is 20% higher than in females (Hollinger and Ticehurst, 1996). HAV infections are more frequent in the colder months (Melnick, 1995; Hollinger and Ticehurst, 1996; Feinstone and Gust, 1997).

1.8.1 Routes of transmission

1.8.1.1 Faecal-oral transmission

HAV is predominantly transmitted by the faecal-oral route with person-to-person contact being the most important route of infection (Ryder, 1999; Cuthbert, 2001). Faecal excretion of the virus is maximal prior to the onset of clinical symptoms (Polish et al., 1999), thus facilitating the spread of the virus (Hollinger and Ticehurst, 1996). Nosocomial infections are common, especially when hygienic standards are poor (Watson et al., 1993; Hollinger and Ticehurst, 1996; Cuthbert, 2001). Faecally contaminated food (Conaty et al., 2000; Cooksley, 2000; Jacobs et al., 2000; Bosch et al., 2001) and water (Mahoney et al., 1992; Chernev et al., 2001) have been implicated in a number of outbreaks of HA (Callahan et al., 1995; Hunter, 1997a).

1.8.1.2 Parenteral transmission

Since viremia can be observed as soon as 17 days prior to the onset of jaundice, it can result in parenteral transmission of HAV (Melnick, 1995; Chudy et al., 1999; Bower et al., 2000). This route of transmission is commonly recognised (Seeberg et al., 1981; Ryder, 1999; Delpech et al., 2000; Gilroy et al., 2000; Cuthbert, 2001) and outbreaks of HA have been

linked to inadequately treated factor VIII concentrate (Kedda et al., 1995; Chudy et al., 1999), blood transfusions (Hollinger et al., 1983; Cuthbert, 2001) and needle-sharing between intravenous drug abusers (Grinde et al., 1997; Delpech et al., 2000; Gilroy et al., 2000). There is no evidence that biting insects transmit the disease to man (Hollinger and Ticehurst, 1996; Hollinger and Emerson, 2001).

1.8.1.3 Sexual transmission

This form of transmission has been documented predominantly in homosexual men (Corey and Holmes, 1980; Centers for Disease Control and Prevention [CDC], 1999; WHO, 2000; Promed, 2004). Oral-anal exposure of HAV seronegative homosexual men to their HAV-infected partners, results in HAV infection in 100% of cases (Corey and Holmes, 1980). The use of condoms during homosexual intercourse does not protect the participants against HAV transmission (Ida et al., 2002). Higher seroprevalence rates of HAV infection are associated with oral-anal contact regardless of sexual orientation (Cuthbert, 2001).

1.8.2 The role of food and water in transmission of HAV

1.8.2.1 Water sources

In developing countries, pollution, high density population and inadequate sanitation results in raw water being contaminated by human waste containing large numbers of enteric viruses (Gerba and Rose, 1990; Callahan et al., 1995; Senouci et al., 1996; Gilgen et al., 1997; Backer, 2002; Lee and Kim, 2002). Wastewater produced in developed countries amounts to approximately 300-600 l/person (Mignotte et al., 1999). The infectious dose for HAV is presumed to be as low as 10–100 virus particles (US Food and Drug Administration/Center for Food Safety and Applied Nutrition [FDA/CFSAN], 2004) and can therefore cause illness even when a small volume of contaminated water is ingested (Backer, 2002). Globally, the role that faecally contaminated treated and untreated drinking water plays in HA outbreaks is

becoming more prominent (Mosley, 1967; Grabow, 1976; Bloch et al., 1990; Gerba and Rose, 1990; Divizia et al., 1993; Friedman-Huffmann and Rose, 1998). Contamination of treated drinking water is usually due to poor management or inadequate treatment processes (Arauz-Ruiz et al., 2001), such as in the case of the 1955-1956 HA outbreak in Delhi, India, which claimed 40 000 victims (Grabow, 1976) and the recent outbreak in Murmansk City in Russia (Chernev et al, 2001). The most frequent reported source of faecal contamination of untreated drinking water, which includes groundwater (Gerba and Rose, 1990) and wells (Gerba and Rose, 1990; Divizia et al., 1993), is the overflow or seepage of sewage from septic tanks (Hunter, 1997a,b). Fertilisation of crops with sewage or sewage sludges, domestic landfills, oxidation ponds and deep well injection of sewage also result in contamination of groundwater with enteric viruses (Gerba and Rose, 1990). As the consumption of fresh water rises, increased stress is placed on available drinking water reserves. This could ultimately lead to ecological damage as a result of over-extraction from rivers and groundwater, more highly polluted water sources and an increased struggle for access to water (Hunter et al., 2001).

Norovirus sequences have been detected in selected bottled waters (Beuret et al., 2002), which imply that other enteric viruses could be present. Mineral or bottled water has been recommended for use during outbreaks of waterborne norovirus gastroenteritis (Boccia et al., 2002). The prolonged survival (up to 300 days at room temperature) of HAV in mineral water (Biziagos et al., 1988; Garin et al., 1996) holds important implications for these recommendations.

The incidence of HAV infection acquired during recreational exposure to water is difficult to access. The long incubation period creates uncertainty about the exact route of transmission involved (Fewtrell, 1991). In many cases, the disease is asymptomatic because of a low dose infection and these individuals act as reservoirs for the virus, excreting large numbers of

infectious HAV, which facilitate the spread of the virus (Goyal, 1983). Recent evidence implicates recreational activity in faecally polluted water as a source of HA (Mahoney et al., 1992; Garin et al., 1994; Taylor et al., 1995; Hunter, 1997a). Canoeists and windsurfers are especially at risk since they unavoidably ingest river water when capsized or inhale aerosols (Taylor et al., 1995; Gammie and Wyn-Jones, 1997). Individuals swimming in public swimming pools (Mahoney et al., 1992) or untreated surface water are also at risk of infection (Hunter, 1997a). Currents and recreational activities break up virus-containing solids, releasing the viruses, which could pose a serious health threat to communities utilising this water for drinking, irrigation and recreational purposes (LaBelle and Gerba; 1979; Divizia et al., 1989a).

1.8.2.2 Food sources

Various foods have been implicated in HAV outbreaks (Koopmans et al., 2002). These included ice-slush beverages, lettuce, oysters, iced tea and frozen strawberries (Cuthbert, 2001; FDA/CFSAN, 2004). Minimally processed foods (MPF) such as strawberries (Bidawid et al., 2000b), lettuce (Rosenblum et al., 1990; Bidawid et al., 2000b), frozen raspberries (Ramsey and Upton, 1989) and green onions (Dentinger et al., 2001) have been implicated in foodborne outbreaks worldwide. HA outbreaks have also been linked to delicatessen foods (Weltman et al., 1996; Schwab et al., 2000). These foods pose as possible vehicles of HAV when served uncooked, undercooked or contaminated after cooking when handled by infected food handlers (Weltman et al., 1996).

Molluscs and shellfish are filter feeders that concentrate human pathogens, such as HAV, in their intestines when their coastal growing waters are contaminated with human sewage (Abad et al., 1996; Cromeans et al., 1997; Mullendore et al., 2001). Major outbreaks of HA have occurred as a result of consumption of raw or inadequately cooked oysters and clams obtained from faecally polluted water sources (Conaty et al., 2000; Kaul et al., 2000; Bosch et al., 2001; Cooksley, 2000), of which the Shanghai outbreak in 1988 is one

of the most prominent, where approximately 300 000 people were infected (Halliday et al., 1991; Cooksley, 2000).

1.8.3 Epidemiological patterns

Three basic epidemiological patterns for HAV are recognized throughout the world (Figure 4) (Feinstone and Gust, 1997; Cuthbert, 2001). The endemic pattern is typically found in densely populated lower socio-economic communities and developing countries such as found in South America, North Australia, Africa and certain parts of Asia (Feinstone and Gust, 1997; Grabow, 1997; Taylor, 1997). In high density, low socio-economic communities where sanitation is inadequate or nonexistent, 100% of children acquire immunity before the age of 10 (Abdool Karim and Coutsoudis, 1993; Martin et al., 1994; Feinstone and Gust, 1997; Cuthbert, 2001; Taylor et al., 2001).

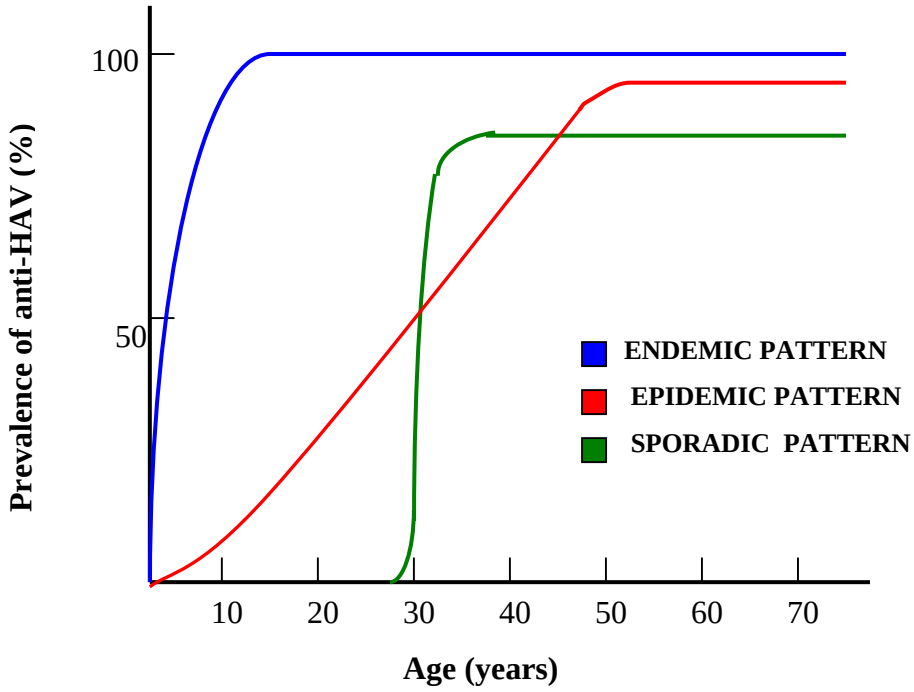


Figure 4: Epidemiological patterns of age specific prevalence of anti-HAV depending on hygienic conditions

(Adapted from Feinstone and Gust, 1997)

This pattern can therefore be applied to the lower socio-economic, predominantly black communities, of South Africa (SA) where all the abovementioned conditions apply (Abdool Karim and Coutsoudis, 1993; Martin et al., 1994; Taylor, 1997). However, with the current trends in urbanization, and as sanitary conditions improve, a change in the epidemic vulnerability of the SA population can be expected (Martin, 1992; Lemon and Stapleton, 1993; Sathar et al., 1994; Melnick, 1995; Taylor et al., 2001). This could result in an increase in the incidence of symptomatic HA in the adult population with associated economic impact (Grabow, 1997).

The sporadic pattern is mostly seen in the developed, higher socio-economic communities and countries where the prevalence of HAV infection increases with age (Feinstone and Gust, 1997; Cuthbert, 2001). Most of these infections are clinical rather than subclinical, with a fatality rate of 1.5% in those over the age of 64 (Martin, 1992; Hollinger and Ticehurst, 1996; Feinstone and Gust, 1997; Cuthbert, 2001). This pattern is typically found in the urbanized predominantly white communities of SA (Martin, 1992).

The epidemic pattern occurs when HAV is introduced into isolated communities. Epidemic infection passes rapidly and so does the virus, since everybody infected in the community develops lifelong immunity against the disease (Hollinger and Ticehurst, 1996; Feinstone and Gust, 1997).

1.9 PREVENTION AND CONTROL

1.9.1 Physical prevention

The most effective control measure to prevent HAV infection is to prevent faecal contamination of food and water sources. This includes personal hygiene, adequate disposal of human waste and securing drinking water reservoirs from faecal contamination (Lemon and Stapleton, 1993; Feinstone

and Gust, 1997; Beard and Lemon, 1999).

1.9.2 Immunological prevention

1.9.2.1 Passive Immunisation

Passive immunisation by means of pooled immunoglobulin (Ig) is a short-term solution to gain temporary immunity to HA (Martin, 1992; Lemon and Stapleton, 1993; Hollinger and Ticehurst, 1996; Cuthbert, 2001). Ig contains anti-HAV antibodies and provides three to five months of protection (CDC, 1999). This means of immunisation is recommended to individuals who need immediate protection such as travellers departing for endemic areas within two weeks and household contacts of infected persons (CDC, 1999; Koopmans et al., 2002).

1.9.2.2 Active Immunisation

Active immunisation with the inactivated virus vaccine provides lifelong immunity is the preferable long-term preventative measure against HAV infection (Hollinger and Ticehurst, 1996; Feinstone and Gust, 1997; Beard and Lemon, 1999). Vaccination is recommended to travellers, homosexual men, intravenous drug users, individuals at occupational risk and those living in endemic areas (Lemon, 1992; CDC, 1999). The vaccine is safe in patients with chronic liver disease, but the immune response is inferior to that observed in healthy individuals (Lee et al., 1997).

1.10 RECOVERY OF HAV FROM WATER AND THE ENVIRONMENT

An effective and sensitive concentration method is fundamental to the successful detection of HAV in water from different sources (Biziagos et al., 1987; Bosch et al., 1991). The recovery and concentration of viruses from water is dependent on the type and nature of the virus and its interaction with

the environment (Gerba, 1987). The electrostatic charge of viruses differs since the proteins that make up their capsids differ in composition. Most viruses, including HAV, are negatively charged in natural waters (Gerba, 1987; Mix, 1987) and therefore require positively charged filters to ensure optimal viral adsorption. The nature of the water from which viruses are to be recovered should also be taken into consideration when choosing a viral recovery and concentration technique (Gerba, 1987).

1.10.1 Factors influencing viral recovery procedures

Waters such as sewage, seawater, drinking water and surface water from rivers and dams, have different compositions and substances dissolved or suspended in them. These dissolved substances influence the choice of viral recovery technique (Gerba, 1987).

The turbidity of the water plays an important role in the recovery and concentration technique. Solids suspended in samples like sewage, river and dam water clog filters, making microporous filter adsorption-elution techniques and ultrafiltration unpractical recovery techniques for viruses in these waters. Solids and soluble organic material can be removed from the water prior to concentration steps by flocculation, adsorption to specific solids (e.g. sand), or by increasing the filter size (Gerba, 1987). Infectious viral particles may be associated with the solids and must be released from these solids prior concentration to facilitate accurate viral enumeration or quantification (Fuhs, 1987; Gerba, 1987; Hurst, 1987). Metallic complexes, formed during pH adjustments in the treating cycle, and precipitates of humic acid could pose a clogging problem during some recovery techniques (Gerba, 1987).

1.10.2 Viral recovery

Filters commonly in use for the recovery of viruses include the following,

nitrocellulose, epoxy fibreglass, fibreglass, charged modified cellulose, and cellulose and glass fibre filters (Gerba, 1987). Various recovery and primary concentration techniques for viruses in water are summarised in Table 2. For HAV, two procedures proved to be efficient for viral recovery; i.e., the adsorption-elution method on flat membranes and ultrafiltration (Panà et al., 1987).

1.10.2.1 Ultrafiltration

The ultrafiltration method refers to the passage of solutions through a cellulose membrane that only permits water and low molecular weight molecules to pass through the pores of the membrane. Viruses and macromolecules are therefore concentrated on the membrane. Tangential flow and hollow fibre configurations reduce clogging of membranes (Gerba, 1987). The two-step tangential flow system is used to process large volumes (>100 l) or as little as 30 ml of water down to 10-15 ml with a 100% recovery rate of HAV (Panà et al., 1987). The ultrafiltration system proved to be efficient for the concentration of viruses from large volumes of environmental samples (Divizia et al., 1989b; Tsai et al., 1993), and can also be applied as a secondary concentration procedure (Rao, 1987).

1.10.2.2 Membrane filters

Filtration of surface water results in reduction of turbidity, as well as pathogens. It also facilitates the removal of organic substances, aggregated viruses and solid associated viruses (Nasser et al., 1995). During viral recovery with membrane filters, being cellulose or any other composition, the viruses in the sample adsorb to the filters. These viruses are then recovered from the filters with an eluting agent that is a slightly alkaline proteinaceous solution. The eluates can be reconcentrated further using a non-proteinaceous solution such as glycine to concentrate the viruses onto a smaller membrane filter (Rao, 1987). This technique can be applied with great success and a recovery rate of 90-100% can be achieved (Passagot et al., 1985; Biziagos et al., 1987; Rao, 1987).

Table 2: Techniques used for recovery and primary concentration of viruses in water.

Method	Volume	Applications	Comments
Filter Adsorption-elution : Negatively charged filters	Large (100-400 l)	All waters, except very turbid samples	• Very efficient for concentrating viruses from large quantities of waters. • Have to adjust pH and cationic concentration before processing
Positively charged filters	Large (<1000 l)	Tap water Seawater Sewage	• No precondition-ing of water necessary before processing of samples
Adsorption-elution: Glass wool, Glass powder or glass beads	Large (<400 l)	Tap water Seawater	• Up to 400 l of water can be pro-cessed through these glass pow-der containing columns

Table 2: Techniques used for recovery and primary concentration of viruses in water (continued)

Method	Volume	Applications	Comments
Organic flocculation	Small (<2 l)	Reconcentration	• Reconcentrates viruses from primary eluates
Protamine sulfate	Small (500 ml-4l)	Sewage	• Efficient for concentration of reo and adeno viruses in sewage
Hydro-extraction	Small (<2 l)	Sewage	• Reconcentration of viruses from primary eluate • Slow processing
Ultra-filtration:			
Soluble Filters	Small (1-10 l)	Clean waters	• Clogs very fast, even when turbidity is very low
Flat membranes	Small (<10 l)	Clean waters	• Clogs very fast, even when turbidity is very low
Hollow fibre or Capillary	Large (>100 l)	Tap water Lake water	• Up to 100 l can be processed • Prefilter sample before processing
Reverse Osmosis	Small (<10 l)	Clean waters	• Cytotoxic compounds also concentrated. This can inhibit assay methods

In an efficient three-step isolation technique for the recovery of enteric viruses, including HAV, seeded water was first filtered with positively charged nylon membranes and eluted with an alkaline buffer (pH 9) containing beef extract. After ultrafiltration and viral RNA purification with a silica-based membrane, viral RNA was detected using molecular techniques (Gilgen et al., 1997).

1.10.2.3 Glass wool and glass powder

Negatively and positively charged glass powder has been used to recover HAV from large volumes of seeded water by adsorption and elution. The charge of the glass powder was modified by polyethylenimine treatment. This is a simple and low cost procedure to perform on location for the recovery and subsequent detection of enteric viruses such as HAV in water. Recovery rates of 61% for sewage to 100% for tap water were achieved (Gajardo et al., 1991). Oiled sodocalcic glass wool have been used for the primary concentration of enteric viruses from seeded water samples in volumes up to 1000 l. Efficiency of recovery (EOR) values of up to 75% was achieved with this recovery technique (Vilaginès et al., 1993).

1.10.2.4 Flocculation

This technique removes the smaller suspended particles and other chemical substances that are too small to settle by gravitational force alone. The chemicals added to the water sample cause particles to stick together as a result of electrostatic and ionic forces (Backer, 2002). Flocculation of HAV can be achieved by the addition of alum to the water sample at a concentration of 30 mg/l, with a recovery rate of up to 88.4%. Recovery is increased to >98% with the addition of a cationic polyelectrolyte at a concentration of 1 mg/l. However, the presence of humic acid in a sample interferes with HAV flocculation (Nasser et al., 1995).

1.10.3 Secondary concentration of HAV from primary eluates

In certain instances it is essential to reconcentrate viruses from primary eluates after the initial recovery method. A few of the methods used to reconcentrate enteric viruses, including HAV, from primary eluates are discussed briefly.

1.10.3.1 Aqueous polymer two phase separation

Composed of a non-ionic polymer, polyethylene glycol (PEG), and an anionic polyelectrolyte, e.g. dextran sulphate. The two polymers are not compatible and the two phases will therefore separate. This separation is temperature, ionic strength and pH dependent. Salts are important in the distribution of viruses between the separate phases. Salt ions distribute unequally in polymer phases e.g. positive ions attract to negatively charged polymers. In this system one phase becomes electrically more negatively charged than the other phase. Negatively charged viruses, such as HAV, favour the more positive upper phase in high salt concentrations (Rao, 1987).

1.10.3.2 PEG hydroextraction

Hydroextraction is the term used when the virus containing sample is placed inside a dialysis tube made of a semipermeable membrane and is surrounded by a hygroscopic material such as PEG. Water is allowed to move freely across the membrane into the PEG, but viruses are trapped and concentrated inside the dialysis tube. Recoveries ranged from 10-100%, depending on modification the investigators used. A 100% recovery has been recorded when this procedure was performed at 4ºC overnight and the membrane rinsed with a 3% beef extract in Tris hydroxymethyl aminomethane solution at pH 9 after 24 h. When sewage or turbid water samples are processed, organic compounds are concentrated with the viruses on the filter membranes. These could flocculate and clog the membranes during the reconcentration steps at low pH levels (pH 3.5). By using a more alkaline buffer, this flocculation can be avoided, so that only viruses are captured on the membrane (Rao, 1987).

1.10.3.3 PEG/sodium chloride (NaCl) concentration

This technique has been used for the concentration and purification of picornaviruses (Minor, 1985) and for the secondary concentration of HAV from water (Biziagos et al., 1987; Vilaginès et al., 1997a). PEG 6000 and NaCl are dissolved in the primary concentrated viral suspension in a ratio of 3:1 and kept at 4ºC overnight. The precipitated virus is concentrated by low speed centrifugation and resuspended in 1:50 to 1:100 of the starting volume.

1.10.3.4 Inorganic precipitates

When a coagulant salt such as aluminium chloride or ferric chloride is added to water sample, the salt dissociates and the metal ion hydrolyses to form positively charged hydroxometal ion complexes (aluminium hydroxide and ferric hydroxide) that adsorbs to negatively charged particles such as viruses. These colloids then aggregate, flocculate, and settle out (Rao, 1987). Recovery rates of these methods range between 80-100%, and subsequent hydroextraction has to be done to reduce the resulting sample volumes to less than 200 ml (Rao, 1987).

1.10.3.5 Organic flocculation

Eluting viruses from membranes with a protein solution, such as a 3% beef extract, gives a very high recovery rate. These primary eluates cannot be concentrated further on another, smaller diameter filter membrane since proteins tend to prevent viruses from adsorbing to the filter. Proteins can be flocculated when lowering pH of the solution to 3.5 (Rao, 1987). The sediment resulting after flocculation and centrifugation at low speed can adsorb viruses and viral recovery rates from these flocs has been documented to be between 60%–91% (Rao, 1987). This method can be used to efficiently recover viruses from wastewater effluents, river water, sludges, soil and sediments (Rao, 1987; Nasser et al., 1995).

1.10.3.6 Centrifugal devices

Different commercial centrifugal devices, e.g. Centricon® concentrators

(Amicon Inc., Beverly, MA), are available for concentration of viruses by ultrafiltration through an anisotropic membrane. Centrifugal force drives solvents and low MW solutes through the membrane into the filtrate vial. Macrosolutes bigger than the membrane's pore size are then concentrated in the sample reservoir and can be recovered.

1.11 ISOLATION, DETECTION AND CHARACTERISATION OF HAV IN FOOD AND WATER SAMPLES

1.11.1 Isolation of HAV in cell culture

Cell cultures are traditionally used to detect potentially infectious viruses in a specimen or sample (Tsai et al., 1993). However, optimal replication of HAV can only be accomplished with the particular combination of cell type, HAV strain and temperature (Siegl et al., 1984; Cromeans et al., 1989; Hollinger and Ticehurst, 1996). HAV is very difficult to adapt and grow in cell culture (Beard and Lemon, 1999) and even cell culture-adapted strains can take weeks to reach optimal concentrations, should optimal conditions apply (Cromeans et al., 1988,1989; Hollinger and Ticehurst, 1996). Fifty per cent tissue culture infectious dose ($TCID_{50}$) values per ml of 10^6-10^9 are typical maximal concentrations for cell culture-adapted HAV strains after mechanical lysis of the cells, since progeny virus are mainly cell-associated and spread to adjacent cells in an as yet unknown manner (Hollinger and Ticehurst, 1996). HAV binds to its host cell in a calcium-dependant manner (Beard and Lemon, 1999). The development of CPE and replication kinetics of HAV in cell culture depends on the interaction between the viral component and the cells at certain passage levels (Anderson, 1987; Cromeans et al., 1988, 1989; Hollinger and Ticehurst, 1996).

Rapid passaging of certain isolates, e.g. HM-175, results in mutational pressure on the viral genome (Gust and Feinstone, 1988; Hollinger and

Ticehurst, 1996) which can shorten the replication cycle dramatically (Cromeans et al., 1989). Certain phenotypes of HM-175 represent the effects of multiple mutations (Hollinger and Ticehurst, 1996) and adaptation to and growth in certain cell lines is dependent on the number and region of viral genes where the mutations took place (Hollinger and Ticehurst, 1996). Mutations in the 2B and 2C gene will lead to possible adaptation to growth in cell culture, but when in the 5′ NCR they are more important for enhancement of growth and altering of host range *in vitro* (Hollinger and Ticehurst, 1996).

1.11.1.1 Cell cultures investigated

A number of cell cultures have been investigated intensively for the isolation and propagation of HAV *in vitro* (Hollinger and Ticehurst, 1996). Amongst these were nonhuman primate cell lines such as the foetal rhesus monkey kidney cell line, FRhK-4, and derivatives thereof (Frp/3, FRhK-6, and FRhK-4R) (Deinhardt et al., 1981; Panà et al., 1987; Gust and Feinstone, 1988; Ashida et al., 1989; Hollinger and Ticehurst, 1996) and an African green monkey kidney cell line, AGMK (Daemer et al., 1981; Gust and Feinstone, 1988). Human cell lines studied include the Detroit-6 cell system (Gust and Feinstone, 1988), a primary hepatocellular carcinoma cell line (PLC/PRF/5 or Alexander cells) (Alexander et al., 1976; Frösner et al., 1979; Hollinger and Ticehurst, 1996), and human fibroblasts (MRC-5) (Flehmig et al., 1981; Siegl et al., 1984). The PLC/PRF/5 (Frösner et al., 1979) and FRhK-4R cell line, a rapidly growing variant of the FRhK-4 cell line (Flehmig, 1981), have been shown to be the most susceptible cell lines for the isolation and propagation of HAV.

A number of nonprimate cell lines were also investigated for susceptibility to HAV infection. Twenty different cell lines, derived from mouse, horse, bovine, rat, hamster, cat, dog, guinea pig, dolphin, pig, turtle, sheep, and quail origin, were inoculated with cell culture-adapted HM-175 strain of HAV. Only cell lines derived from guinea pig (GPE), dolphin (SP 1K) and pig (IB-RS-2 D10) origin supported the growth and replication of a cell culture-

adapted HM-175 strain, but not the WT HM-175 strain (Dotzauer et al., 1994).

1.11.2 Detection of HAV in water samples

There is a definitive need for rapid detection and identification techniques for viruses in environmental food and water sources (Koopmans et al., 2002). The major interests in these fields are to determine whether the detected viruses are viable and potentially infectious. Techniques not based on infectivity cannot distinguish between viable and nonviable viruses (Hermann, 1987).

1.11.2.1 HAAg detection techniques

The detection of HAAg does not necessarily correlate with the presence of infectious virus (Cromeans et al., 1989). Different immunological-based HAAg detection techniques include EM, RIA, EIA, IF and agglutination tests (Hermann, 1987). These techniques have a detection limit of 10^5–10^6 particles/ml are not sensitive enough for the detection of HAV in environmental samples (Taylor et al., 2001). Recently an IF technique has been described that can be used to detect and quantitate HAV in sewage effluent and agri-food surfaces (Kukavica-Ibrulj et al., 2003). Modified immunological-based assays such as RIFA (Lemon et al., 1983; Bishop and Anderson, 1997) and Blue-Cell ELISA (Samuel et al., 2000) are useful for the detection of HAV and HAAg in infected cell cultures.

1.11.2.2 HAV nucleic acid detection

Another approach, also not based on detecting infectious viruses, is the detection of viral nucleic acids in the samples by molecular-based techniques such as cDNA/RNA (Divizia et al., 1989a), RNA/RNA (Shieh et al., 1991) and RT-PCR hybridisation assays using specific probes (Metcalf et al., 1988, Gilgen et al., 1997; Hermann, 1987; Legeay et al., 2000). The RT-PCR oligonucleotide probe hybridisation technique is an extremely sensitive technique and ideal to detect small quantities HAV RNA directly in

environmental samples or after cell culture infection (Dubrou et al., 1991).

RT-PCR techniques are very sensitive, rapid and specific and are able to detect very small quantities of viral nucleic acid in water samples (Morace et al., 1993; Lewis et al., 2000). As few as 0.002 plaque forming units (pfu) per reaction could be detected by PCR techniques (Tsai et al., 1993). PCR techniques give more rapid results than the lengthy procedures of cell culture, but a positive RT-PCR result does not necessarily indicate viable, infective HAV particles that could pose a threat to public health (Tsai et al., 1993). A number of RT-PCR and modified RT-PCR techniques for the rapid detection of HAV have been described (Agnès et al., 1994; Lees et al., 1994; Taylor et al., 1997; Schwab et al., 2001). Modified techniques include an AC-PCR method (Jansen et al., 1990; Graff et al., 1993; Deng et al., 1994) and immunomagnetic capture (IC)-PCR technique (Jothikumar et al., 1998). The ideal method for the detection of infectious HAV would be to integrate cell culture with immunological-based or molecular-based techniques.

1.11.3 Characterisation of HAV

HAV are classified into seven genotypes based on the nucleotide sequences of the VP3/VP1 terminus and VP1/VP2A junction of the genome. Three of these are associated with simian infections and the other four with human infection (Robertson, 2001; Mbayed et al., 2002). In some geographical regions such as Japan, China and USA, the clustering of isolates indicates endemic spread of HAV, while the patterns seen in Western Europe suggest imported cases (Taylor, 1997). Sequence analysis of HAV strains can thus be a useful tool in tracing the source of contamination or route of infection during water- and foodborne outbreaks (Costa-Mattioli et al., 2001a; Diaz et al., 2001; Koopmans et al., 2002). HAV is hyperendemic in SA (Robertson et al., 1992), but there are limited data available on the genotypes circulating in the different socio-economic communities (Kedda et al., 1995; Taylor, 1997).

1.12 HAV QUANTIFICATION TECHNIQUES

Two basic procedures can be implemented for the quantification of viruses in cell culture: quantal methods such as $TCID_{50}$ and the most probable number (MPN) techniques, or enumerative methods such as plaque assays (Cromeans et al., 1987; Sobsey, 1987). HAV replicates slowly and inefficiently in cell culture, resulting in low yields of viral particles and often causing persistent, non-cytopathic infection (Dotzauer et al., 1994; Bishop and Anderson, 1997; Klinger et al., 2001). The virus remains strongly cell-associated when grown in cell culture, and spread between cells by an unknown mechanism (Bishop et al., 1994; Gosert et al., 2000).

Progeny virus particles of the fast growing, cell culture-adapted strains, such as pHM-175, rarely exceed titres of 10^7 $TCID_{50}$ (Gosert et al., 2000). Typically, less than 30% of infectious virus, WT HAV or cell culture-adapted strains, may be released in the supernatant during propagation, although up to 50% has been documented (Bishop et al., 1994). This, together with the absence of CPE in most cell systems, makes the quantification of HAV in cell culture a difficult task (Cromeans et al., 1989). Infection and replication can therefore only be monitored by the detection of viral antigens (Cromeans et al., 1989) or nucleic acid in the infected cell (Agnès et al., 1994; Chaves et al., 1994).

1.12.1 Quantal methods

$TCID_{50}$ and MPN both qualify as quantal methods for the determination of viral titres (Sobsey, 1987; Hierholzer and Killington, 1996). Quantal techniques do not enumerate infectious particles in the original viral stock suspension, only the dilution that will give CPE in 50% of inoculated cells (Hierholzer and Killington, 1996).

1.12.1.1 $TCID_{50}$ technique

$TCID_{50}$ is defined as the dilution of a virus required to infect 50% of a given batch of inoculated cell cultures (Levy et al., 1994; Hierholzer and Killington, 1996). The data from each infected tube or well is used for the calculation of the $TCID_{50}$ of the initial virus stock suspension. This can be done applying the Reed-Muench, Kärber or Spearman-Kärber methods (Grist et al., 1979; Hierholzer and Killington, 1996).

1.12.1.2 MPN technique

The MPN methods can be calculated by various designed calculations, and is an estimate of the number of infectious organisms present per volume in a specific sample at a specific dilution (American Public Health Association [APHA] et al., 1998).

1.12.2 Enumerative methods

1.12.2.1 Plaque assays

The plaque assay is an infectivity assay that enumerate the number of infectious viral particles present in a given virus suspension, since every foci, or plaque, originates from a single infectious virus. Viral titration is therefore very accurate and precise (Guttman-Bass, 1987; Sobsey, 1987; Hierholzer and Killington, 1996). Plaque assays are divided into suspension assays and monolayer assays (Hierholzer and Killington, 1996). Infectivity titre is expressed as pfu/ml (Hierholzer and Killington, 1996).

Plaques generated by HAV in cell culture are small and not well defined, and immunological-based Ag detection techniques can be used to enhance their visibility with a detector such as radioactivity (Lemon et al., 1983; Wheeler et al., 1986; Gust and Feinstone, 1988), fluorescence (Nadalah and Loh, 1990) or peroxidase (as for immunoperoxidase [IP] and ELISA techniques) (Nasser and Metcalf, 1987; Nadalah and Loh, 1990; Usuba et al., 1990; Yap and Lam, 1994). IP has three important practical advantages compared to IF which add

to the cost effectiveness and simplicity of the procedure. Staining in IP is permanent and can therefore be stored indefinitely, results can be read under an ordinary light inverted microscope and cells can be grown in plastic wells instead of glass when doing peroxidase staining (Nadala and Loh, 1990). It is important to note that for the cell culture-adapted strain pHM-175, only one virus particle per 200 physical particles is infectious (Chudy et al., 1999). This could lead to inaccuracies when quantitating total viral titres in seeded water samples.

1.13 RISK ASSESSMENT

Water has been associated with outbreaks of a wide spectrum of viral infections worldwide (Hunter, 1997a,b). The waterborne transmission of HAV is well documented (Mahoney et al., 1992; Hunter, 1997a). HAV can survive a broad range of temperatures (-20ºC to 60ºC) (Feinstone and Gust, 1997; Koopmans et al., 2002), standard chlorination practices of drinking water supplies (Feinstone and Gust, 2002), low RH (42%) (Mbithi et al., 1991) and pH values (pH 1) (King et al., 2000). HAV has been shown to survive for months in experimentally contaminated fresh water, seawater, marine sediments, wastewater, soils and oysters (Hollinger and Emerson, 2001). Consequently, depending on conditions, the virus could be stable in the environment for months (Department of Water Affairs and Forestry [DWAF], 1996; CDC, 1999), thereby posing a possible health threat to the human population.

A basic need of any person is to know that the food and water they ingest is safe. Thus, should pathogens be detected in the food or water sources, the possible risk of infection to the consumer should be determined. Drinking water supplies worldwide are being contaminated with enteric viruses as a result of water pipes leaking and insufficient or faulty treatment processes (Haas et al., 1993; Gale, 2001), posing a possible risk of HAV infection to

consumers (Hunter, 1997a). The detection, quantification and characterisation of pathogens in drinking water is essential for quantitative risk assessment and is considered essential for the monitoring of microbial safety and quality of source and treated waters (Haas et al., 1999). Even though viral concentrations may be low, drinking water is used by every member of a population, so that even a low risk of infection may affect a significant number of consumers.

Recreational exposure to polluted water has often been linked to HA outbreaks (Mahoney et al., 1992; Gammie and Wyn-Jones, 1997; Hunter, 1997a). Risk of infection increases with increased immersion in contaminated water (Taylor et al., 1995; Gammie and Wyn-Jones, 1997). Enteric viruses, such as HAV, are excreted by infected individuals in numbers of up to 10^{11}/g faeces. Faecal contamination of recreational water can occur as a result of storm water discharge, runoff, sewer overflow, or sewage discharge directly into the water source (DWAF, 1996).

There are four basic steps to quantifiable risk assessment (Haas and Eisenberg, 2001): i) hazard assessment, ii) exposure assessment, iii) dose-response analysis, and iv) risk characterisation. Variables that can influence the assessment include the EOR, infectious dose, estimated volume of water ingested per person, the immune status of the population studied, etc. (Haas et al., 1999). An important factor in risk assessment is the acceptable risk of infection in a given community. At present there are no such data available for SA. In the USA, the acceptable risk of infection value for drinking water as stated by the USA Environmental Protection Agency (US EPA) is 1 infection per 10 000 consumers per year (EPA, 1986; Regli et al., 1991; Macler, 1993; Hunter et al., 2003) based on dose-response data for rotaviruses (Ward et al., 1986) and *Giardia* (Regli et al., 1991; Macler and Regli, 1993). There are few data on the acceptable limit for viruses in recreational water (Guidelines for Canadian Recreational Water Quality, 1992), and water quality guidelines and acceptable risks are mainly based on levels of indicator

organism (EPA, 1986; Guidelines for Canadian Recreational Water Quality, 1992; López-Pila and Szewzyk, 2000). Since the infectious dose of HAV is unclear, but considered to be 10-100 particles (FDA/SCAN, 2004), even low levels of faecal pollution could pose a significant risk of infection (DWAF, 1996). The criteria for risk of infection from enteric viruses in full-contact recreation in water in SA are set out by DWAF (1996) (Table 3). The criteria are based on the low infective dose of viruses and on the assumption that ingestion of water during recreational activities does not exceed 100 ml/activity (DWAF, 1996).

Table 3: Full contact recreation: effects of enteric viruses on human health (DWAF, 1996)

Enteric Viruses ($TCID_{50}$/10 l)	Effects
Target Water Quality Range 0	Negligible risk of enteric virus infection expected for single samples
1-10	Meaningful risk of infection may exist, particularly if virus counts in this range are recorded for consecutive samples. Minimal risk expected if occasional/isolated samples yield counts in this range
>10	Significant risk of infection expected, particularly if virus counts in this range are obtained for consecutive samples or >50% of the samples tested. Risk of infection increases as number of viruses increases.

However, with the advent of molecular technology for the detection of enteric viruses in water, guidelines defining water safe for recreational purposes (DWAF, 1996) have the same shortcomings as those for drinking water (Grabow et al., 2001). Water quality guidelines and the health risk of the potential hazard should therefore be interpreted in the context of the country in which they are applied (Dawson and Sartory, 2000).

Since HAV has a clearly defined clinical picture, with complete recovery and subsequent life-long immunity, the virus has been identified as a potential model for risk assessment studies with regard to the virological quality of water. HAV has been detected, by means of cell culture and RT-PCR-probe hybridisation assay, in recreational water sources as well as in drinking water supplies in SA (Taylor et al., 2001). These qualitative results indicate the presence of viable, potentially infectious viruses indicating a possible health risk to consumers. There are no data available in SA on the risk of infection constituted by HAV in recreational surface or drinking water sources to water users.

1.14 MOTIVATION FOR THIS INVESTIGATION

Prevention of HA worldwide is a political, socio-economic and engineering dilemma. Providing communities with clean, fresh drinking water, proper waste disposal programs and improving living conditions will reduce the incidence of HA (Lemon and Stapleton, 1993; Feinstone and Gust, 1997). Due to multiple political and socio-economic changes in SA, there is an improvement in the living standards of the lower socio-economic communities. As potable water and adequate sanitation become more available to these communities a gradual increase in the prevalence of HA is expected (Martin, 1992).

HAV has been detected in rivers and dams used as source water for water purification plants providing drinking water to different regions, and for recreational activities in SA (Taylor et al., 2001). HAV has been implicated in HA in recreational water users e.g. canoeists (Taylor et al., 1995). Although HAV has been detected in surface water in SA there are no data on the actual risk of infection posed by HAV to persons exposed to these water sources through domestic and recreational activities.

In order to develop a reliable cost-effective strategy for the sensitive detection of HAV in drinking and recreational water samples for further risk-assessment studies, HAV recovery, cell culture amplification and molecular-based detection assays will be evaluated to determine which combination of assays is the most sensitive and practically feasible for the routine monitoring of water. To ascertain whether the water could be the source of infection in selected patients, HAV isolates from both water sources and clinical specimens will be characterised to determine their molecular identity and relationship. As there are no data on the role of water in the transmission of HAV in SA, these data will provide valuable information with regard to the contribution of contaminated water to the overall incidence of HA in SA.

1.15 AIMS OF THE INVESTIGATION

The hypothesis for this investigation was that HAV detected in water samples were viable and posed a significant risk of infection to individuals using contaminated water sources for domestic and recreational purposes. To confirm or disprove this hypothesis, the objectives for this investigation were multifold:

1) To reassess and compare the sensitivity of methods for the recovery and detection of HAV in water samples;
2) To investigate techniques for the possible quantitation of HAV in a variety of water sources;
3) To characterise viral isolates from water sources and clinical material to determine whether or not the genotypes associated with human infection, are present in water sources used for human consumption and recreational purposes.
4) To determine the risk of infection constituted by HAV in drinking and recreational water.

CHAPTER 2

ASSESSMENT OF HAV ISOLATION AND DETECTION TECHNIQUES

2.1 INTRODUCTION

There is a need for techniques for the rapid isolation and detection of HAV in environmental sources such as food, surface water and drinking water. The major interests in these fields are to determine whether viruses that can be detected, are viable with the potential to cause infection. Techniques not based on infectivity cannot distinguish between viable and nonviable viruses (Hermann, 1987). The majority of methods for the isolation and detection of enteric viruses from water sources rely on animal cell culture techniques (Gajardo et al., 1991; Schwab et al., 1993). Conventional cell culture procedures used for enteroviruses are, however, inadequate for the isolation of HAV (Grabow, 1997; Hollinger and Emerson, 2001). Although various primary and continuous cell cultures of primate origin support the replication of HAV, WT HAV grows slowly or not at all in conventional cell cultures (Hollinger and Ticehurst, 1996; Hollinger and Emerson, 2001). The success of HAV isolation depends on various factors of which viral strain, cell type, incubation time and temperature are the most important (Hollinger and Ticehurst, 1996). Incubation periods from two weeks up to 12 months (Crance et al., 1987) with incubation temperatures ranging between 32ºC and 37ºC have been reported (Flehmig, 1980; Siegl et al., 1984; Crance et al., 1987; Dotzauer et al., 1994). Selected cell culture systems, namely AGMK, PLC/PRF/5 and FRhK-4R, have been shown to be more susceptible for the isolation and propagation of WT HAV as well as cell culture-adapted strains of HAV (Daemer et al., 1981; Flehmig, 1981; Gust and Feinstone, 1988;

Hollinger and Emerson, 2001). Primary vervet monkey kidney (VK) cells and the Vero cell line have also been reported to be of value, but were not as commonly used as previously mentioned cell cultures (Flehmig, 1981; Wheeler et al., 1986; Crance et al., 1987; Ashida et al., 1989; Stapleton et al., 1991; Bishop and Anderson, 1997; Taylor et al., 2001).

As most WT HAV isolates do not produce CPE in cell culture, alternative techniques to detect HAV infection must be applied. These include immunological-based Ag detection techniques such as IF (Divizia et al., 1989a; Flehmig, 1981; Taylor, 1985; Nadala and Loh, 1990; Blank et al., 2000), RIA (Daemer et al., 1981; Flehmig, 1981; Stapleton et al., 1991), RIFA (Lemon et al., 1983; Bishop and Anderson, 1997) and EIA (Yap and Lam, 1994; Beales et al., 1996; Samuel et al., 2000) as well as molecular-based techniques such as RT-PCR-oligonucleotide probe assay (Robertson et al., 1991, 1992; Agnès et al., 1994; Taylor et al., 2001) and AC-PCR (Jansen et al., 1990; Robertson et al., 1992). The EIA (Nasser and Metcalf, 1987), RT-PCR (Shieh et al., 1995; Cromeans et al., 1997; Taylor, 1997; Legeay et al., 2000) and oligoprobe hybridisation (Jaykus et al., 1996) techniques can be adapted and applied to cell-free systems. ssRNA probes have also been used for the detection of HAV directly from environmental and drinking water sources (Shieh et al., 1991).

In order to ascertain which was the most susceptible cell culture for the routine isolation of WT HAV from different sources, a number of cell cultures were infected with the cell culture-adapted strain, pHM-175, and a WT HAV isolate. Cell cultures of both human, i.e. PLC/PRF/5, and non-human primate origin, i.e. VK, Buffalo green monkey kidney (BGM), FRhK-4R and Vero C1008, were selected for comparative purposes. Viral infection of the cell cultures was monitored by IF (Gust and Feinstone, 1988), RT-PCR (Robertson et al., 1991, 1992; Taylor 1997) and a RT-PCR-oligonucleotide probe assay (Taylor et al., 2001).

2.2 AIMS OF THE INVESTIGATION

The objectives of this investigation were threefold:

i) To determine which cell cultures were the most suitable for the optimal isolation and amplification of WT HAV from water samples;

ii) To determine the optimal incubation period for the early detection of WT HAV in these cell cultures;

iii) To determine the sensitivity of the RT-PCR-oligonucleotide probe hybridisation assay and immunological-based Ag detection techniques.

2.3 MATERIALS AND METHODS

2.3.1 Hepatitis A viruses

2.3.1.1 Cell culture-adapted strain HM-175 43c

The HM-175 strain was isolated from a faecal specimen from a 35 year old male during a HA outbreak in Australia in 1976 (Daemer et al., 1981; Hollinger and Ticehurst, 1996). This HM-175 isolate is the type strain of the genus *Hepatovirus* and is classified as genotype I, subgenotype IB (Robertson et al., 1992). This WT strain of HAV was adapted to grow in cell culture by 17 passages in primary and secondary AGMK cell cultures. The cell culture-adapted strain was then used to infect the BGM cell line with blind passages every three or four days (Gust and Feinstone, 1988). This mutation pressure resulted in viral variants that were either attenuated, associated with persistent infection, cytopathogenic or resistant to neutralisation (Hollinger and Ticehurst, 1996). The HM-175 43c variant (referred to as pHM-175 in this dissertation) used during this investigation causes a CPE when grown in susceptible cell cultures such as AGMK (Daemer et al., 1981), BS-C-1 (a African green monkey kidney cell line) and FRhK-4 (Cromeans et al., 1989).

The HM-175 43c viral stock culture used for this investigation was obtained from Prof A Bosch, Department of Microbiology, Facultat de Biologia, University of Barcelona, Barcelona, Spain.

2.3.1.2 South African WT HAV

The WT HAV containing specimen (923200) used in this investigation was a faecal specimen collected from a HAV IgM positive male patient at a Care and Rehabilitation Centre, Cullinan, SA. The virus was initially detected in the stool specimen by EIA (Hepanostika HAV Microelisa system; Organon Teknika, The Netherlands) and subsequently by RT-PCR (Taylor, 1997). The strain was characterised as genotype I, subgenotype IA (Taylor, 1997).

A 100% (w/v) suspension of stool was prepared in serum-free Eagle's minimal essential media with Earle's salts (E-MEM) to which penicillin (50 µg/ml), streptomycin (50 µg/ml) and neomycin (100 µg/ml) (PSN Antibiotic Mixture [100X], GIBCOBRL, Life Technologies, Paisley, Scotland) was added and homogenised using glass beads. After centrifugation at 1000 x *g* (Biofuge Primo, Heraeus Instruments, Kendro Laboratory Products, Hanau, Germany) for 30 min the supernatant was decontaminated by filtration through a 0.45 µm filter (Ministart® CE, Sartorius AG, Goettingen, Germany). The resultant clear suspension served as WT HAV stock.

2.3.2 Cell cultures

A number of different cell cultures, of both human and non-human primate origin were included in this investigation.

2.3.2.1 PLC/PRF/5 human hepatoma cell line

The PLC/PRF/5 human hepatoma cell line (American Type Culture Collection [ATCC] CRL 8024), also known as Alexander cell, was derived from the liver of a chronic carrier of hepatitis B virus (HBV) who died of primary hepatocellular carcinoma (Alexander et al., 1976). These cells carry copies of

the entire HBV genome but secrete only HB surface Ag (HBsAg) into the culture media (Frösner et al., 1980; Deinhardt et al., 1981; Gust and Feinstone, 1988). The PLC/PRF/5 cell line was found to support the replication of WT HAV, but an incubation period of at least seven weeks at 32°C was needed before any HAAg could be detected and maximal HAV titres could only be demonstrated approximately 10 weeks post infection (p.i.) (Deinhardt et al., 1981; Crance et al., 1987). Cell cultures between passages 98 and 102 were used.

2.3.2.2 FRhK-4R cell line

The FRhK-4 cell line (ATCC CRL 1688), derived from foetal rhesus monkey kidney cells, is similar to the FRhK-6 cell line, but retains it's sensitivity and susceptibility to HAV infection for a longer period (Gust and Feinstone, 1988). Three strains of HAV (GBG, GBM, GJA) were isolated in the FRhK-4 and later FRhK-4R cells (Flehmig, 1980, 1981). The FRhK-4R cell line is a rapidly growing variant of this FRhK-4 cell line (Flehmig, 1981). Serial passaging of the abovementioned virus in the FRhK-4 and FRhK-4R cell lines reduced the time in which viruses could be detected, with some isolates being detected, by RIA, as early as six days p.i. (Flehmig, 1981). Cell cultures, between passages 70 and 100, were used during the investigation.

A clone of the FRhK-4R cell line was kindly supplied by Prof. Dr. B Flehmig, Hygiene-Institut der Eberhard Karls Universität, Tübingen, Germany.

2.3.2.3 BGM cell line

The BGM cell line (European Collection of Cell Cultures [ECACC] 90092601) was derived from African green monkey (C*ercopithecus aethiops*) kidney cells in Buffalo, USA (Dahling et al., 1974). Three strains of HAV, namely MS-1, SD-11 and HM-175, were used to investigate the propagation of HAV in this cell line. The HAAg could be detected, by IF, as early as seven days p.i. in cells infected with HM-175, but an incubation period of up to 21 days at 37°C was needed for the detection of SD-11. MS-1 could not be

detected after 21 days incubation (Daemer et al., 1981). Cell cultures, between passages 80 and 100, were used during this investigation.

2.3.2.4 Vero African Green Monkey cell line (Vero C1008)

The Vero C1008 cell line is a clone of the Vero 76 cell line (ATCC CRL 1587), which is a derivative of the original Vero cell line (ATCC CCL 81). The Vero cell line was initiated from the kidney of a normal adult African Green Monkey in Japan, 1962. Vero C1008 was cloned in 1979 by Price (Hay et al., 1992) and displays some contact inhibition after forming a monolayer and could therefore be useful in growing slow replicating viruses such as HAV. Vero C1008 cell cultures, between passages 20 and 29, were used during the investigation.

2.3.2.5 VK cells

Primary VK cell cultures, prepared from the kidneys of freshly sacrificed vervet monkeys (*C. pygerythrus*), were kindly supplied by the National Institute for Communicable Diseases (NICD) (formerly known as the National Institute for Virology [NIV]), Sandringham, SA, and passaged prior to use. When primary cells are passaged, they become secondary cells, which are more susceptible to viral infection and amplification than primary cells, but have a tendency to degenerate spontaneously due to the presence of endogenous simian viruses (Grist et al., 1979). These endogenous viruses could also interfere with isolation and amplification of infecting viruses (Grist et al., 1979).

Secondary VK cells, between passage four and 10, were used during the investigation.

2.3.3 Media and reagents

Each cell line or cell culture has its own unique requirements regarding growth and maintenance media (Grist et al., 1979; Ham and McKeehan, 1979).

2.3.3.1 Foetal calf serum

Foetal calf serum (FCS) contains a large number of different growth promoting factors and is therefore an ideal supplement for promoting cellular multiplication. FCS protein neutralises trypsin and other proteases and helps the cells to overcome the damage done to them during subculturing procedures. Serum also acts as a buffer between the cells and toxic concentration of nutrients by binding to the nutrient molecules and releasing them in small non-toxic quantities for cellular growth (Ham and McKeehan, 1979).

The FCS (Delta Bioproducts, Johannesburg, SA), which had been screened for mycoplasmas and bovine viruses and contained <2 EU, was decomplemented for 30 min at 56ºC and stored at -20ºC.

2.3.3.2 Growth media

E-MEM with Earle's salts, L-glutamine, non-essential amino acids, penicillin (100 U/ml) and streptomycin (100 µg/ml) (NICD) supplemented with 5-7% FCS was used for the propagation of the VK, Vero and BGM cell cultures. As the PLC/PRF/5 cell line has an endogenous mycoplasma, the same medium, with the addition of 60 µg/ml tylocine (anti-PPLO, GIBCOBRL), was used. The FRhK-4R cell line was propagated in a 1:1 mixture of E-MEM with Earle's salts and E-MEM with Hank's salts (EMEM-HMEM), with L-glutamine, non-essential amino acids, penicillin (100 U/ml) and streptomycin (100 µg/ml) (NICD) supplemented with 5-10% FCS.

2.3.3.3 Maintenance media

E-MEM supplemented with 0.5-2% FCS was used for the maintenance of the VK, Vero, PLC/PRF/5 and BGM cell cultures. Tylocine was added to the maintenance media used for the PLC/PRF/5 cell line at a final concentration of 60 µg/ml. The FRhK-4R cell line was maintained in EMEM-HMEM supplemented with 1% FCS.

2.3.3.4 Cryopreservation media

The cryopreservation medium for the VK, Vero, PLC/PRF/5 and BGM cell cultures was prepared with double strength E-MEM (Highveld Biological [Pty] Ltd, Lyndhurst, SA) with the addition of 20% FCS, 10% glycerol (Sigma Chemical Co., St. Louis, MO) and penicillin and streptomycin (Penicillin-Streptomycin 100X, GIBCOBRL). For the FRhK-4R cell line, double strength 1:1 EMEM-HMEM (Highveld Biological) was used as the base medium. The glycerol is added to protect the cells' membranes against crystal formation during the cryopreservation process (Coriell, 1979).

2.3.3.5 Starvation media

Serum-free E-MEM was used to starve the VK, PLC/PRF/5, Vero and BGM cell cultures prior to infection. FRhK-4R cell cultures were starved with serum-free EMEM-HMEM.

2.3.3.6 Activated trypsin-versene

The trypsin-EDTA solution used contained 0.25% trypsin and 0.05% EDTA diluted in $Ca^{2+}Mg^{2+}$-free Dulbecco buffer, supplemented with 200 U/ml penicillin and 100 µg/ml streptomycin (NICD).

2.3.3.7 Agar overlay

The agar overlay for the plaque assays contained prewarmed (37°C) double strength EMEM-HMEM containing 100 U/ml penicillin, 100 µg/ml streptomycin and 2% FCS to which 26 mM $MgCl_2$ was added. This was mixed thoroughly with an equal volume autoclaved sterile 1% agarose (SeaKem® ME agarose, FMC BioProducts, Rockland, ME), which had been cooled to 55ºC. The agarose-media mixture was cooled to 37ºC before overlaying infected cell culture monolayers.

2.3.4 Cell culture techniques

Standard published cell culture techniques for the propagation, maintenance and infection of the cell cultures (Grist et al., 1979; George et al., 1996) were used.

2.3.4.1 Subculturing of cell cultures

Cells were subcultured in a certain ratio depending on the density of the monolayer, the volume of the flask used and purpose for which the cells were required. The cells were detached from surface of the flask by the addition of trypsin-EDTA followed by incubation for 1-3 min at 37ºC. The trypsin-EDTA was neutralised by the addition of an equal volume of growth media. The cells were centrifuged at 300 x *g* (Rota Uni II, BHG, Germany) for 2 min to form a loose pellet. The supernatant was discarded and the resulting pellet was resuspended in growth media at a concentration of 10^5 cells/ml and seeded into flasks as required. The cells were incubated at 37°C in a humidified 5% CO_2 atmosphere. Fresh growth media was added to the cells in their logarithmic phase to ensure a healthy confluent monolayer.

2.3.4.2 Maintenance of cell cultures

For the maintenance of infected cell cultures maintenance media, with reduced concentrations of FCS, was used. For HAV propagation maintenance media was changed every 7-10 days p.i. to ensure cell survival.

2.3.4.3 Cryopreservation of cell cultures

After detachment of the cells from the surface of the flask by trypsinisation, the cells were pelleted by gentle centrifugation at 300 x *g* (Rota Uni II) for 2 min. The resultant loose pellet was resuspended in 1 ml cryopreservation medium and transferred to cryovials. Cryopreservation of cell cultures was done gradually to avoid cell membrane-damaging crystals forming in the cells (Coriell, 1979). The cells were first held for 2 h at 4ºC, then for 2 h at -20ºC and finally transferred to -70ºC.

2.3.4.4 Revival of cryopreserved cell cultures

Frozen cell cultures were thawed rapidly at 37ºC to prevent damage to the cell membranes. The thawed cell culture was resuspended in growth media, centrifuged at 300 x *g* (Rota Uni II) for 2 min, and the resulting loose cell pellet resuspended carefully in 2 ml growth media and seeded into a 25 cm^2 cell culture flask thinly coated with FCS. The cells were incubated undisturbed for 48 h at 37ºC, after which the appropriate volume of prewarmed growth medium was added to the flask.

2.3.4.5 Preparation of microtitre trays and tissue culture dishes

For infection experiments, 25 cm^2 cell culture flasks, 6-well NUNCLON™ Multidishes and 96-well NUNCLON™ MicroWell™ plates (NUNC™ Brand Products, Nalge Nunc International, Roskilde, Denmark), or 16-well Lab-Tek® tissue culture chamber slides (Nalge Nunc International, Naperville, IL) were seeded with the appropriate volume of 10^5 cells/ml and incubated at 37ºC in a humidified 5% CO_2 atmosphere for 24-48 h.

2.3.4.6 Infection of cell cultures

Prior to infection the growth medium was withdrawn from semi-confluent (70-80%) monolayers and the cells were starved for 60 min at 37ºC in serum-free starvation medium. After withdrawal of the starvation medium, the appropriate volume of virus or treated sample was added and allowed to adsorb to the cells for 1 h at 37ºC, with gentle swirling every 15 min. The appropriate volume of maintenance media was then added followed by incubation at 37ºC in 5% CO_2 in air in a humidified atmosphere.

For plaque assays, confluent (100%) monolayers of FRhK-4R cells were infected with the appropriate volume of virus inoculum and after adsorption, the inoculum was withdrawn. The infected cells were covered with 5 ml of the overlay agar, followed by incubation at 37ºC in 5% CO_2 in air in a humidified atmosphere.

2.3.5 Virus detection

2.3.5.1 Immunological-based assays

a) IF assay

Immunofluorescent assays have been used by a number of investigators for the detection of HAAg in infected cell cultures and animal tissue (Flehmig, 1980; Daemer et al., 1981; Siegl et al., 1984). These assays can be subdivided into two categories: direct IF (DIF) and indirect IF (IIF). The DIF technique uses a virus-specific fluorescein-isothiocyanate (FITC)-labelled antibody as a detector, while IIF uses an unlabelled virus-specific antibody as the primary antibody with a secondary fluorescein-labelled antibody as the detector.

In this investigation the IIF method was used to detect HAAg in the infected cell cultures. In brief, infected cell cultures were rinsed with $Ca^{2+}Mg^{2+}$-free phosphate buffered saline (PBS) (PBS [10X] w/o calcium and magnesium; GIBCOBRL), air dried, and fixed with 100% acetone at -20ºC. A 1:50 dilution of human convalescent phase anti-HAV positive serum was used as the primary antibody and goat anti-human IgG-FITC conjugate (Zymed Laboratories, Inc., San Francisco, CA) as the detector. A counter stain, Evans blue (Merck, Darmstadt, Germany) (1% in PBS), was applied to enhance the fluorescence. The HAAg was visualised under a fluorescence microscope as clusters of fine immunofluorescent granules in the cytoplasm of the HAV infected cells (Gardner and McQuillin, 1974; Gust and Feinstone, 1988). Uninfected or negative cells appear red under the UV illumination.

b) IP focus assay

The IP focus assay (IPFA) has all the advantages of IF but with the benefit of using a light microscope to visualise the colour reaction. It is a sensitive, easy to apply method capable of detecting antigens or plaques in infected cell cultures. The assay is essentially the same as the IF assay (section 2.3.5.1a) except that the secondary detector antibody is linked to horseradish peroxidase (goat anti-human HRP conjugate; Zymed) and presence of HAAg is visualised by a permanent colour reaction.

A number of different insoluble chromagens, namely 3,3'-diaminobenzidine tertrahydrochloride (DAB), 4-chloro-1-naphthol (4CN) and 3-amino-9-ethylcarbazole (3AEC), were compared to determine which was most suitable for the visualisation of HAAg in infected cell cultures. A recently described modified 3,3′,5,5′-tetramethylbenzidine (TMB) method (Blue-Cell ELISA) (Samuel et al., 2000) was also assessed. In brief: The infected cell culture plates were incubated with a 1:20 dilution of monoclonal human anti-HAV antibodies made in sterile PBS supplemented with 2% bovine serum albumin (BSA) (pH 7.4) and incubated for 60 min at 37ºC. The unbounded antibody solution was removed by consecutive washing steps with sterile PBS (pH 7.4). A 1:500 dilution of goat anti-human HRP conjugate (Zymed), made with sterile 2% BSA/PBS (pH 7.4), was added to the infected plates and incubated for 30 min at 37ºC. After washing the excess conjugate off with sterile PBS (pH 7.4), the uninfected cells were blocked with a sterile 2% BSA/PBS (pH 7.4) solution for 30 min at 37ºC. The excess blocking solution was washed off thoroughly with PBS (pH 7.4), and the chromogen added. Plaques or infected foci could then be visually documented and used for quantification purposes.

2.3.6 Viral nucleic acid detection

2.3.6.1 RNA extraction

Total RNA was extracted from harvested cell culture material, stool specimens, water samples and serum using the method most suitable for the type of specimen being processed. A total of three different RNA extraction methods were applied. A negative extraction control (distilled water) was included with each extraction to ensure that there was no contamination during the extraction procedure.

a) TRIZOL® RNA extraction

RNA present in treated stool specimens, infected cell culture material and environmental water samples was extracted using the TRIZOL® (GIBCOBRL) RNA extraction method following manufacturer's instructions. The TRIZOL®

reagent is a mono-phasic solution of phenol and guanidine isothiocyanate that facilitates rapid total RNA extraction from a variety of samples.

In short, TRIZOL® reagent homogenises the starting material while maintaining the integrity of the RNA present. Cellular material is disrupted and cell components dissolved. Chloroform is added and after centrifugation the solution separates into an organic phase and an aqueous phase. RNA is precipitated from the upper aqueous phase with absolute ethanol and isopropanol. For this study the method was modified and instead of using isopropanol for the final precipitation as recommended by the manufacturer, 3 M sodium acetate (pH 8.3) (seeDNA™ co-precipitant; Amersham International plc, Little Chalfont, Buckinghamshire, United Kingdom [UK]) was used to improve visualisation of the pellet. The precipitated protein-free RNA was resuspended in 30 µl sterile nuclease-free water (Promega Corp., Madison, WI) containing 0.1 µl (4 U) ribonuclease inhibitor (rRNasin®; Promega).

b) QIAamp® Viral RNA Mini Kit

Viral RNA was extracted from cell free material such as serum and cell culture supernatant using spin column technology (QIAamp® Viral RNA Mini Kit; QIAGEN GmbH, Hilden, Germany) according to the manufacturer's protocol.

Briefly the principle of this method of RNA extraction is: The viral particles in the serum or cell-free specimen are lysed in a denaturing buffer which contains a chaotropic salt, which also inactivates RNases and preserves intact viral RNA. The lysate is then applied to the membrane. Carrier RNA, contained in the buffer enhances the binding of the viral RNA to the silica-gel based membrane. Buffering conditions of the sample lysate are then adjusted so that the isolated RNA binds optimally to the membrane. The salt concentrations and pH values of the lysate ensure that protein and other contaminants are not retained on the membrane but rinsed away during the two washing steps by buffers. The RNA is eluted in a final volume of 60 µl

low salt buffer provided. The viral RNA can be concentrated further by precipitation with seeDNA™ (Amersham).

c) RNeasy® Mini Kit

The principle of the RNeasy Mini Kit (QIAGEN) is based on the combination of microspin technology and the selective binding properties of a silica-gel based membrane.

Briefly the principle of this method of RNA extraction is: Harvested cell culture suspensions were disrupted in the presence of a lysating buffer and homogenised in a QIAshredder (QIAGEN). Total RNA was extracted from the homogenate using spin columns provided according to manufacturer's instructions. DNA was removed by using RNase-free DNase set protocol (QIAGEN) according to manufacturer's recommendations. A specialised high-salt buffer system in which the homogenised lysate is diluted only allows RNA longer than 200 bases to bind to the silica-gel based membrane. Smaller, unwanted RNA molecules such as ribosomal RNA (rRNA) and transfer RNA (tRNA) are able to pass through the column and are effectively rinsed away by washing steps, leaving only high quality viral and cellular mRNA in the spin column. This RNA is eluted in 30 µl nuclease-free water provided.

2.3.6.2 RT-PCR protocol

The genomic region of HAV selected for the RT-PCR detection of HAV from water samples, infected cell cultures and clinical specimens was the variable region within the capsid protein, namely the C terminus of VP3 through the N terminus of VP1 (VP3/VP1). The region was selected as it was found to detect HAV strains from different regions of the world (Robertson et al., 1991, 1992), including SA (Taylor, 1997).

a) Oligonucleotide primers

A published set of primers: reverse primer: 5′–CAAAGAAAGTACCTGAG ACATTTCC-3′; forward primer: 5′-GTTTTGCTCCTCTTTATCATGCTA TG- 3′, amplifying the VP3/VP1 capsid region (nt 2176-2414) with an expected product size of 247 base pairs (bp) was used (Robertson et al., 1991). The primers were synthesised by Sigma-Genosys Ltd., Pampisford, UK.

b) Amplification by RT-PCR

To exclude the possibility of cross-contamination, reagents for the RT-PCR were prepared in a laminar flow cabinet. The RT-PCR reactions were carried out in rooms separate from those used for the initial processing of the water samples and clinical specimens, and for the analysis of the amplicons.

The RT-PCR reaction, as described by Taylor (1997), was applied. Reverse transcription was performed in a 48 µl reaction cocktail. The cocktail contained extracted RNA (5-10 µl for clinical specimens; 15 µl for water samples and cell culture extracts), 5 U of avian myeloblastosis virus (AMV) RT (Promega Corp.), 1X PCR buffer (10 mM Tris-HCl [pH 8.8], 25 mM potassium chloride [KCl], 3.5 mM $MgCl_2$) (Opti-Prime™ 10X PCR buffer #7, Stratagene, La Jolla, CA), 100 ng reverse primer and 4 U ribonuclease inhibitor (rRNasin®, Promega Corp.) and 0.5 mM dideoxy nucleotide triphospates [dNTP] (PCR grade) (Roche Diagnostics GmbH, Mannheim, Germany). The reaction mix was incubated for 1 h at 42ºC. After completion of the RT-reaction, 100 ng of the forward primer and 2.5 U *Taq* DNA polymerase (Roche) was added and the reaction mix overlaid with nuclease-free mineral oil (Sigma). The PCR cycles included: denaturation for 1 min at 95ºC, annealing for 1 min at 45ºC, extension for 1.5 min at 72ºC for 30 cycles and a final extension for 10 min at 72ºC. A positive control, namely cell culture-adapted HAV (pHM-175) RNA, and negative control (sterile nuclease-free water) were included in each RT-PCR reaction.

2.3.6.3 Detection of RT-PCR amplicons

a) Agarose gel electrophoresis

Amplicons (20 µl) were analysed in a 2% agarose gel (SeaKem® LE agarose, FMC BioProducts) with the addition of 5 µl of 10 mg/ml ethidium bromide (EtBr) solution (Sigma) per 100 ml of gel. Amplicons were visualised by UV illumination. A MW marker (DNA MW Marker V, Roche) was included on each gel to determine and confirm the size of the amplicons. The amplicons, which co-migrated with that of the positive control, were considered to be presumptively positive.

b) Oligonucleotide probe hybridisation

Hybridisation was used to detect and confirm the specificity of the DNA amplified from the viral RNA by RT-PCR. A virus-specific 3′-end labelled oligonucleotide 20 bp positive sense oligonucleotide probe, complementary to a region within the abovementioned RT-PCR product (nt 2232-2251), was used (Robertson et al., 1991). The probe, with the sequence, 5'-TCAACAACAGTTTCTACAGA-3' was synthesised by Sigma-Genosys Ltd.

The positive sense oligonucleotide probe was non-radioactively labelled with digoxigenin (DIG)-dUTP using the DIG oligonucleotide 3′-end labelling kit (Roche). Amplicons (5 µl) were added to 15 µl 1X TE buffer, pH 8.0 (Amresco), denatured for 10 min at 99ºC and kept on ice. Denatured amplicons were spotted onto a positively charged nylon membrane (Roche) using a vacuum manifold apparatus (Bio-Dot Apparatus, Bio-Rad, Hercules, CA) and left to adsorb for 30 min at room temperature. The membrane was air-dried, and the DNA fixed to the membrane by UV cross-linking for 5 min on each side of the membrane followed by baking for 15 min at 80°C. The pre-hybridisation and hybridisation solutions and conditions as described by Robertson et al. (1992) were applied. After hybridisation a modified version of the washing procedure described by Robertson et al. (1991) was used (Taylor et al., 2001). Chemiluminescent detection of the amplicon-oligonucleotide probe hybrids was done using the DIG nucleic acid detection

kit (Roche) and DIG wash and block buffer set (Roche) according to the manufacturer's recommendations. Positive results, i.e. amplicons containing HAV DNA, appear as dark spots on the chemiluminescent film (Roche). Amplicons not detected by dot-blot hybridisation analysis were considered to be negative.

2.3.7 Viral quantification

In order to ascertain the susceptibility of different cell lines to HAV infection and the sensitivity of the different viral detection assays, the titre of the viral stock suspensions used had to be calculated.

2.3.7.1 $TCID_{50}$ assay

The $TCID_{50}$ assay is an endpoint assay. $TCID_{50}$ is defined as the highest dilution of virus causing CPE in 50% of inoculated cell cultures. It is a quantitative assay that will only give an indication of the dilution of virus which will infect 50% of the cells inoculated, and not an enumeration of total number of infectious units in the original viral suspension (Hierholzer and Killington, 1996).

Microtitre plates were seeded with FRhK-4R, BGM and PLC/PRF/5 cell suspensions respectively. Ten-fold dilutions of the stock suspension of the pHM-175 strain were made in serum-free MEM. A fixed volume (100 µl) of each dilution was inoculated into the appropriate number of wells (minimum of three) of a 96-well microtitre plates. As a negative cell culture control, 100 µl of serum-free MEM was used. The infected microtitre plates were incubated at 37°C for 10 days in a humidified atmosphere in the presence of 5% CO_2 in air. The plates were examined daily by light microscopy for the appearance of CPE and the number of wells showing CPE out of the total number of replicates for each dilution were recorded. In addition, the plates were stained 10 days p.i. with crystal violet (0.5% crystal violet in 100% ethanol) to visualise the CPE colourimetrically. Results were read

spectrophotometrically (ICN Flow Titertek® Multiskan PLUS absorbance reader [Version 2.03, LabSystems, Finland]) at two wavelengths (492 nm and 615 nm). The $TCID_{50}$ was calculated using the Kärber formula (Appendix A) (Grist et al., 1979).

2.3.7.2 Plaque assay

The plaque assay is an infectivity assay that quantifies the number of infectious units in a virus suspension and is more accurate than the $TCID_{50}$ endpoint assay. Infectious viruses producing CPE are enumerated by counting the resulting clear zones. Every clear zone is called a pfu and represents an infective virus (or cluster of viruses) present in a given dilution. As HAV plaques are very small and often difficult to enumerate, immunological-based assays can be used to improve the visualisation of plaques (Lemon et al., 1983; Bishop and Anderson, 1997; Samuel et al., 2000; Richards and Watson, 2001).

Confluent monolayers of FRhK-4R cell cultures were prepared in 6-well cell culture plates. Ten-fold dilutions of the pHM-175 viral stock solution was prepared in serum-free MEM and the duplicate cell monolayers were infected with 100 µl of the appropriate virus dilution, overlaid with agar and incubated for 10 days at 37ºC. In this investigation, IPFA (section 2.3.5.1b) was used to improve the visualisation and enumeration of the plaques. The infectivity titre is expressed as the number of pfu/ml and calculated as follows:

pfu X reciprocal of dilution X reciprocal of inoculum volume (ml) (Hierholzer and Killington, 1996).

2.4 EXPERIMENTAL PROCEDURE

2.4.1 Assessment of susceptibility of different cell cultures, optimal incubation period and temperature for the early detection of HAV amplification

To determine which cell cultures are most suitable for the optimal isolation and amplification of WT HAV from water samples, FRhK-4R, BGM, Vero C1008, VK and PLC/PRF/5 cells were infected with the cell culture-adapted strain, pHM-175, and WT HAV. Infected cells were harvested every seven days p.i. for a period of 21 days and viral detection was done by RT-PCR-oligonucleotide probe assay and IIF.

The optimal incubation temperature for isolation and amplification of HAV was assessed using the FRhK-4R, PLC/PRF/5 and BGM cell lines. Two temperatures, i.e. 37°C and 32°C, were selected since WT HAV has been isolated at both these temperatures (Frösner et al., 1979; Flehmig, 1980). Although an incubation period of 21 days was considered to be the maximum incubation time for relevant and economical routine surveillance of water samples for HAV, infected cell cultures were monitored for six weeks p.i.

2.4.1.1 Experiment 1: Uninterrupted incubation

Aliquots (100 µl) of the stock suspension of each virus (pHM-175 and WT HAV) were inoculated onto 12 semi-confluent monolayers in 25 cm^2 flasks of each of the three cell lines. Six of the infected flasks were incubated for six weeks at 37°C and the other six at 32ºC, with replenishment of the maintenance medium every seven days. One flask of each cell type was harvested weekly by scraping the cells off the surface of the flask into the surrounding medium. The harvested cell culture suspensions were stored in 1 ml aliquots at -70°C. Harvested cells were freeze-thawed twice and ten-fold dilutions (up to 10^{-9}) of each cell culture suspension were prepared in sterile PBS (pH 7.4). A 120 µl of each dilution was analysed for HAV RNA by RT-

PCR-oligonucleotide probe hybridisation assay as described previously (section 2.3.6.2 and 2.3.6.3).

2.4.1.2 Experiment 2: Blind-passage experiment

In order to determine whether or not blind-passaging could shorten the period between infection and first detection of the virus, a second set of cell cultures was infected and incubated at 37ºC and 32ºC as described previously (section 2.4.1.1). However, in this experiment, 1 ml of infected cell culture suspension was blind-passaged onto a freshly prepared semi-confluent monolayer at days seven and 14 p.i. Infected cells were harvested at day seven, 14 and 21 p.i. The harvested cell cultures were stored in 1 ml aliquots at -70°C. Harvested cells were freeze-thawed twice and ten-fold dilutions (up to 10^{-9}) of each cell culture suspension were prepared in sterile PBS (pH 7.4). A 120 µl of each dilution was analysed for HAV RNA by RT-PCR-oligonucleotide probe hybridisation assay as described previously (section 2.3.6.2 and 2.3.6.3).

2.4.2 Determination of sensitivity of molecular- and immunological-based techniques

This investigation was designed to ascertain which of the following assays. namely RT-PCR, RT-PCR-oligonucleotide probe hybridisation assay, IIF, and plaque assay, was the most sensitive for the detection of HAV. Ten-fold dilutions of a stock suspension of pHM-175 (2 X 10^5 $TCID_{50}$/ml) was made in sterile PBS (pH 7.4) and used for all assays. For each assay, the highest dilution at which HAV could be detected was noted. This was then used as an indication of the sensitivity of the technique.

2.4.2.1 Plaque assay

Monolayers of FRhK-4R were prepared in 6-well multidishes (section 2.3.4.5) and infected in duplicate with 100 µl of the appropriate dilution of pHM-175. The infected cells were overlaid as described previously (section 2.3.3.7) and incubated at 37ºC for 10 days. After 10 days the agar overlay was removed

and plaques visualised with the IPFA with DAB as a chromagen and enumerated.

2.4.2.2 IIF

Semi-confluent monolayers of FRhK-4R were prepared in 16-well multidishes (section 2.3.4.5) and infected in duplicate with 100 µl of the appropriate dilution of pHM-175. Infected cells were incubated for 10 days at 37ºC, after which the maintenance medium was withdrawn and the infected cells washed, fixed and stained as described previously (section 2.3.7.1).

2.4.2.3 Molecular detection

RNA was extracted from 100 µl of the ten-fold dilutions using the TRIZOL® method (section 2.3.6.1a), and analysed for HAV by RT-PCR and RT-PCR-oligonucleotide probe hybridisation assays as described in section 2.3.6.2 and 2.3.6.3.

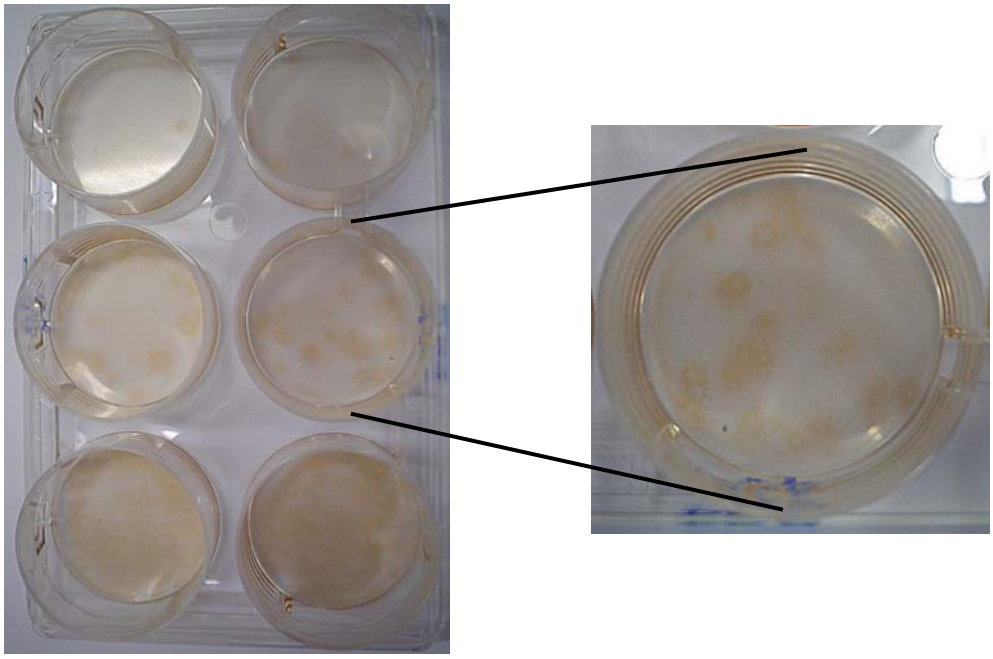
2.5 RESULTS

2.5.1 Determination of HAV stock culture titres

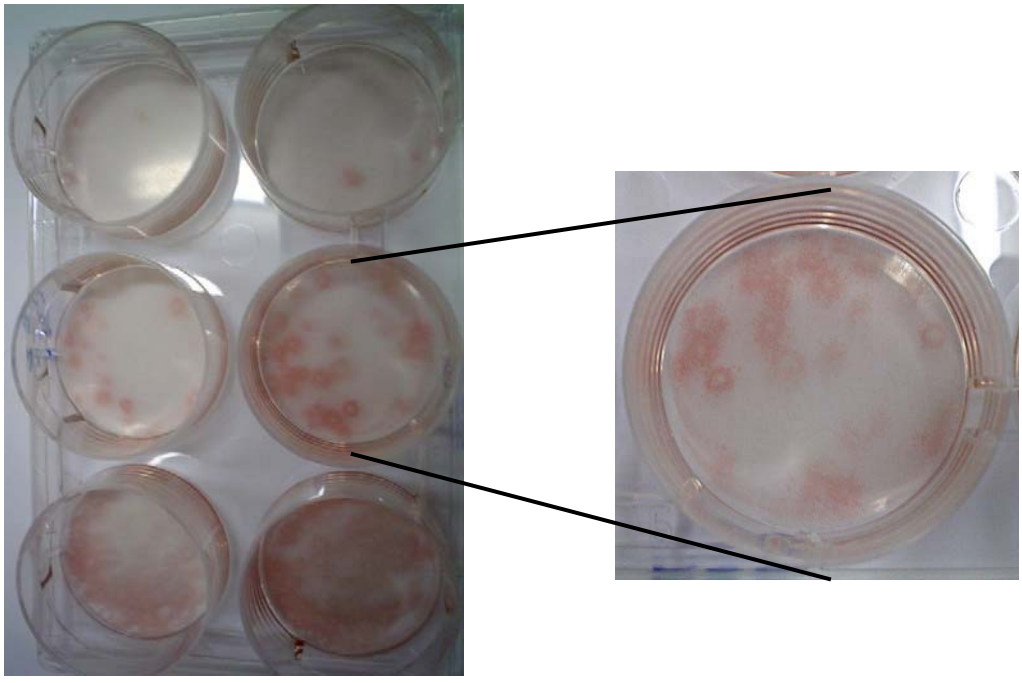
The titre of the pHM-175 stock culture, after visual examination of infected FRhK-4R cell culture, was calculated to be 2 X 10^5 $TCID_{50}$/ml.

2.5.2 Assessment of different chromagens used in IPFA

The results of the chromagen comparisons used during IPFA for the detection and enumeration of HAV are presented in Figures 5A and B.



A) 3,3'-Diaminobenzidine tertrahydrochloride (DAB)



B) 3-amino-9-ethylcarbazole (3AEC)

Figure 5: Comparative results obtained for 10-fold serial dilutions of HAV with the immunoperoxidase focus assay when DAB (A) and 3AEC (B) were used as chromagens

From these results it was evident that 3AEC produced clearer foci of infection compared to DAB, TMB and 4CN. However, as 3AEC is toxic and expensive, DAB was used throughout the rest of the investigation as a safe cost-effective alternative.

2.5.3 Sensitivity of molecular- and immunological-based techniques

Results depicting the sensitivity of each of the molecular- and immunological-based assays investigated are presented in Figure 6.

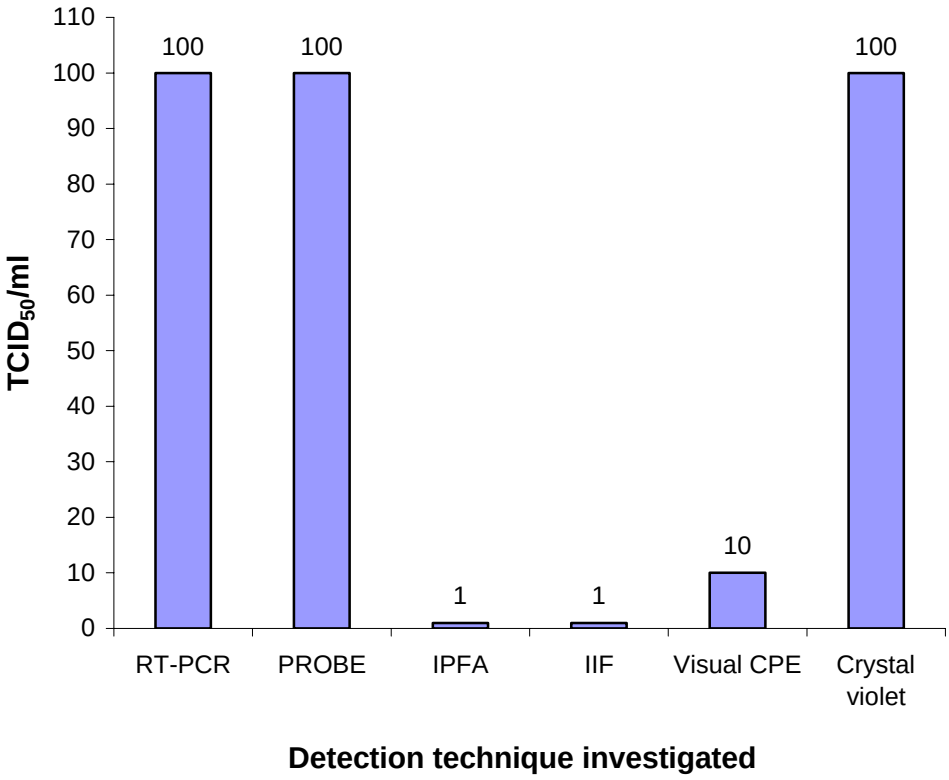


Figure 6: Assessment of the sensitivity of molecular- and immunological-based detection techniques

The results indicate that the IPFA and IIF techniques were able to detect 1 $TCID_{50}$/ml of the pHM-175 with which the FRhK-4R cells were infected. Visual reading of CPE was able to detect as few as 10 $TCID_{50}$/ml, while RT-PCR and RT-PCR-oligonucleotide probe assay both detected 10^2 $TCID_{50}$/ml.

2.5.4 Assessment of susceptibility of different cell cultures, optimal incubation period and temperature for the early detection of HAV infection

The results of the comparison of cell culture susceptibility are presented in Figure 7. Replication of HAV in the VK cells and the Vero C1008 cell line was unsatisfactory (results not shown), consequently these cells were excluded from further studies.

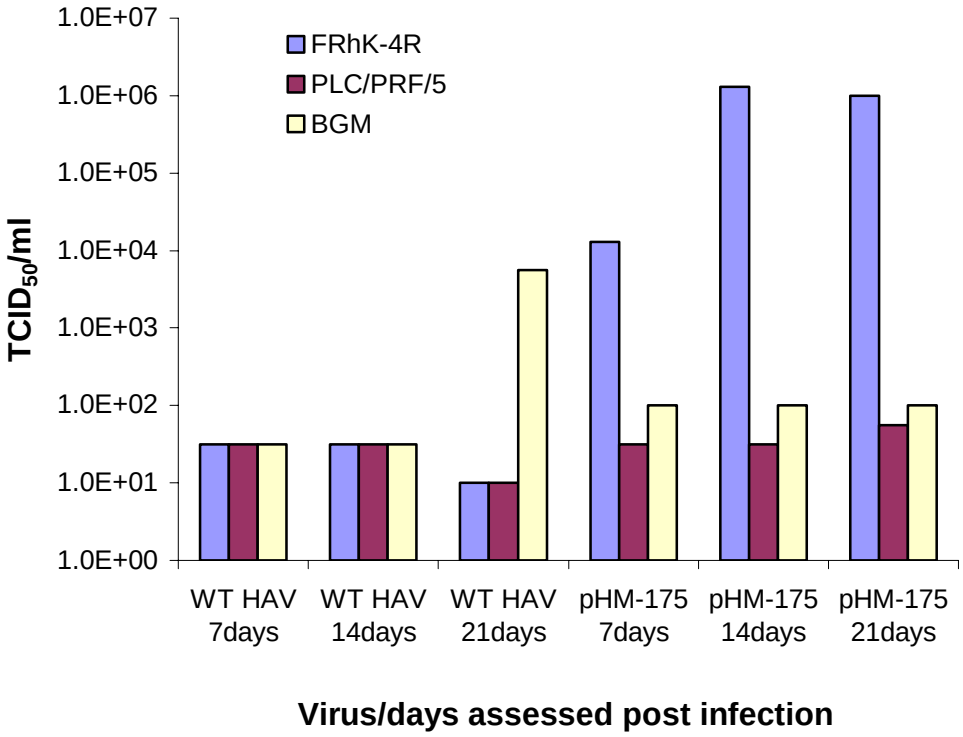


Figure 7: Comparison of susceptibility of cell cultures infected with wild type hepatitis A virus (WT HAV) and the cell culture-adapted strain, pHM-175. Infected cells were left undisturbed for 21 days and analysed by immunofluorescence

From the results presented in Figure 7 it is evident that the FRhK-4R cell line was the most susceptible to infection with pHM-175, with optimal replication within 14 days p.i. after which a plateau was reached. The BGM cells supported the replication of the particular WT HAV strain used in this

investigation more efficiently than the other cell lines with the titre rapidly increasing between day 14 and 21 p.i. The PLC/PRF/5 cell line was not susceptible to viral infection of either of the HAV strains used. The susceptibility of the three cell cultures to infection with the pHM-175 and WT HAV strain, with blind passaging every seven days for a period of 21 days is presented in Figure 8.

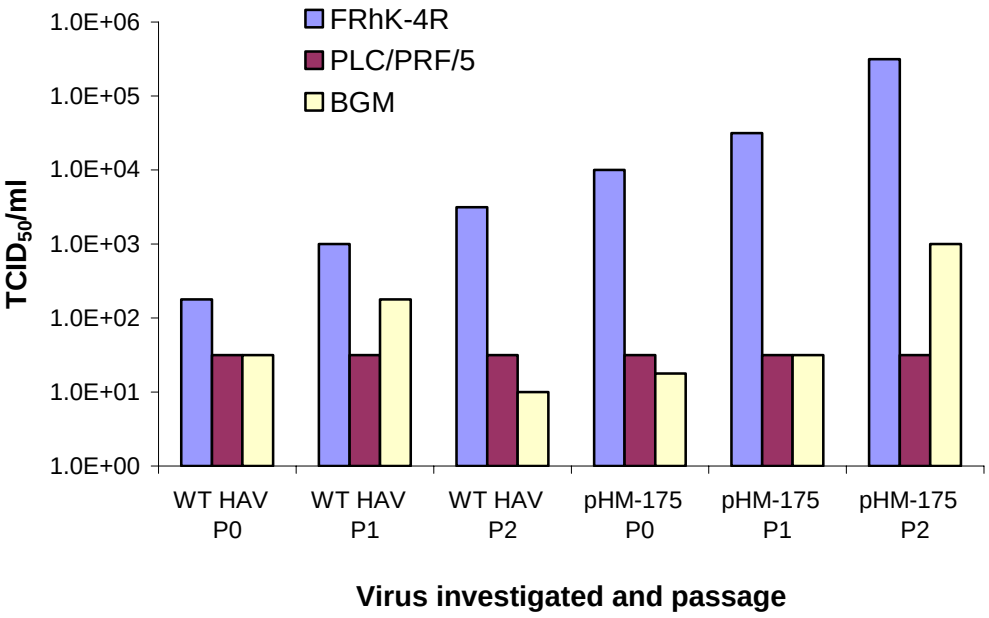


Figure 8: Comparison of susceptibility of cell cultures infected with wild type hepatitis A virus (WT HAV) and the cell culture-adapted strain, pHM-175. Infected cell cultures were passaged at seven day intervals for 21 days post infection and analysed by immunofluorescence

The FRhK-4R cell line proved to be the most efficient cell line to support growth of both the WT HAV and pHM-175 strain when blind passaged at seven day intervals p.i. The titre of the WT HAV strain increased after a single passage, but decreased again after the second blind passage. No increase in viral replication could be detected in the PLC/PRF/5 cell line for either of the HAV strains used for infection.

2.5.5 Assessment of optimal incubation temperatures with and without weekly blind passaging of infected cell cultures

Results from the study to determine the optimal incubation period for both HAV strains are presented below. Infected cells were left undisturbed for six weeks (Table 4) or blind passaged at seven day intervals for 21 days (Table 5).

Table 4: Assessment of optimal incubation temperatures for HAV isolation when left uninterrupted for six weeks post infection (p.i) and analysed by reverse transcriptase-polymerase chain reaction

Temperature: 32ºC						
Virus:	**WT HAV**			**pHM-175**		
Day p.i.	**FRhK-4R**	**PLC/PRF/5**	**BGM**	**FRhK-4R**	**PLC/PRF/5**	**BGM**
7	+	+	+	+	+	+
14	+	-	-	-	-	-
21	+	-	-	-	-	-
28	Weak +	-	-	-	-	-
35	-	-	-	-	-	-
42	-	-	-	-	-	-
Temperature: 37ºC						
Virus:	**WT HAV**			**pHM-175**		
Day p.i.	**FRhK-4R**	**PLC/PRF/5**	**BGM**	**FRhK-4R**	**PLC/PRF/5**	**BGM**
7	-	-	-	+	++	+
14	-	-	-	++	+	-
21	-	-	-	+++	+	-
28	-	-	-	+++	+	-
35	-	-	-	+++	+	-
42	-	-	-	+++	+	-

\+ = Positive HAV amplicon signal visible on agarose gel
++ = Strong positive HAV amplicon signal visible on agarose gel
+++ = Intense positive HAV amplicon signal visible on agarose gel

The cell culture-adapted strain, pHM-175, did not replicate at 32ºC in any of the cell cultures investigated, and only the original inoculum could be detected by RT-PCR. The WT HAV strain used in this investigation could however be detected at 32ºC on the FRhK-4R cell line, but once again only the input inoculum could be detected on the BGM and PLC/PRF/5 cell lines.

At 37ºC the FRhK-4R and PLC/PRF/5 cells supported the replication of pHM-175, but only the input inoculum could be detected in the BGM cells. The WT HAV strain did not replicate in any of the cell cultures investigated at 37ºC.

Table 5: Assessment of optimal incubation temperatures for HAV isolation when blind passaged every seven days post infection (p.i.) and analysed by reverse transcriptase-polymerase chain reaction

Temperature: 32ºC						
Virus:	**WT HAV**			**pHM-175**		
Day p.i.	**FRhK-4R**	**PLC/PRF/5**	**BGM**	**FRhK-4R**	**PLC/PRF/5**	**BGM**
7	++	+	+	-	-	-
14	+	-	-	-	-	-
21	-	-	-	-	-	-
Temperature: 37ºC						
Virus:	**WT HAV**			**pHM-175**		
Day p.i.	**FRhK-4R**	**PLC/PRF/5**	**BGM**	**FRhK-4R**	**PLC/PRF/5**	**BGM**
7	++	-	+	+++	+++	++
14	+	-	-	++	++	+
21	-	-	-	+	+	-

\+ = Positive HAV amplicon signal visible on agarose gel
++ = Strong positive HAV amplicon signal visible on agarose gel
+++ = Intense positive HAV amplicon signal visible on agarose gel

2.6 DISCUSSION

In the past, the isolation, detection and characterisation of enteric viruses in environmental sources relied heavily on cell culture methods (Schwab et al., 1993). These methods are time-consuming and labour intensive, and WT HAV grows slowly or not at all in conventional cell cultures (Beard and Lemon, 1999; Hollinger and Emerson, 2001). The cell line chosen can influence the amplification of the particular WT HAV strain to be isolated (Gust and Feinstone, 1988; Hollinger and Emerson, 2001). Cell lines derived from monkey kidney cells have been used successfully in previous investigations for the propagation of cell culture-derived HAV strains. These include cell lines from African green monkey kidney cells such as BS-C-1 (Cromeans et al., 1989; Stapleton et al., 1991; Bishop et al., 1994; Bishop and Anderson, 1997), Vero (Widell et al., 1988; Hay et al., 1992) and AGMK (Daemer et al., 1981). Rhesus monkey kidney derived cell lines e.g. FRhK-4 (Wheeler et al., 1986; Cromeans et al., 1989; Stapleton et al., 1991; Dotzauer et al., 1994) and FRhK-4R (Flehmig, 1981) have been applied with equal success. Cell lines derived from human origin such as the hepatoma cell line, PLC/PRF/5 (Frösner et al., 1979; Deinhardt et al., 1981; Siegl et al., 1984; Crance et al., 1987), diploid embryonic lung cells, MRC-5 (Siegl et al., 1984; De Chastonay and Siegl, 1987) and embryonic kidney or fibroblast cells (Flehmig et al., 1981) have also been used in studies of HAV. Since no single cell line has ever been specifically targeted as being the most susceptible to HAV infection, different human and non-human primate cells (VK) and cell lines (PLC/PRF/5, BGM, FRhK-4R and Vero) were used in this investigation to determine which was the most suitable for the isolation of the specific WT HAV strain used.

Factors such as incubation period, incubation temperature (Crance et al., 1987; Dotzauer et al., 1994) and blind passaging (Beard and Lemon, 1999; Hollinger and Emerson, 2001) can influence the isolation of HAV (Hollinger and Ticehurst, 1996). For these reasons, the suitability of the PLC/PRF/5, BGM

and FRhK-4R cell lines infected with WT HAV were investigated at two incubation temperatures, 32ºC and 37ºC. Cells were incubated for different periods of time, with and without serial passaging, to determine the optimal combination for isolation of the virus. Although it was evident that the FRhK-4R cell line was the most susceptible cell line for the propagation of the WT HAV strain used in this study and the cell culture-adapted strain, pHM-175, BGM cells also supported growth of the particular WT HAV strain used in this investigation (Figure 7 and 8). The WT HAV strain could be detected by RT-PCR up to 28 days p.i. at 32ºC (Table 4), although the intensity of the positive HAV signal obtained after molecular analysis decreased after 21 days incubation, as well as after serial passage (Table 5). No WT HAV could be detected by RT-PCR after incubation at 37ºC (Table 4) when left uninterrupted for six weeks p.i. The signal intensity of the WT HAV amplicons observed (Table 5) decreased after serial passage, indicating that it was the inoculum virus being diluted with every passage. It is important to mention though that another strain of WT HAV could require totally different propagation conditions and/or cell line for optimal amplification.

Since WT HAV has little or no visible CPE on infected cell cultures (Beard and Lemon, 1999; Hollinger and Emerson, 2001), the sensitivity of different detection techniques not dependent on CPE, had to be investigated. Early detection of HAAg, using immunological-based assays, can be used to indicate viral replication. Previous investigators successfully applied luminescent immunofocus assay (LIFA) for the quantitation of both non-CPE and CPE-forming cell-culture adapted HAV strains, and concluded that quantitation using plaque assays was not as accurate as LIFA (Richards and Watson, 2001). In earlier studies the quantal IF assay and IP assay, using DAB as chromogen, were successfully used to titrate a cell culture-adapted HAV strain in cell culture (Nadala and Loh, 1990). From the results of this study (Figure 5 and 6), the IPFA, using either DAB or 3AEC as chromogen, and IIF were the most sensitive techniques to apply for detection of both WT HAV and pHM-175 in cell cultures. IIF could be applied for the visualisation

of WT HAV as well as cell culture-adapted strains. Results from IIF were obtained within 10 days, but it has to be borne in mind that, depending on the strain, WT HAV may need a longer incubation period for replication. Analysis of IIF results are operator dependent, and non-specific fluorescence could be misinterpreted as positive, due to human error. Fluorescent staining is not permanent, as in the case of IPFA, and a UV microscope is needed for visualisation. The IPFA detection technique described in this investigation, although sensitive, is labour intensive, expensive and results rely heavily on precise technique and pH of reagents used. IPFA can be used for the detection of infected foci where no visual CPE is evident or for the visualisation of plaques which for HAV are small and not readily visible in unstained or conventionally stained (e.g. crystal violet) cell cultures. Results are however more difficult to observe than those obtained from IIF. Results obtained from the manual visualisation of CPE can also be biased since this technique is operator dependent. Only one in 200 physical viral particles of the cell culture-adapted strain, pHM-175, are infective for cell culture (Chudy et al., 1999), and it is unclear as to whether the same apply to WT HAV. Quantal results for the assessment of the susceptibility of cell cultures to HAV infection, using cell culture-adapted strains such as pHM-175, should therefore be interpreted with caution, since this indicates that the results obtained during this investigation could be a major underestimate of the true number of viruses present in a sample.

Molecular-based techniques, such as RT-PCR (Croci et al., 2000; Legeay et al., 2000; Soule et al., 2000) and RT-PCR-oligonucleotide probe assay (Mullendore et al., 2001; Schwab et al., 2001; Taylor et al., 2001) have facilitated the detection of HAV in food and water sources. The RT-PCR and oligonucleotide probe assay used in this investigation, as described by Taylor et al. (2001), have a sensitivity limit of 100 $TCID_{50}$/ml, indicating that low numbers of HAV usually found in the environment, could be missed when these techniques are applied for the routine surveillance of environmental samples. These molecular techniques are expensive and do not distinguish

between viable and non-viable viral particles, so no conclusion as to the pathogenicity of HAV detected can be made. This can however be a valuable epidemiological tool to assess whether or not HAV is present in the environment.

2.7 RECOMMENDATIONS

A combination of cell cultures, incubation times and temperatures would have to be used for optimal isolation and detection of WT HAV circulating in the environment. This would however, be impractical for the routine surveillance of environmental water samples. From this investigation it was concluded that a combination of FRhK-4R cell culture, infected and incubated unpassaged for 21 days at 32ºC and 37 ºC, followed by RT-PCR-oligonucleotide probe assay, should be used for the detection of WT HAV.

CHAPTER 3

ASSESSMENT OF TECHNIQUES FOR THE RECOVERY OF HAV FROM WATER SAMPLES

3.1 INTRODUCTION

A wide spectrum of human enteric viruses, including HAV, excreted in human faeces, can be transmitted via the faecal-oral route and are potential water pollutants (Nasser et al., 1995; Grabow, 1996; Hunter, 1997a; Soule et al., 2000). Since the infectious dose of enteric viruses is low (<100 viral particles in the case for caliciviruses and HAV), they pose a possible health risk to communities utilising polluted water sources (Gilgen et al., 1997; Huang et al., 2000). As viruses in water sources are usually present in low concentrations, large volumes (>50 l) of water need to be analysed in order to detect waterborne viruses (Senouci et al., 1996; Gilgen et al., 1997; Huang et al., 2000; Soule et al., 2000). To facilitate detection, a variety of techniques have been described for the recovery of viruses from water. These include ultrafiltration (Divizia et al., 1989a,b; Garin et al., 1994; Soule et al., 2000), adsorption-elution using filters or membranes (Passagot et al., 1985; Senouci et al., 1996; Gilgen et al., 1997; Bidawid et al., 2000b), glass wool (Vilaginès et al., 1993; Vilaginès et al., 1997a,b) or glass powder (Gerba, 1983; Gajardo et al., 1991; Menut et al., 1993), two-phase separation with polymers (Schwab et al., 1993) and flocculation (Nasser et al., 1995; Backer, 2002). The recovered, and therefore more concentrated, viruses can be detected using conventional cell culture techniques (Vilaginès et al., 1997a,b; Soule et al., 2000), RT-PCR (Tsai et al., 1993; Gilgen et al., 1997; Vilaginès et al., 1997a), IF (Kukavica-Ibrulj et al., 2003), LIFA (Richards and Watson, 2001), RIA

(Lemon et al., 1983), and nucleic acid hybridisation (Gajardo et al., 1991; Cromeans et al., 1997). Each of the recovery, isolation and detection techniques has its own advantages and disadvantages. To monitor the virological quality of water, an efficient combination of techniques has to be assessed for the optimal recovery and detection of the low titres of viruses present in water (Gilgen et al., 1997; Soule et al., 2000).

3.2 AIMS OF THE INVESTIGATION

The aim of this investigation was to assess recovery techniques with regard to EOR and cost effectiveness. In the first part of the investigation, three primary recovery techniques were assessed, namely two glass wool-adsorption-elution techniques and ultrafiltration using a commercial flat membrane. In an independent series of experiments, using molecular based assays, the efficiency of secondary concentration techniques, namely PEG/NaCl (Minor, 1985; Biziagos et al., 1987; Vilaginès et al., 1997a) and the Centricon® Plus-80 Biomax-100 filter devices (Amicon Inc.), in relation to viral RNA extraction methods were assessed. The standard glass wool adsorption-elution technique was used for primary recovery of HAV in the latter investigation.

3.3 EXPERIMENTAL PROCEDURE

Two independent experiments were done in this investigation:

Series A (Figure 9): The EOR of the standard glass wool adsorption-elution technique, the modified glass wool adsorption-elution technique and ultrafiltration as primary recovery procedures were assessed. Evaluation of the standard glass wool and ultrafiltration techniques were repeated in triplicate. In both the glass wool techniques, PEG/NaCl secondary

concentration was applied, while the ultrafiltration procedure did not require a secondary concentration step (Figure 9). Viral RNA from serial dilutions of the water samples, taken during the individual steps in the viral recovery process, was extracted from 140 µl of each sample using the QIAamp® Viral RNA Mini Kit (section 2.3.6.1b) according to the manufacturers' recommendations

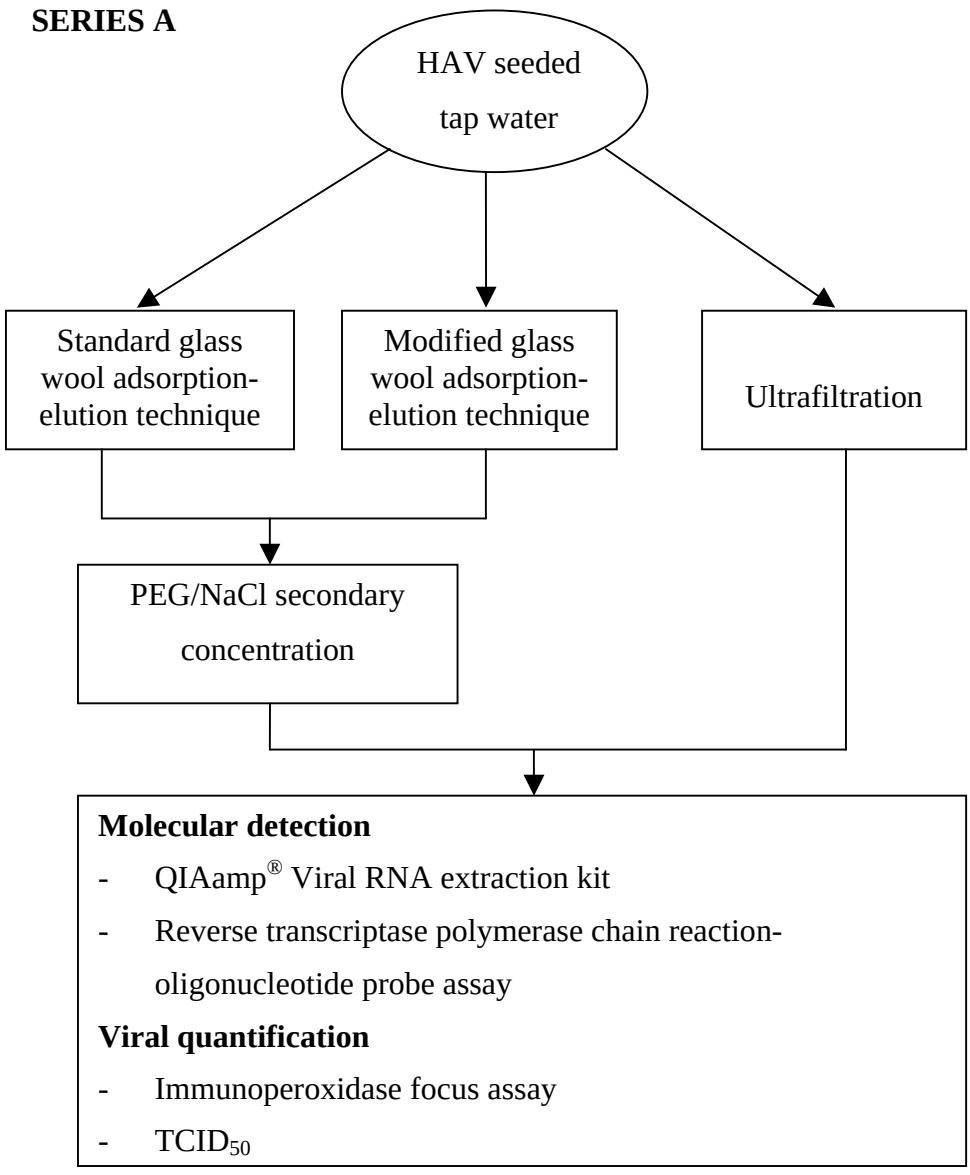


Figure 9: Series A - Flowchart of the experimental procedure where the efficiency of recovery of the three primary recovery techniques was assessed.

Series B (Figure 10): Primary recovery was done using the standard glass wool adsorption-elution technique followed by one of two secondary procedures, namely PEG/NaCl or the Centricon® Plus-80 Biomax-100 centrifugal procedure.

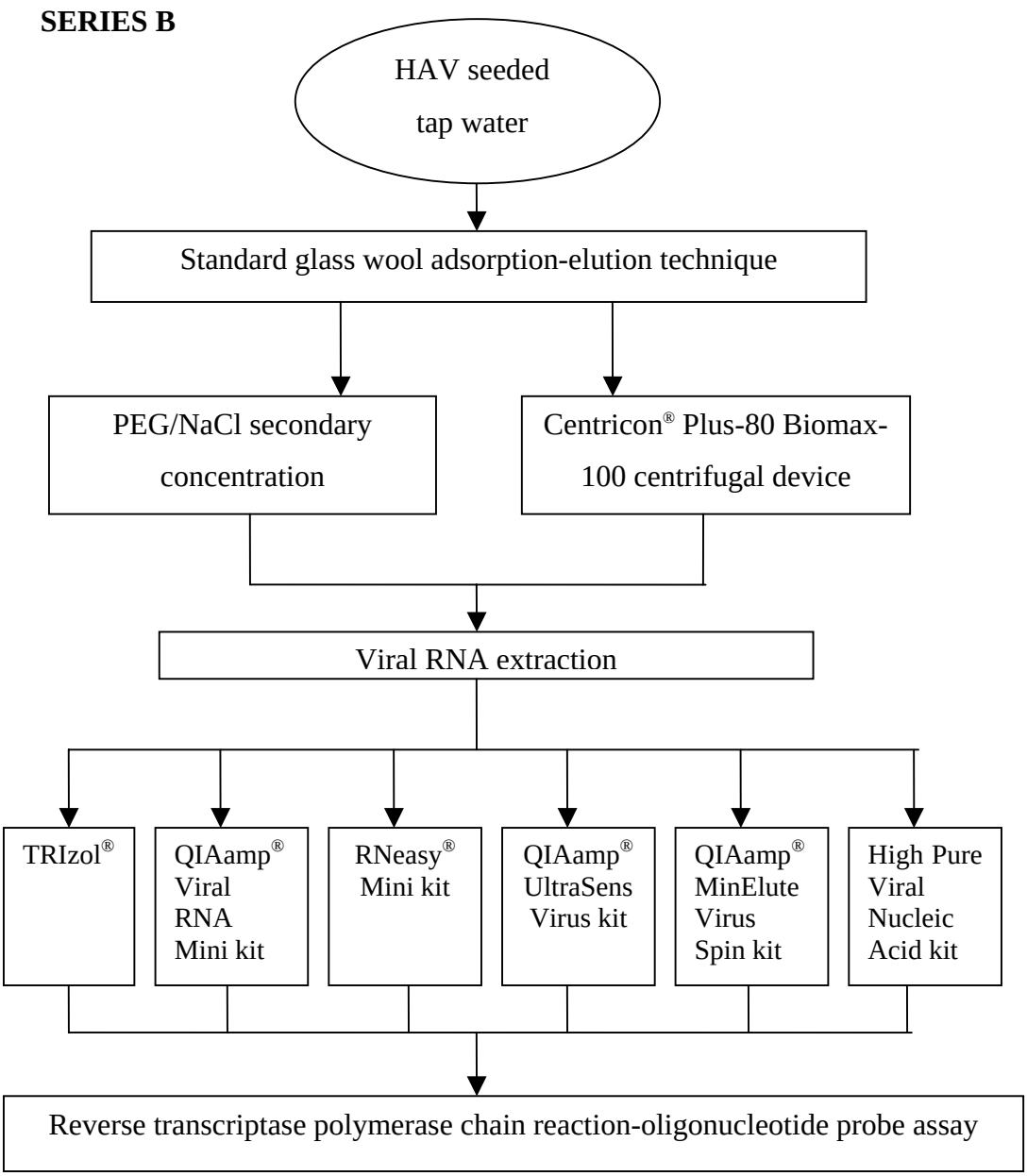


Figure 10: Series B - Flowchart of the experimental procedure where different combinations of secondary concentration procedures and RNA extraction techniques were assessed.

In this series of experiments, different combinations of secondary concentration procedures and a variety of RNA extraction techniques were compared. The sample input volume for all RNA extraction procedures was 180 µl and extracted viral nucleic acid was eluted in 60 µl nuclease-free water or a low-salt buffer provided.

3.4 MATERIALS AND METHODS

3.4.1 Virus stock

The cell culture-adapted HAV strain, pHM-175, was propagated in the FRhK-4R cell line. The propagated viruses were partially purified from the cell culture extract, resuspended in sterile PBS (pH 7.4) and quantified using the $TCID_{50}$ technique (section 2.3.7.1) and IPFA (section 2.3.5.1b). Virus stock, aliquoted into 1 ml volumes, was stored at -70°C.

3.4.2 Seeding of water samples

Water samples for the seeding experiment were prepared as follows: 10 l of tap water was dechlorinated with 1.2 ml/l 1 M sodium thiosulfate ($Na_2S_2O_3$) and mixed thoroughly for ± 10 min. The pH and temperature of the water were noted. A 1 ml aliquot of viral stock was mixed with 1 l of the dechlorinated tap water, returned to the remaining tap water in the aspirator and mixed thoroughly to ensure random distribution of the virus in the seeded water. A 10 ml sample of the seeded water was drawn for quantification and molecular detection of the input HAV.

3.4.3 Glass wool column preparation

3.4.3.1 Standard glass wool column preparation

The standard column used in this investigation was a modification of the column described by Vilaginès et al., (1993). Essentially, 10 g glass wool (R.725, St. Gobain, Isover-Orgel, France) was compressed into a perspex column (260 mm x 30 mm) (Figure 11). The glass wool was divided into three equal amounts and each section teased and compressed separately into the column to form a glass wool plug approximately 10 cm high with a final density of 0.5 g/cm^3 (glass wool dry w/v basis). A metal/plastic coupling link was attached to each end of the column. The glass wool was soaked with sterile distilled water and pre-treated consecutively with 40 ml 1 M HCl, 100ml sterile distilled water, 40 ml 1 M NaOH and 100 ml sterile distilled water to adjust the pH to pH 7.0 and to positively charge the glass wool. To remove any chlorine not neutralised by the $Na_2S_2O_3$, 15 g of hydrochlorex (USF Wallace and Tiernam, Günzburg, Germany) was added to the column above the glass wool.

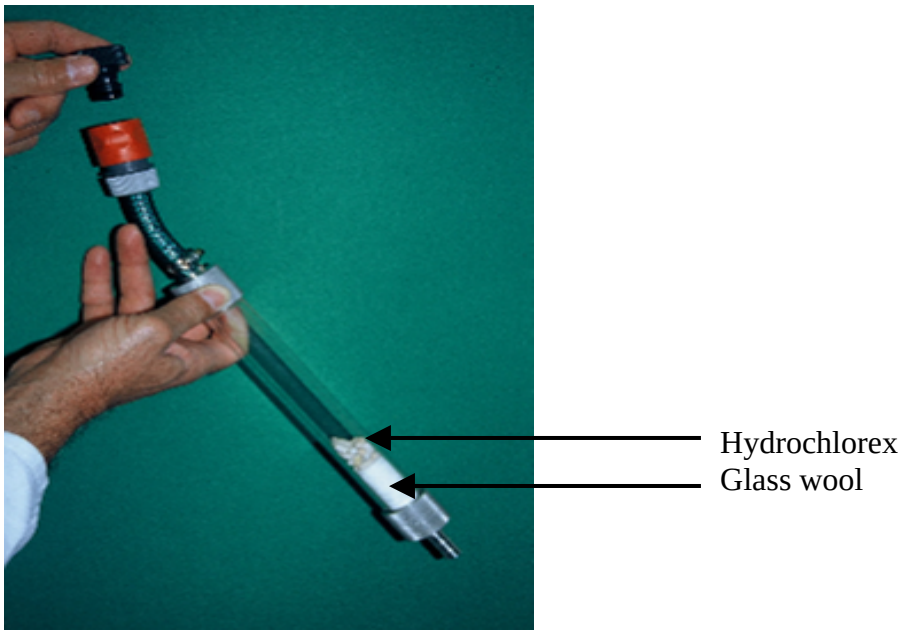
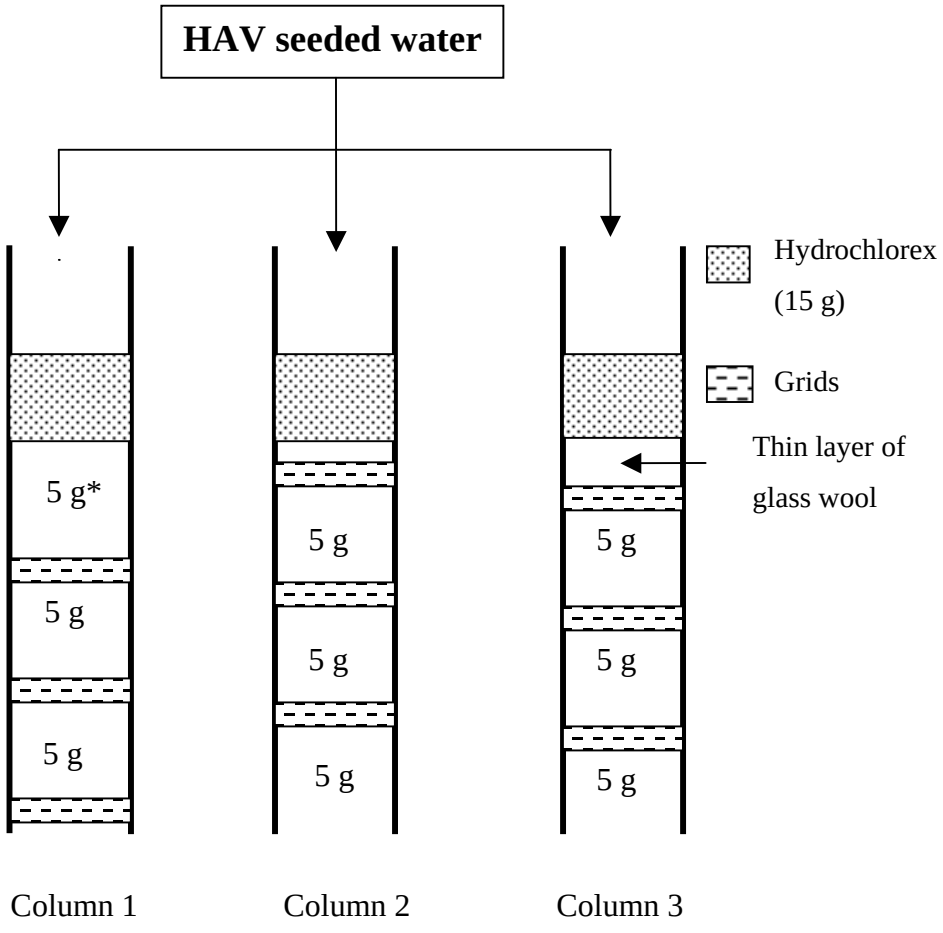


Figure 11: A packed standard glass wool column with hydrochlorex for recovery of viruses from large volumes of water

3.4.3.2 Modified glass wool column preparation

The modified glass wool column was prepared as described previously (section 3.4.3.1), with the following modifications: three portions of 5 g of glass wool was used and three steel gauze grids (pore size = 1 mm^2, 30 mm in diameter) were inserted between the glass wool sections. The grids were placed at different positions to assess whether the placing could influence the EOR (Figure 12). Each portion of glass wool was teased and compressed into the column at a different angle, as was done for the standard column, pre-treated as described previously and 15 g hydrochlorex pellets added.



* Glass wool

Figure 12: Modified glass wool columns showing the various positions of the metal gauze grids

3.4.4 Primary concentration procedures

3.4.4.1 Glass wool adsorption-elution

The seeded dechlorinated tap water (10 l) was filtered through the positively charged glass wool columns by negative pressure at a rate of 10 l/h. The negatively charged viruses, which adsorbed to the glass wool, were eluted twice with 50 ml glycine-beef extract buffer (GBEB) (3.754 g/l glycine [Merck], 5 g/l beef extract V [Becton Dickinson and Co., Cockeysville, MD]), pH 9.5, which reverses the ionic charge of the viruses and releases them from the glass wool. Immediately after elution, the pH of the eluate was adjusted to pH 7 with 1 M HCl (pH 1) (Merck). A 10 ml sample of the eluate was drawn for viral quantitation and molecular detection. The remaining 90 ml of the eluate was subjected to secondary concentration using PEG/NaCl.

3.4.4.2 Ultrafiltration

The seeded dechlorinated tap water (10 l) was passed through 150 mm diameter flat polyacrylonitrile/polyvinyl chloride copolymer membrane filters (Amicon XM50, Amicon Inc.) using the Diaflo ultrafiltration system. This process was performed at 4ºC at a flow rate of 1 l/h with 450 kPa pressure. After filtration, the membrane was washed four times with 5 ml sterile PBS supplemented with 2% BSA. The resulting 20 ml viral suspension was stored at -70ºC in 1 ml aliquots prior to quantification and molecular analysis for HAV.

3.4.5 Secondary concentration procedures

3.4.5.1 PEG/sodium chloride concentration

Secondary concentration of viruses from the eluate was done with PEG 6000 (Merck) in the presence of NaCl (Merck) as described by Minor (1985) and Vilaginès et al., (1997b). PEG 6000 (7.5%) and NaCl (2.5%) was added to the 90 ml neutralised eluate, dissolved and maintained for 18 h at 4ºC with continual slight stirring. After centrifugation at 2000 x *g* (Sorvall® Super T21,

DuPont Co., DE) for 2 h at 4ºC, the supernatant was discarded and the pellet resuspended in 10 ml sterile 2% BSA/PBS. The resuspended pellet was sonicated (Soniprep 150, MSE) at 20 amplitude microns for 30 s to dissociate any fragments or clumps, where after the sample was again centrifuged at 3000 x *g* for 10 min at 4ºC to precipitate any solids. The resulting virus-containing supernatant was stored in 1 ml aliquots at -70ºC for subsequent viral quantification and molecular analysis.

3.4.5.2 Centrifugal devices procedure

In an independent experiment, Centricon® Plus-80 Biomax-100 centrifugal filter units were assessed as an alternative secondary virus concentration procedure. In brief, the sample filter cup of the unit was pre-rinsed with 80 ml sterilised distilled water as per manufacturer's instructions. A maximum of 80 ml of the primary eluate (pH 7) was added to the sample filter cup and centrifuged at 3500 x *g* (Beckman GS-6R, Beckman Instruments, Inc., CA) for 30 min at 4ºC. This procedure was repeated with the remaining eluate until the desired volume (5-10 ml) was achieved. This concentrate was removed, and a retentate cup was placed into the sample filter cup. The unit was inverted and centrifuged at 1000 x *g* for 2 min at 4ºC. The resulting volume of concentrate was removed from the retentate cup and added to the 5-10 ml concentrate. The virus suspension was stored in 1 ml aliquots at -70ºC for molecular analysis by RT-PCR.

3.4.6 Isolation and quantification

Confluent FRhK-4R cell monolayers in 6-well NUNCLON™ Multidishes and semi-confluent monolayers (75-80%) in 96-well NUNCLON™ MicroWell™ plates were infected, in duplicate, with 100 µl of serial 10-fold dilutions (up to 10^{-9}) of samples taken during each of the three steps during viral recovery. The plates were examined daily by light microscopy for CPE up to 14 days p.i. In addition to visual examination, the 96-well plates were stained with a 0.5% crystal violet-ethanol solution as described previously (section 2.3.7.1) and

results were read spectrophotometrically at two wavelengths (492 nm and 615 nm). From these results, the $TCID_{50}$ values were calculated and compared to the $TCID_{50}$ values determined for the optically read CPE results. Plaques present in the 6-well plates were stained using the IPFA technique (section 2.3.5.1b) with either DAB (Sigma) or 3AEC (Sigma) as chromogen, and enumerated.

The EOR was calculated for the IPFA and $TCID_{50}$ methods as follows:

$$\% \text{ Recovery} = \frac{\text{Number of pfu/ml after recovery X dilution factor}}{\text{Number of pfu/ml directly after seeding X dilution factor}} \text{ X } 100$$

3.4.7 Molecular detection

3.4.7.1 RNA extraction

a) TRIZOL® RNA extraction: Refer section 2.3.6.1(a)

b) QIAamp® Viral RNA Mini Kit: Refer section 2.3.6.1(b)

c) RNeasy® Mini Kit: Refer section 2.3.6.1(c)

d) QIAamp® UltraSens™ Virus kit (QIAGEN)

This kit is designed for the effective recovery of viral RNA and DNA from cell-free substances such as serum, plasma or as in this case, a cell-free virus suspension. The recommended input volume of sample required in this protocol is 1 ml, but for comparative purposes, 180 µl of sample was adjusted to 1 ml with nuclease-free water and used as input. The denaturing buffer lyses the viruses and forms complexes with the nucleic acids. Carrier RNA, added to the denaturing buffer, enhances the binding of viral nucleic acids to the silica-membrane and also protects the viral RNA against RNases which escaped the denaturing buffer. Complexes are sedimented at low speed (1200 x *g*) centrifugation, resuspended in buffer with proteinase K and incubated at 40ºC to allow full proteinase K digestion. Binding conditions are adjusted by an ethanol-containing buffer, and the proteinase K digested sample is transferred to the silica-membrane where the nucleic acids bind to the

membrane during centrifugation and contaminants simply pass through. Remaining enzyme inhibitors and contaminants which could affect downstream molecular techniques, are efficiently removed by consecutive washing steps. The nucleic acids are eluted with a low-salt buffer.

e) QIAamp® MinElute™ Virus Spin kit (QIAGEN)

This kit simultaneously purifies viral RNA and DNA. The viruses are lysed under highly denaturing conditions at elevated temperatures in the presence of protease and a denaturing buffer containing chaotropic salts and detergent. Together, these reagents inactivate RNases present in the sample. Carrier RNA, has the same function as described previously. Binding of viral nucleic acids to the silica-membrane is optimised with the addition of ethanol to the lysate. The lysate is then transferred to the spin column where viral RNA and DNA adsorb to the silica-membrane during centrifugation. Salt and pH conditions ensure that protein, salts and other contaminants which could influence downstream molecular reactions, are not retained on the membrane, but washed from the sample during the consecutive washing steps with buffers. Viral nucleic acids are eluted in a low-salt buffer provided.

f) High Pure Viral Nucleic Acid kit (Roche)

Viral RNA and DNA can be extracted simultaneously with this protocol. Viral lysis is accomplished by incubation of samples with a lysis buffer, containing chaotropic salt facilitating specific binding of nucleic acids to the glass fibres, in the presence of proteinase K. Contaminants are removed from the bound nucleic acids by consecutive washing steps. Purified nucleic acids are eluted in a low salt buffer.

3.4.7.2 RT-PCR-oligonucleotide probe assay

Viral RNA was amplified by RT-PCR (section 2.3.6.2), analysed by gel electrophoresis (section 2.3.6.3a) and results confirmed for specificity by oligonucleotide probe hybridisation (section 2.3.6.3b).

3.5 RESULTS

3.5.1 Quantification of viral stock

Using the different viral quantification techniques (section 2.3.7), the titre of the HAV stock was calculated to be 4 X 10^4 $TCID_{50}$/ml and 10^5 pfu/ml respectively.

3.5.2 Efficiency of primary recovery techniques

The EOR results of the standard glass wool column, modified glass wool column and ultrafiltration techniques investigated for the use for primary recovery of HAV from water samples are presented in Table 6, 7 and 8.

3.5.2.1 Standard glass wool column

The results (Table 6) indicated that irrespective of the quantification technique applied, the final EOR of the standard glass wool column was <10%. The EOR calculated for the primary recovery of HAV ranged from 1.78-10%, and after secondary concentration from 0.3-10%. In each case, there was a reduction in the EOR between the primary recovery and secondary concentration steps.

3.5.2.2 Modified glass wool column

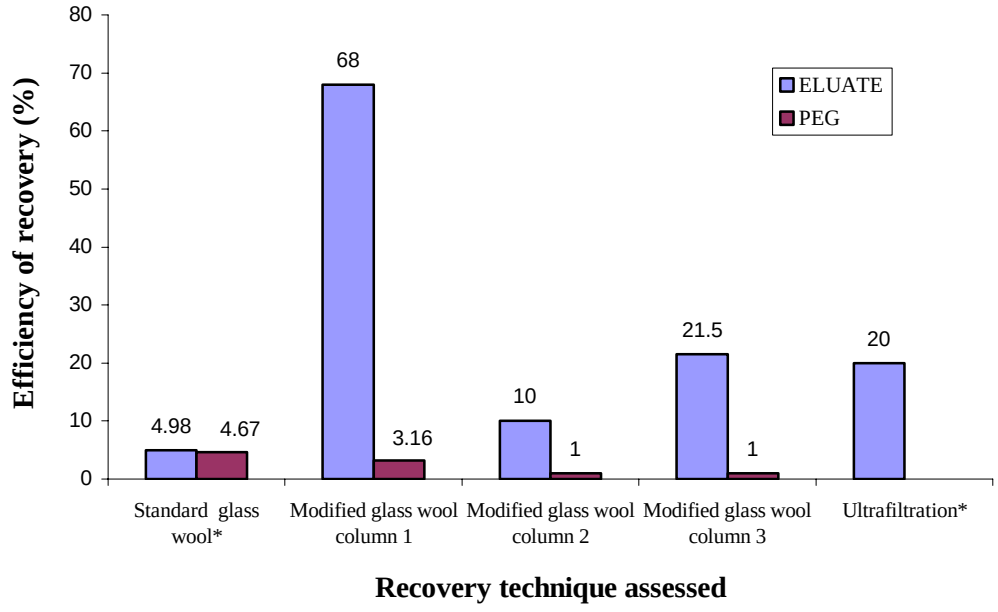
The EOR values for the three variant modified glass wool columns are presented in Table 7. Column 1 (Figure 12) showed a final EOR of 3.16% (Table 7), which was better than the 1% recovery (Table 7) calculated for columns 2 and 3 (Figure 12). Once again, a loss of viruses was incurred during the secondary concentration procedure. A broad range of EOR values was observed for the crystal violet and IPFA techniques indicating that the EOR values based on $TCID_{50}$ values were the most accurate (Table 7).

3.5.2.3 Ultrafiltration

Based on $TCID_{50}$ calculations done using visual CPE documentation, the final EOR was calculated at a constant 20% (Table 8). From the results it is evident that the crystal violet and IPFA quantification techniques gave a large spectrum of results, making these techniques unreliable for the quantification of HAV in seeded water samples.

3.5.2.4 Comparative analysis

A comparative analysis between the final EOR of the various recovery techniques assessed, based on $TCID_{50}$ calculations from visually read CPE results, is presented in Figure 13. The mean EOR of the standard glass wool columns is <5%, making this method the most inefficient of the three techniques assessed. The EOR after primary recovery using the modified glass wool column 1 (Figure 12) was the highest at 68%, but after secondary concentration the EOR decreased to 3.16%, indicating loss of virus. This loss of virus was noted in all the modified glass wool columns. The ultrafiltration technique gave a consistent 20% EOR.



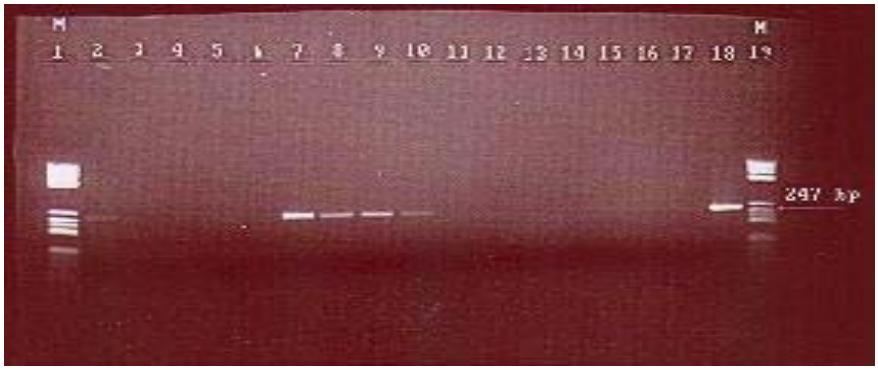
* Mean values of triplicate experiments

Figure 13: Efficiency of recovery for the three recovery techniques based on $TCID_{50}$/ml

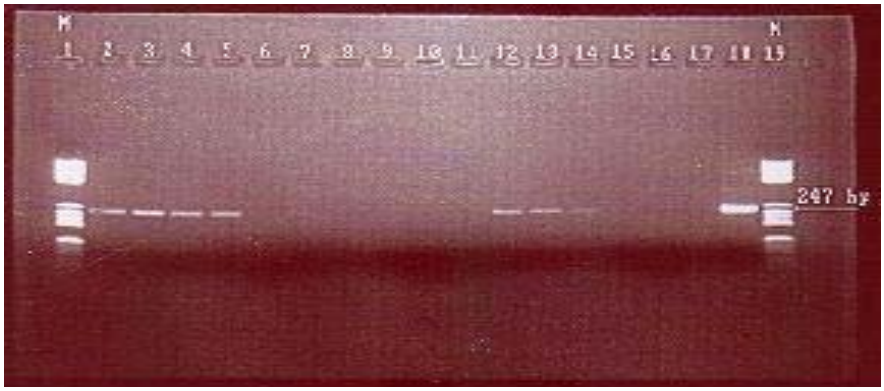
3.5.3 Comparison of RNA extraction protocols in combination with secondary concentration techniques

From Figure 14 it was evident that the most effective nucleic acid extraction kit to be used in combination with the PEG/NaCl secondary concentration procedure for the isolation and detection of HAV from water samples, was the High Pure Viral Nucleic Acid kit, followed by the QIAamp® UltraSens™ and QIAamp® Viral RNA Mini kits.

The results presented in Figure 15, indicated that the use of the High Pure Viral Nucleic Acid kit was the most effective nucleic acid extraction protocol to be used in conjunction with the Centricon® Plus-80 Biomax-100 centrifugal device. The QIAamp® UltraSens™ extraction kit gave weaker positive signals compared to the High Pure Viral Nucleic Acid kit. Even though the High Pure Viral Nucleic Acid kit can be applied in combination with both secondary concentration techniques, the intensity of the amplicon bands, as seen on the agarose gels, differed. In Figure 14 (A), the intensity of the bands in lanes 7-10 decreased with the dilution of the RNA, but this was not the case for the Centricon® Plus-80 Biomax-100 system.

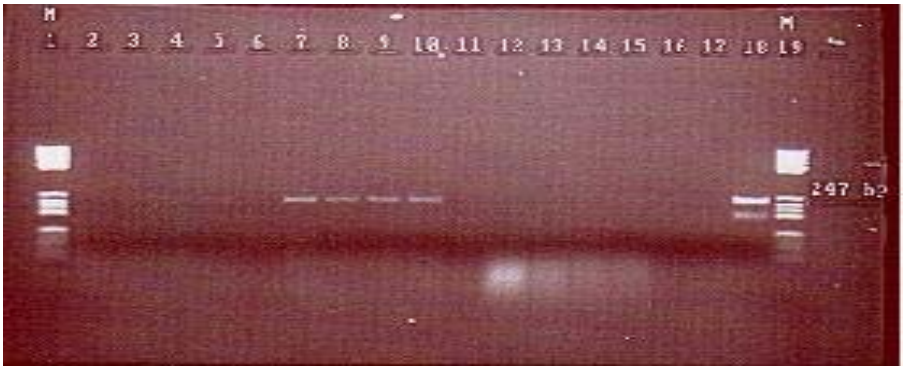


(A)



(B)

Figure 14: Comparative reverse transcriptase polymerase chain reaction (RT-PCR) results of the PEG/NaCl secondary concentration technique in combination with different RNA extraction procedures. Dilutions of RNA loaded in the following order for each extraction procedure: 1, 1:2, 1:4 and 1:8 and a negative extraction control. (A) lanes 2-6: QIAamp® MinElute™ (QIAGEN), lanes 7-11: High Pure Viral Nucleic Acid (Roche) kit, and lanes 12-16: TRIzol® RNA extraction procedures, (B) lanes 2-6: QIAamp® Viral RNA Mini kit (QIAGEN), lanes 7-11: RNeasy® Mini RNA (QIAGEN), and lanes 12-16: QIAamp® UltraSens™ (QIAGEN) RNA extraction procedures. Lanes 17 and 18 on gels represents a negative RT-PCR control and a positive HAV (pHM-175) RT-PCR control respectively. MW marker V (Roche) was loaded in lanes 1 and 19



(A)

(B)

Figure 15: Comparative reverse transcriptase polymerase chin reaction (RT-PCR) results of the Centricon® Plus-80 Biomax-100 secondary concentration technique in combination with different RNA extraction procedures. Dilutions of RNA loaded in the following order for each extraction procedure: 1, 1:2, 1:4 and 1:8 and a negative extraction control. (A) lanes 2-6: QIAamp® MinElute™ (QIAGEN), lanes 7-11: High Pure Viral Nucleic Acid (Roche), and lanes 12-16: TRIzol® RNA extraction procedures, (B) lanes 2-6: QIAamp® Viral RNA Mini kit (QIAGEN), lanes 7-11: RNeasy® Mini RNA (QIAGEN), and lanes 12-16: QIAamp® UltraSens™ (QIAGEN) RNA extraction procedures. Lanes 17 and 18 on gels represents a negative RT-PCR control and a positive HAV (pHM-175) RT-PCR control respectively. MW marker V (Roche) was loaded in lanes 1 and 19

3.5.4 Cost analysis

The basic costs for consumables used in the standard and modified glass wool techniques are of the same order. The modified glass wool technique uses an additional 5 g of glass wool and grids are inserted, which would increase the cost minimally. The infrastructure needed includes the aspirators, perspex columns, coupling links, sonicator and centrifuge of which the latter is the most expensive piece of equipment. Recovery can be performed at room temperature or can be done in-line. The advantage of the in-line procedure would be that large volumes (>100 l) of water could be passed through the system and transportation costs are minimised.

With the ultrafiltration system, substantial capital outlay is needed to set up the infrastructure which includes a cold room (4ºC), pressure tanks, air pressure tanks, water units and a centrifuge. The commercially available flat membranes are expensive (>R 500 each), and although they can be re-used up to 5 times, once damaged by impurities found in polluted water, they are no longer of use. This recovery technique therefore is not a practical choice for turbid water samples. The system cannot be set up in-line and the water tank can only accommodate a maximum of 20 l of water, necessitating extra transportation costs. Consumables used in this procedure are cheaper than those needed for the glass wool systems, and once the infrastructure is in place, running costs are low, except for the cost of the membranes.

For the Centricon® Plus-80 Biomax-100 secondary concentration procedure, the unit cost is substantially greater and single-use, which makes PEG/NaCl the cheaper alternative. Additionally, a separate centrifuge dedicated only to this recovery procedure is required, making this system more expensive than the glass wool techniques in combination with PEG/NaCl secondary concentration.

3.6 DISCUSSION

The detection of viruses in water depends on factors such as i) the recovery method, ii) recovery efficiencies, iii) virus type, iv) the volume of water sampled, v) storage and transport conditions, and lastly, the viral assay used in the laboratory for analyses (Environment Agency Report NC/99/40, 2000). In the past glass wool has been used with varying EOR rates for the recovery of enteric viruses from aqueous environments (Menut et al., 1993; Vilaginès et al., 1993; Vilaginès et al., 1997a,b). Studies investigating the EOR for glass wool and glass powder, using tap water samples seeded with poliovirus, found that the efficiency of glass wool adsorption-elution recovery varied from 16.3-36.4% with a mean of 25.5 ± 6.1% (Menut et al., 1993). In another investigation, the EOR of glass wool was assessed using treated drinking water, untreated surface as well as treated waste water spiked with poliovirus type 1 and 2, coxsackievirus B2 and B4, echovirus 11 and rotavirus SA11 (Vilaginès et al., 1993). An EOR of 77% was obtained for 100 l poliovirus-spiked tap water, and rates of 72% and 62% respectively for 400 l and 1000 l water volumes, indicating that the volume of water used does not significantly influence the EOR. When the experiment was repeated with the other viruses mentioned, varying EOR results were obtained, ranging from 62.5-75.2%. Glass wool adsorption-elution recovery experiments done with surface drinking water and treated wastewater spiked with poliovirus, demonstrated EORs of 62.7% and 57%, respectively (Vilaginès et al., 1993). In an investigation for the recovery of poliovirus from drinking and seawater, average EOR rates of 72% for drinking water and 75% for seawater was obtained respectively, but the repeatability of these results were <20% (Vilaginès et al., 1997b). When compared to these previous EOR studies for other viruses, the standard glass wool adsorption-elution procedure used in this investigation had a poor EOR of HAV (<5%) from seeded tap water. It must be borne in mind though that these investigations never included HAV in the evaluation of the EOR of various recovery techniques. These investigators also used commercially manufactured stainless steel columns, not the perspex

columns as were used in this study. The material composition of the glass wool columns could possibly play a role in the EOR of viruses. Whether or not HAV differs significantly in structure and surface charge from other enteroviruses is unclear, and this could influence the virus' behaviour during recovery procedures in a yet unknown way and consequently the EOR.

Previous investigations have shown that the density of the packed glass wool in the column affects the outcome of the recovery efficiency of enteroviruses and rotavirus (Vilaginès et al., 1993; Environment Agency Report NC/99/40, 2000). The EOR for poliovirus was 99.4% with a glass wool density of 0.5 g/cm^3, which dropped to 43.1% when packing density was halved. This could be explained by the fact that with a lower packing density, the viral contaminated water is not forced to flow through the glass wool itself, but can easily form canals through and around the glass wool column, minimising viral adhesion surface and time to the packing material (Environment Agency Report NC/99/40, 2000). With densely packed glass wool these water canals are dispersed and forced through the whole glass wool surface. Width and length of the columns do not affect the outcome of viral recovery (Environment Agency Report NC/99/40, 2000), therefore the only concern in this respect should be cost effectiveness of the column of choice. In this investigation, 10 g of glass wool was compressed in perspex columns at a density of 0.5 g/cm^3 for the standard glass wool columns, and metal gauze grids inserted at 5 g glass wool intervals in the modified glass wool columns to disperse the flow of water even more. During this experiment, the flow rate of the water samples was kept at 10 l/h, since this was calculated as the ideal flow rate for optimal viral recovery in previous studies (Environment Agency Report NC/99/40, 2000). As evident from Table 7, the grids' positioning (Figure 12) in the columns appeared to influence the EOR with column 1 being the most efficient. This phenomenon was however not investigated in detail and no conclusions as to the optimal placement of grids could be made. A dramatic (>50%) loss of viruses was noted during secondary concentration in both the standard and modified glass wool column procedures described

(Figure 13). Although 100% concentration is expected with PEG/NaCl the loss of virus could possibly explained by virus clumping, or attaching to the solid material, even after sonication, and being discarded together with the final pellet. In an in-house investigation, human astroviruses were detected in the final PEG/NaCl pellet which is usually discarded after sonication (Nadan S, personal communication).

In order to make the most effective choice between using glass wool or ultrafiltration as a viral recovery method, one should look at various factors: (1) costs involved, (2) EOR, (3) volume size to be processed, (4) the practical application of the method, and (5) degree of difficulty. When one looks at the cost analysis done for this study, the modified glass wool adsorption-elution technique using PEG/NaCl as secondary concentration procedure would be the most cost effective technique for routine virological screening of environmental and drinking water. This method of primary recovery is easy to operate and was proven to have a higher EOR than the standard glass wool adsorption-elution technique as well. The larger volume of glass wool and insertion of grids at the positions illustrated for column 1 (Figure 12), improves the EOR since the flow of water is dispersed additionally at regular intervals, maximising the viral adhesion surface and time to the positively charged glass wool. The system can be implemented in-line, making it practical for the analysis of large volumes of water at a sample point and no additional transportation costs for the samples are involved. Although the commercially available ultrafiltration membranes proved to be the most constantly efficient method of viral recovery in this investigation, the method had a few drawbacks. The costs involved to set up the basic infrastructure are substantial. In addition, this system cannot be implemented in-line and large volumes of water have to be transported, contributing to the costs. This procedure was time-consuming since a maximum flow rate of 1 l of tap water per hour could be achieved, making the technique impractical for viral recovery from turbid water samples. A maximum of 20 l can be analysed at a time, making it difficult for the detection of low viral titres usually found in

water environments. The 20% EOR obtained for the ultrafiltration method during this investigation, does not compare well with the findings of other researchers (Divizia et al., 1989b; Soule et al., 2000) where recoveries of up to 100% were noted for HAV. The low EOR results of this study could be ascribed to virus clumping and inadequate washing of viruses from the membranes, resulting in an underestimate of viruses present. In addition, the washing procedure used, may not have been optimal for the recovery of HAV from the membranes in this study.

Although IPFA staining is an accurate way of enumerating the pfu of the pHM-175 strain used during this investigation, the technique is time-consuming, labour intensive and expensive. Also, the clear visualisation of plaques depends on accuracy of technique and pH of reagents used during the staining process. In all the experiments, the IPFA gave a spectrum of results, making this quantification technique less reliable than the visual assessment of CPE. Although the interpretation of HAV CPE is objective and operator dependent, this technique for the calculation of $TCID_{50}$ proved to be the most reliable quantification technique to be applied according to this study. It must be borne in mind though that most strains of WT HAV do not cause CPE in cell culture, therefore this technique will be of no use when attempting to quantify WT HAV from environmental water sources. In this scenario, the immunological-based IIF and IPFA techniques could be of use.

In combination with an efficient and practical recovery technique, one needs an effective virus detection protocol as well. From the second set of independent experiments, it was clear that the High Pure Viral Nucleic Acid, QIAamp® Viral RNA Mini and QIAamp® UltraSens™ viral nucleic acid extraction kits are all suitable candidates to be used in combination PEG/NaCl. The High Pure Viral Nucleic Acid and to a lesser extent the QIAamp® UltraSens™ extraction kits are effective for isolating HAV RNA in combination with the Centricon® Plus-80 secondary concentration protocol. Once again, costs will influence the RNA extraction procedure to be applied.

The High Pure Viral Nucleic Acid kit can be used effectively in combination for both the secondary concentration techniques described, but the intensity of the RT-PCR amplicon signals between the two methods differ. PEG/NaCl removes RT-PCR inhibitors, whereas the Centricon® Plus-80 Biomax-100 filter devices concentrate all large MW particles (>100 kD), which could influence the downstream molecular detection techniques. Although the viral nucleic acid extraction kits are designed to eliminate these contaminants, the concentration of inhibitors during the ultrafiltration procedure may have been too great to overcome. Contaminants blocking the silica-membranes in the spin columns, prohibits RNA adsorption to the membranes, compromising the RNA extraction and subsequently the RT-PCR assay.

3.7 RECOMMENDATIONS

From the results a general recommendation can be made that primary recovery of HAV from water should be done using the modified glass wool adsorption-elution technique described, with grids inserted as for column 1 (Figure 12). Secondary concentration using PEG/NaCl is the most cost effective, practical and simple technique that can be applied. Effective viral RNA extraction techniques to use in combination with PEG/NaCl are the High Pure Viral Nucleic Acid, the QIAamp® Viral RNA Mini or the QIAamp® UltraSens™ extraction kits. Since the latter require an input volume of 1 ml sample, this protocol would probably be the most practical choice for detection of low titres of HAV from water samples.

It must be borne in mind that these results are for HAV recovery and detection only, and results could differ when another virus is used. Therefore, the optimal combination of recovery and molecular detection techniques should be investigated for each independent virus.

CHAPTER 4

CHARACTERISATION OF HAV STRAINS DETECTED IN WATER SAMPLES AND CLINICAL SPECIMENS

4.1 INTRODUCTION

The limited antigenic variability of HAV has urged scientists to do comparisons of nucleotide sequences within a limited region of the genome to define the genetic relatedness between different strains (Hollinger and Emerson, 2001; Pina et al., 2001). Based on limited segments of the genome, namely the VP3 C-terminus through the VP1 amino terminus, and the VP1/VP2A junction area, nucleotide sequence comparisons have been used to classify HAV into seven different genotypes. Genotypes IA, IB, II, IIIA, IIIB, and VII are closely associated with human infection, while genotypes IV, V and VI are associated with simian infections (Robertson, 2001; Ching et al., 2002; de Paula et al., 2002; Mbayed et al., 2002). Human antigenic variants, which escape antibody neutralisation, were only recently discovered. These variants were the consequence of mutations within the VP1 region, resulting in amino acid modifications in the surface protein, thus changing its antigenic properties (Costa-Mattioli et al., 2003). The sequence analysis of HAV strains contributes to the molecular epidemiology of the virus. Most human strains cluster in genotypes IA and IB (Mbayed et al., 2002). Genotypes IA and IB appear to be associated with infections in North and South America, Europe, and SA, and IB with infections originating in North Africa. Genotypes IA and IIIA circulate in South-east Asia, and a single representative of genotype VII has been identified in Sierra Leone (Robertson, 2001; Ching et al., 2002). Very little is known about the epidemiology of the other human genotypes.

The complete sequence of genotype VII, designated SLF88 and responsible for two fulminant hepatitis cases in Sierra Leone, was only recently published (Ching et al., 2002).

HAV is excreted in faeces for two to three weeks prior to the onset of clinical symptoms (Zuckerman and Zuckerman, 1999; Cuthbert, 2001), and faeces can remain infectious up to four weeks thereafter (Polish et al., 1999). HAV RNA has also been detected in faeces for up to ten weeks after the onset of symptoms (Robertson et al., 2000). This makes it difficult to conclusively link HAV outbreaks to specific sources on epidemiological grounds. Two regions within the HAV genome are commonly used for genotyping during epidemiological studies, namely the VP3/VP1 capsid region (Robertson et al., 1991, 1992; Taylor, 1997; Taylor et al., 2001) and the VP1/2A putative junction region (Roberston et al., 2000). The VP1 terminal region spans a region of intermediate genetic variability and can be used for genetic assignment and identifying the route of transmission (Costa-Mattioli et al., 2001b). During epidemiological studies and outbreak situations, the VP1/2A region has been successfully used to distinguish between genotypes circulating in a community (Costa-Mattioli et al., 2001a,b; Pina et al., 2001; de Paula et al., 2002; Mbayed et al., 2002). This region has been used in a heteroduplex mobility assay to establish a mixed infection of a childcare provider with both subgenotype IA and IB, and the sources of both infections could be established (de Paula et al., 2003). During an outbreak among drug abusers, the same region was used to identify the source of infection, namely needle sharing (Grinde et al., 1997). Both the VP3 C terminus and VP1/2A junction has been used during investigations of HA outbreaks among haemophiliacs who received HAV contaminated clotting factors (Kedda et al., 1995; Chudy et al., 1999). Foodborne HAV outbreaks are well documented and in many of these cases sequence analysis was applied to identify the source of HAV contamination (Hutin et al., 1999; Kaul et al., 2000; Kingsley et al., 2002). Sequence comparisons between HAV strains isolated from patients and suspected water sources have been used successfully during waterborne

outbreaks to trace the source of infection (Arauz-Ruiz et al., 2001; Lappalainen et al., 2001). Propagation and consecutive passaging of WT HAV strains in cell cultures could result in mutations, either insertions or deletions, which enable the virus to adapt to grow in cell culture (Robertson et al., 1989), but may consequently loose their virulence in the process (Tedeschi et al., 1993; Graff et al., 1994). For epidemiological studies the best scenario would therefore be to use WT HAV strains for sequence analysis to detect the presence of induced mutations that could influence epidemiological data (Robertson et al., 1992; Taylor, 1997).

4.2 AIMS OF THE INVESTIGATION

The objective for this part of the study was to ascertain whether hospitalised cases of HA could be associated with the consumption of water from sources used for recreational and domestic purposes. In order to accomplish this, HAV strains from clinical specimens and water sources will be sequenced, genotyped and compared to one another to determine the percentage of similarity between clinical and water isolates.

4.3 MATERIALS AND METHODS

4.3.1 Clinical specimens

Between June 1999 and June 2002, a total of 85 HAV IgM positive serum specimens were collected from the Pretoria and Kalafong Academic Hospitals, Pretoria, SA. Autopsy specimens of liver, spleen, small and large intestine, heart and whole blood from a patient with fulminant hepatitis were screened for the presence of HAV. A total of 327 stool specimens referred from paediatric patients presenting with gastroenteritis in 2001, were analysed for HAV. Specimens were stored at -20ºC prior to analysis. These specimens

were examined retrospectively and results did not impact on the management of the patient, consequently no informed consent was required. To maintain confidentiality of patients, the patients were given unique coded identifiers.

Ethical approval (S6/2001, 25 January 2001) has been obtained from the Ethical Committee at the University of Pretoria, Pretoria, SA, to include the HAV-positive serum, stool and other relevant clinical specimens in this study.

4.3.2 Water samples

From June 1999 to June 2002, a total of 765 untreated drinking water and 662 treated drinking water samples were collected from water sources in six different geographical settings in SA, as well as from the neighbouring country of Namibia. Primary viral recovery was done using the standard glass wool adsorption-elution technique (section 3.4.4.1). Viruses were concentrated further using PEG/NaCl precipitation (section 3.4.5.1). Recovered viruses were propagated on FRhK-4R and PLC/PRF/5 cell cultures (section 2.3.4.6) in preference to the BGM cell line, as in the literature these cell lines were used more commonly for HAV isolation from environmental samples. Recovered viral concentrations and infected cell cultures were screened for the presence of HAV RNA using molecular techniques.

4.3.3 RNA extraction and detection of HAV RNA

4.3.3.1 RNA extraction

RNA was extracted from clinical specimens using the QIAamp® Viral RNA Mini Kit (section 2.3.6.1b). Stool suspensions (10% w/v) were prepared in sterile PBS and treated with chloroform and PSN Antibiotic Mixture (100X) (GIBCOBRL) before RNA extraction. The RNeasy Mini Kit (section 2.3.6.1c) was used for the extraction of viral RNA from infected FRhK-4R and PLC/PRF/5 cell cultures. RNA was eluted in 30 µl RNase-free water and stored at -70ºC for analysis.

4.3.3.2 HAV detection

All water samples and infected cell cultures were screened for the presence of HAV RNA by RT-PCR-oligonucleotide probe assay (section 2.3.6.2 and 2.3.6.3). In addition a second RT-PCR using a published primer set, with an expected product size of 360 bp, was used to amplify the VP1/2A putative junction (nt 2949-3308) of selected HAV-positive samples (Robertson et al., 1992). Primer sequences were as follows: reverse primer: 5′-AAGTTTTCCTGGAGAGGTGTGACT-3′; forward primer: 5′-TATTTGTCTGTCACAGAACAATCAG-3′. Primers were synthesised by Sigma-Genosys Ltd. The reaction mix, amplification conditions and detection protocol were the same as that described for the VP3/VP1 capsid region (section 2.3.6.2b and 2.3.6.3a).

4.3.4 Sequencing of viral amplicons

Amplicons were sequenced using manual and/or automated sequencing, but where results indicated a possible mix of HAV strains within the same sample, products were cloned and re-sequenced directly.

4.3.4.1 Manual sequencing

a) Sequencing reactions

Laboratory accreditation specifications for care and safety precautions were observed when working with radioactive material. The sequencing of the RT-PCR amplicons was carried out by the dideoxynucleotide chain termination method (Sanger et al., 1977), using the T-7 Sequenase Version 2.0 Product Sequencing Kit (USB Corp., Cleveland, OH) according to the manufacturer's instructions. In short: RT-PCR amplicons were first treated with the enzyme exonuclease 1 to remove residual ss-primers and extraneous ssDNA produced by the RT-PCR reactions. Shrimp alkaline phosphatase removed the remaining dNTPs from the PCR mixture, which would interfere with the labelling step of the sequencing process. Both the forward and reverse primers were used for sequencing. The primer annealed to the template and

the reaction proceeded by incorporating radioactive S^{35}-labelled bases into the synthesised nucleic acid. The time of the T-7 polymerase activity was determined by the size of the amplicon product being sequenced. Placing the reaction onto ice stopped the polymerase activity. A termination reaction with each of the four DNA bases took place separately. An analogue for dGTP, 7-deaza-dGTP was used. The 7-deaza-dGTP formed weaker secondary structures, enabling more linear DNA to be formed. This eliminated some compression of the nucleic acid and resulted in a better separation pattern. The addition of a stopping solution halted the reaction.

b) Preparation of sequencing gel

The sequencing reactions were resolved on an 8% polyacrylamide (BioRad Laboratories, Hercules, CA)-6 M urea (Merck) gel in 1 X Tris-Borate-EDTA (TBE) buffer (Amresco).

c) Electrophoresis of sequenced samples

The samples were denatured for 2 min at 75ºC before loading 3 µl of the sample into the allocated well. All samples were loaded in the sequence G-A-T-C. Electrophoresis was allowed for a period of time as determined by the size of the initial PCR product.

d) Development and analysis of sequencing gel

The gel was fixed and vacuum-dried before being exposed to X-ray film (Hyperfilm™-ßmax, AEC/Amersham [Pty] Ltd., Kelvin, SA) for 12-24 h at room temperature. The film was developed and results analysed by reading the bands exposed on the film in the ascending order of G-A-T-C from the lower end. The profiles generated by long and short electrophoresis periods were consolidated to provide a single sequence to be analysed.

4.3.4.2 Cloning of amplicons

HAV amplicons, derived from water samples which gave uninterpretable sequencing data after direct sequencing, were cloned to establish whether

there was more than one HAV strain within the sample. Cloning was performed as described by Page and Steele (2004). White colonies (5 colonies per plate), containing the cloned products, were picked from the plates with a sterile pipette tip, each suspended separately in 15 µl of sterile nuclease-free water and amplified in a 50 µl PCR reaction mix, excluding the RT step, as described previously (section 2.3.6.2). The PCR cycles used were as described in 2.3.6.2b.

4.3.4.3 Automated sequencing

RT-PCR amplicons were purified with Exonuclease 1/Shrimp Alkaline Phosphatase (Fermentas, Lithuania). Both strands of the DNA amplicons were sequenced using the Spectrumedix SCE2410 genetic analysis system with the ABI BigDye Terminator cycle sequencing kit (version 3.1, Applied Biosystems, Foster City, CA) as specified by the manufacturer. Nucleotide sequences were manipulated and interpreted using CHROMAS (version 1.45, Griffith University, Queensland, Australia). Automated sequencing was performed by Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, SA. The PCR amplicons derived from the cloned HAV nucleic acid fragments, were sequenced using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) and compiled with the accompanying sequence analysis software module.

4.3.5 Sequence analysis and genotyping

A 177 bp (nt 2208-2384) region within the 247 bp VP3/VP1 amplicons, and a 168 bp (nt 3024-3191) region within the 360 bp VP1/2A amplicons were used for sequence analysis and genotyping of HAV strains.

Nucleotide sequences were entered into a database in PC/GENE (version 6.85, IntelliGenetics Inc., Geneva, Switzerland). New sequences were translated and genotyped using the CLUSTALX (version 1.8) program by pairwise comparison of nt of the isolates and reference strains (Thompson et al., 1997).

In addition, nucleotide sequences of the SA clinical specimens were compared, by pairwise analysis, to HAV strains isolated from the water samples from different geographic regions for the same time period. A summary of the reference strains used for the sequence analysis is presented in Table 9.

Table 9: Reference strains used for pairwise analysis based on the VP1 region of the HAV genome

Strain	Genotype	GenBank accession no.	Reference
HM-175	IB	M14707	Cohen et al., 1987
MBB	IB	M20273	Paul et al., 1987
MS-1	IA	M22821	Robertson et al., 1987
CF53	II	AY64476	Lu et al., 2004
GA76	IIIA	M66695	Robertson et al., 1991
PA21	IIIA	M34084	Brown et al., 1989
CY145	IV	M59286	Nainan et al., 1991
AGM27	V	D00924	Tsarev et al., 1991
JM55	VI	N/A	
SLF88	VII	AY032861	Ching et al., 2002
SA strains previously isolated included in sequence analysis			
923200	IA	U66481	Taylor, 1997
VDM	IA	U66489	Taylor, 1997
406808	IA	U68695	Taylor, 1997
406909	IB	U66483	Taylor, 1997
2334	IB	U66487	Taylor, 1997
412991	IB	U68689	Taylor, 1997
504184	IB	U66485	Taylor, 1997
6100	IB	N/A	Taylor, 1997
JVR	IB	U68692	Taylor, 1997
SLUDGE	IB	U66488	Taylor, 1997

N/A = not available

Reference strain JOR88, isolated from Amman, Jordan, was included for sequence analysis (Robertson et al., 1992; Taylor, 1997). Dendograms were generated using the CLUSTALX version 1.8 program.

4.4 RESULTS

4.4.1 HAV strains from surface and drinking water samples

A total of 11 amplicons from HAV strains from surface water samples and 9 strains from treated drinking water samples were of adequate quality to be sequenced. The HAV strains and their source are summarised in Table 10.

4.4.2 HAV strains from clinical specimens

HAV RNA was detected in 56.47% (48/85) of the anti-HAV IgM serum specimens by RT-PCR-oligonucleotide probe assay. Of these RT-PCR positive specimens, 14 amplicons were of satisfactory quality for direct sequencing. HAV RNA was detected in 0.92% (3/327) of paediatric stool samples of HAV asymptomatic children presenting with diarrhoea. These three amplicons were of adequate quality to be sequenced directly.

Demographic data of patients and sources of HAV are presented in Table 11. Most (83%) of the adult patients were of white Caucasian origin within the higher socio-economic population, while 50% of paediatric patients were from the lower socio-economic communities.

Table 10: HAV RNA detected in treated drinking and surface waters sources

Strain	Source	Cell culture or Direct water	Genomic region amplified
BL3	Surface	FRhK-4R	VP3/VP1 & VP1/2A
VAALD	Dam	FRhK-4R	VP3/VP1& VP1/2A
WH3	Drinking water	FRhK-4R	VP3/VP1& VP1/2A
WH4	Raw sewage	FRhK-4R	VP3/VP1& VP1/2A
K19K	River	FRhK-4R	VP3/VP1& VP1/2A
A18_F	Dam	FRhK-4R	VP3/VP1
A18_P		PLC/PRF/5	VP3/VP1
A18_DIR		Direct water	VP3/VP1
A15_F	Drinking water	FRhK-4R	VP3/VP1
A15_DIR		Direct water	VP3/VP1
A19	Drinking water	Direct water	VP3/VP1
A20	Drinking water	Direct water	VP3/VP1
B4	Drinking water	Direct water	VP3/VP1
N1	River	FRhK-4R	VP3/VP1
N6_F	River	FRhK-4R	VP3/VP1
N6_P		PLC/PRF/5	VP3/VP1
N6_DIR		Direct water	VP3/VP1
S2_F	Drinking water	FRhK-4R	VP3/VP1
S2_P		PLC/PRF/5	VP3/VP1
WH31P	Drinking water	PLC/PRF/5	VP3/VP1

Table 11: Demographic data of clinical specimens

Isolate	Specimen	Patient data		Epidemiological data
		Age (years)	Ethnicity	
20X9564	Serum	13	W	Sporadic HAV
20X7915	Serum	?	W	Sporadic HAV
20X6073	Serum	17	A	Sporadic HAV
20X4464	Serum	3	A	Sporadic HAV
20X3009	Serum	10	W	Sporadic HAV
20X26133	Serum	35	W	Sporadic HAV
20X24214	Serum	13	W	Sporadic HAV
20X195	Serum	Adult	W	Fulminant HAV
99X7724	Serum	8	A	Sporadic HAV
99X539	Serum	40	W	Sporadic HAV
99X4337	Serum	29	W	Sporadic HAV
99X1953	Serum	?	A	Sporadic HAV
99X18636	Serum	21	W	Sporadic HAV
99X13311	Serum	7	A	Sporadic HAV
STOOL_E9	Stool	<5	?	Gastroenteritis
STOOL_U10	Stool	<5	?	Gastroenteritis
STOOL_W2	Stool	<5	?	Gastroenteritis

W = white Caucasian

A = African ethnic origin

? = data not available

4.4.3 Sequence analysis

4.4.3.1 Clinical specimens

Analysis of the 177 bp fragment of the VP3/VP1 region enabled the successful genotyping of 17 clinical specimens isolated during this investigation. The relatedness of these strains to each other, HAV reference strains and

previously isolated SA HAV strains (Taylor, 1997), is presented in Figure 16. Although all clinical isolates were identified as genotype IB, two distinct clusters were evident (Figure 16).

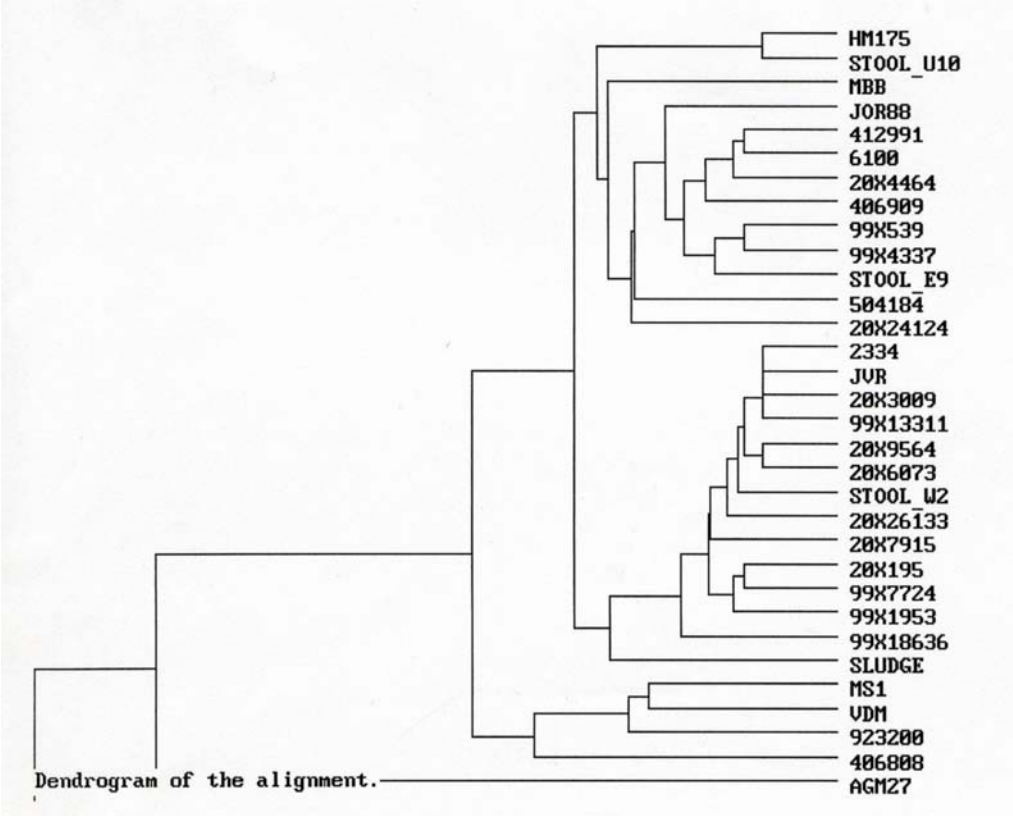


Figure 16: Pairwise comparison of 177 bp nucleotide sequences within the VP3/VP1 region of clinical HAV isolates in relation to SA isolates from an earlier study (Taylor, 1997) and selected HAV reference strains

Reference strains HM-175, MBB and JOR88 grouped together in a cluster with six SA strains isolated during this study. The percentage nt identity of specimen STOOL_U10, was 100% to that of reference strain HM-175. Sequences derived from serum specimens, 99X539, 99X4337, 20X4464, and 20X24214, and the stool specimen, STOOL_E9, clustered closely to the JOR88 strain with 95-97% nt identity to this strain, compared to 93-96% nt

identity to the type strain HM-175. These clinical strains clustered together with previously identified SA strains 412991, 6100, 406909, 504184 (Taylor, 1997).

The remaining 10 HAV strains, from serum specimens and stool specimen, STOOL_W2, clustered separately, with 92-93% nt identity to type strain HM-175. Within this cluster a number of strains, namely showed a high percentage (100%) of nt identity to SA HAV strains, JVR and 2334, isolated in 1983 and 1984 respectively. Strains 20X7915, 99X1953, 99X7724, 20X195 and 99X18636 showed >97% nt similarity to each other and 92-94% nt identity to HM-175.

4.4.3.2 Water samples

The pairwise comparative analysis of nt sequences, based on the 177 bp VP3/VP1 region, of HAV RNA detected in water samples, in relation to the HAV reference strains, is presented in Figure 17.

All HAV strains from the surface and drinking water samples clustered within subgenotype IB. Based on the 177 bp fragment from the VP3/VP1 region of the HAV genome, nt sequences from 17 amplicons derived from 13 water samples showed 100% identity to the type strain HM-175 and cell culture-adapted strain pHM-175. The nt sequences of the A18_F, A18_P, and A18_DIR strains, amplified from water samples immediately after viral concentration, and from infected FRhK-4R and PLC/PRF/5 cell cultures respectively, were identical to one another and to HM-175 and pHM-175. Strain A15_F, derived from FRhK-4R cell culture, differed by only three bases (98.3% nt sequence identity; 96.6% amino acid identity) to the A15_DIR strain, which was directly amplified from the same water sample.

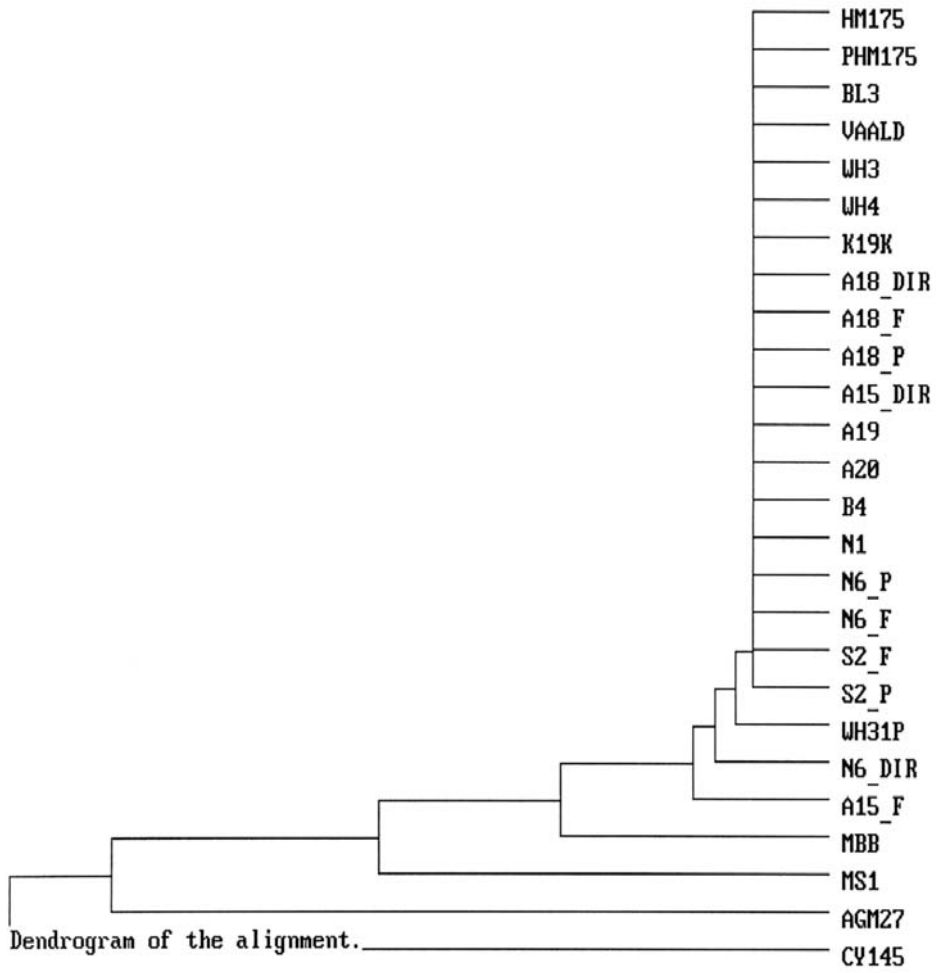


Figure 17: Dendogram of pairwise comparison of nucleotide sequences derived from drinking and surface water samples based on the 177 bp fragment from the VP3/VP1 region of the HAV genome

Nucleotide sequences from amplicons derived from N6_F and N6_P, isolated from FRhK-4R and PLC/PRF/5 cell culture extracts respectively, showed 100% similarity to each other and to HM-175 and pHM-175. However the nt sequence of the amplicon derived directly from the same water sample (N6_DIR) immediately after viral recovery, differed by two bases (98.8% nt sequence similarity; 96.6% amino acid identity) from the strains derived from cell culture.

Since a number of the sequences showed 100% nt sequence identity to the pHM-175 strain which was used as a control, selected strains (Table 10) were analysed using a 168 bp fragment within the VP1/2A region. The five strains analysed showed 100% nt identity to the pHM-175 strain (results not shown).

The relationship between isolates from water samples and those from clinical specimens are presented in Figure 18. Isolates N6_F and A15_DIR represents isolates with 100% nt sequence identity to HM-175 and pHM-175. From the results it was evident that the water isolates clustered together with HM-175 and a single clinical specimen, STOOL_U10. All the other clinical specimens clustered separately from this cluster.

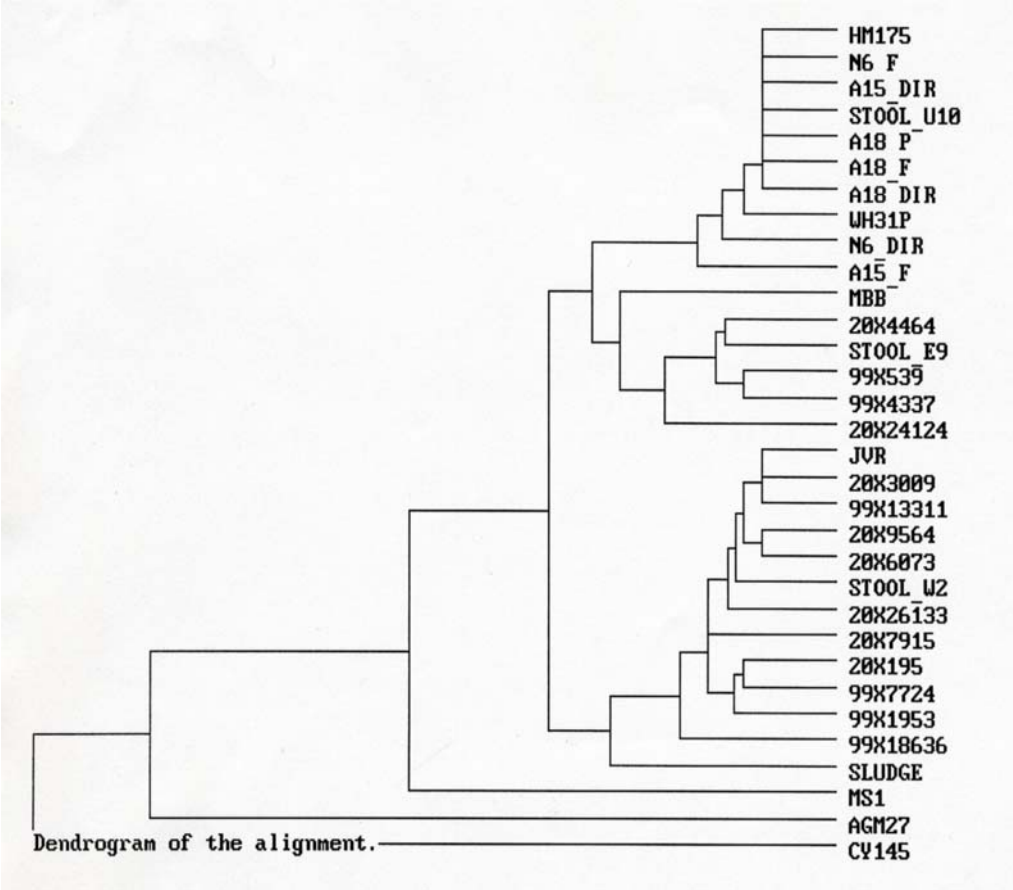


Figure 18: Dendogram depicting the relationship, over a 177 bp fragment of the VP3/VP1 terminus region, between the surface and drinking water and clinical HAV strains in relation to other isolates characterised previously over a 177 base pair fragment of the VP3/VP1 terminus region

4.5 DISCUSSION

To date, few studies investigating the molecular epidemiology of HAV in SA have been published (Kedda et al., 1995; Taylor, 1997). In one of the molecular epidemiological studies performed on clinical specimens, collected 1982 to 1996, subgenotype IB was demonstrated to be the predominant genotype circulating in the SA community (Taylor, 1997). Kedda et al., (1995) reported HAV genotype II from SA strains from an outbreak at a crèche and in sporadic HAV cases, but during the present investigation, no genotype II strains were detected. All the clinical specimens investigated in the present study grouped into subgenotype IB. Data from the current investigation corresponds to findings of Taylor (1997). The low detection rate in 56.47% of anti-HAV IgM positive serum specimens, could be ascribed to the fact that many of the specimens had been frozen and thawed at least once prior to analysis. Repeated freezing and thawing leads to denaturation and precipitation of proteins, causing reduced viral titres and subsequently reduced yields of the isolated viral RNA. In addition, cryoprecipitates formed by freeze-thawing will cause clogging of the QIAamp membranes if not removed prior to processing by brief centrifugation (6800 x *g*, 3 min).

One of the clinical specimens, STOOL_U10, showed 100% nt identity to the type strain HM-175. This type has not been described previously in Africa. This specimen was collected from a HAV asymptomatic child presenting with diarrhoea. This suggests that the HM-175 strain, which was initially described in Australia, also circulates in the SA community. This phenomenon has also been encountered in Germany (Robertson, 1992) and Spain (Pina et al., 2001), where sequences identical to HM-175 were identified in the community. In most of these studies though, only one region of the genome has been used for analysis, and recent findings suggest sequencing of the whole 900 nt VP1 region to accurately genotype HAV strains (Costa-Mattioli et al., 2003). The other clinical specimens clustered into two distinct clusters within genotype IB, some (99X4337, 99X539, 20X24214, 20X4464, STOOL_E9) being closer

related to the reference strains HM-175, MBB and JOR88. The other cluster, comprising the remaining of the serum specimens and stool specimen STOOL_W2, had a nt identity of >92% to HM-175. The serum specimens are in many cases closely related to stool and serum specimens used by Taylor (1997), indicating the continuous circulation of these HAV strains in the SA communities. This observation underlines the antigenic stability of HAV.

Since many of the initial sequences of the water isolates were identical to the cell culture-adapted strain pHM-175, a second region, the VP1/2A junction region, was investigated to clarify whether or not this was due to amplicon contamination from the screened RT-PCR products. According to analysis of the VP3/VP1 region, the HM-175 and pHM-175 strains are identical, but when the VP1/2A region was investigated, these two strains differed in a single base position. Once again, the selected water isolates were identical to the pHM-175 strain, but since this region has not been used in molecular screening of any water samples or clinical specimens for the past decade, amplicon contamination was excluded. The results indicated HAV viral contamination during either water sampling, handling, transport or recovery procedures. All these steps before the samples are inoculated onto cell culture are performed by different groups or individuals, which make it difficult to pinpoint the source of contamination. Sufficient controls were included in cell culture infection, RNA extraction and detection techniques to exclude these steps as points of contamination. In future, the whole VP1 region should be sequenced as suggested by Costa-Mattioli et al. (2003), to eliminate false results, since HM-175, but not pHM-175, is usually included for comparative purposes. By sequencing the complete VP1 region, the possibility of contamination with cell culture-adapted strains could be excluded. Alternatively the 5' NCR region should be sequenced as the pHM-175 strain has a duplication which facilitates differentiation from WT HAV strains (Sánchez et al., 2002).

Initial nt sequences from amplicons derived from water isolates, WH31P and N6_DIR, gave uninterpretable results, hence the amplicons were cloned to elucidate whether there was more than one strain present or not. These two samples were not identical to the HM-175, pHM-175 or other water isolates. Even though there were nt sequence differences, the WH31P isolate's amino acid sequence was identical to that of HM-175, relating to the antigenic stability of HAV. N6_DIR differed in two amino acid sequences from HM-175 and the two isolates derived after cell culture propagation. This indicates that possible cell culture adaptation has occurred. Nucleotide similarity between A15_F, a cell culture derived isolate, and A15_DIR, isolated directly after viral concentration and 100% identical to HM-175, was 98.3%, while amino acid similarity was 96.6%. This suggests that possible nt deletions or insertions have taken place during cell culture adaptation process.

Since a clinical specimen was found to be identical to the majority of strains isolated from water sources, the possibility of water being a source of human infection could not be excluded. However as the clinical strain was from a paediatric patient presenting with gastroenteritis and not hepatitis *per se*, it could be speculated that the paediatric patient had an asymptomatic infection, which in case of HAV is common in an endemic region, such as SA. The other clinical strains isolated were however associated with clinical infections and it could be speculated that these strains were more pathogenic, but there is no conclusive evidence to support this theory. Our inability to detect other strains of HAV in water sources poses questions as to the stability and survival of different strains in the environment.

CHAPTER 5

HEPATITIS A VIRUS IN SURFACE WATER IN SOUTH AFRICA: WHAT ARE THE RISKS?

This chapter was written in the format as required by "The Journal of Applied Microbiology".

5.1 ABSTRACT

Aims: To assess the potential risk of infection constituted by HAV to persons using surface dam and river water for domestic and recreational purposes. **Methods and Results:** This investigation estimates the potential risk using a deterministic exponential risk assessment model which works with mean values and conservative assumptions. HAV was detected in 17.5% of river and 14.9% of dam water samples tested. The probability of infection (P_{inf}) to the higher socio-economic population using the river water for recreational purposes was 1.09 X 10^{-3} per day and 3.28 X 10^{-1} per annum if 100 ml was ingested per day. For recreation in the dam water the P_{inf} value was 1.17 X 10^{-4} per day and 4.18 X 10^{-2} per annum. For the lower socio-economic population, risk values for drinking purposes (2 L day^{-1}) were ten-fold greater. The number of indicator organisms in these sources exceeded drinking and recreational water quality guidelines.

Conclusion: These surface waters did not conform to the US EPA acceptable risk of 1 infection per 10 000 consumers per year for drinking water. Neither of the water sources conformed to US EPA bacterial quality guidelines for recreational water indicating possible health risks to recreational water users.

Significance and impact of study: This is the first risk assessment study addressing the risk of infection constituted by HAV in surface water to different socio-economic populations in South Africa.

Keywords: hepatitis A virus, surface water, risk assessment, drinking water, recreational water

5.2 INTRODUCTION

A wide spectrum of human enteric viruses excreted in faeces is potential water pollutants (Grabow, 1996). Faecally polluted natural surface water used for recreational activity could therefore pose a potential health risk to the public (Fewtrell, 1991; López-Pila and Szewzyk, 2000). Different analytical methods, namely epidemiological studies and mathematical models based on dose-response relationships, have been applied to determine the risk of infection posed by viruses in recreational (Fewtrell, 1991; Fewtrell et al., 1992; Garin et al., 1994; Gammie and Wyn-Jones, 1997) and drinking water (Gerba and Haas, 1988; Bosch et al., 1997; Lee and Kim, 2002; Vivier et al., 2002). Contaminated drinking water has been implicated in outbreaks of hepatitis A (HA) (Hunter, 1997) and recreational exposure to faecally polluted water has unequivocally been linked to HA outbreaks (Mahoney et al., 1992; Hunter, 1997), with the risk of infection increasing with increased immersion in contaminated water (Taylor et al., 1995; Gammie and Wyn-Jones, 1997). To date, a limited number of epidemiological studies have been applied to determine the risk posed by hepatitis A virus (HAV) infection after recreational exposure to polluted surface water sources (Phillip et al., 1989; Gammie and Wyn-Jones, 1997). There is however a dearth of guidelines, both worldwide and in South Africa (SA), as to what an acceptable risk of infection is for waterborne pathogens. In the Surface Water Treatment Rule of the United States of America (US) Environmental Protection Agency (EPA) 1 waterborne infection per 10 000 consumers per year for drinking water is considered an acceptable risk (EPA, 1986; Regli et al., 1991, Macler, 1993;

Hunter et al, 2003). This acceptable risk level is based on dose-response data for rotaviruses (Ward et al., 1986) and *Giardia* (Regli et al., 1991; Macler and Regli, 1993). As there are very few data on the maximum acceptable limit for viruses in recreational water (Guidelines for Canadian Recreational Water Quality, 1992), water quality guidelines and risk values are based on levels of indicator organisms, namely faecal coliforms, enterococci and *Escherichia coli* (EPA, 1986; Guidelines for Canadian Recreational Water Quality, 1992; López-Pila and Szewzyk, 2000). These levels of risk may be too lenient with regard to HAV infection as the burden of disease and economic impact of HA is substantial (Macler and Regli, 1993; Centers for Disease Control and Prevention [CDC, 1999]; Cuthbert, 2001). It is therefore important to determine the risk posed by HAV to persons exposed to faecally polluted surface water.

Hepatitis A virus is endemic in SA with epidemiological features of both the developed and developing countries being present (Martin et al., 1994; Schoub et al., 2000). Routine vaccination for HAV is not included in the childhood immunization schedule currently recommended in SA (Department of Health, 1995), consequently in the high density, low socio-economic communities where sanitation is inadequate, nearly 100% of children acquire immunity before the age of ten years (Abdool Karim and Coutsoudis, 1993; Martin et al., 1994; Taylor, 1997; Taylor et al., 2001). However, with the current trends in urbanization, and as sanitary conditions improve, a change in the epidemic vulnerability of this population group can be expected (Martin, 1992; Taylor et al., 2001). This could result in an increase in the incidence of symptomatic HA in the adult population with associated economic impact (Grabow, 1997). The sporadic pattern of disease is seen in the urbanized, higher and predominantly white socio-economic community where the prevalence and severity of clinical HAV infection increases with age (Martin, 1992), and by 40 years of age 50-70% of this community will be immune to HAV infection (Sathar et al., 1994; Taylor et al., 2001).

Although there are little data on water-related viral illnesses in SA (Grabow, 1996), contaminated river water was identified as the possible source of HAV infection in canoeists (Taylor et al., 1995). Hepatitis A virus has been detected in surface river and dam (impoundment) water used for recreational and domestic purposes in SA (Taylor et al., 2001). These water sources are used by the non-immune higher socio-economic communities for recreational activities while the predominantly immune lower socio-economic population uses the same water for domestic, irrigation and recreational purposes. Data regarding the burden of HAV infection and disease in SA is inadequate (Schoub et al., 2000), consequently the contribution of treated and untreated drinking water, and recreational water to the burden of HAV infection in SA is unknown. In this study the possible risk of infection constituted by HAV to individuals in different socio-economic communities using the same surface water sources for domestic and recreational purposes was determined. To quantify the possible risk of infection posed by HAV, a risk assessment approach based on the following four steps was applied: (1) hazard identification, (2) dose-response assessment, (3) exposure assessment, and (4) risk characterization (Haas et al., 1999).

5.3 MATERIALS AND METHODS

5.3.1 Hazard identification

Hepatitis A virus is a small (27 nm in diameter), icosahedral, non-enveloped, single-stranded (ss), positive-sense RNA virus belonging to the family Picornaviridae (Hollinger and Emerson, 2001). The two biotypes of HAV, namely human HAV and simian HAV, are the only members of the genus *Hepatovirus* (King et al., 2000). There is only one serotype (Hollinger and Emerson, 2001), with infection conferring lifelong immunity (Appleton, 2000; Hollinger and Emerson, 2001). Based on primary sequence variability there are four human and three simian genotypes (Feinstone and Gust, 1997; Taylor,

1997; King et al., 2000; Robertson, 2001). The infectious dose of HAV is unknown but is presumed to be of the order of 10 to100 particles (US Food and Drug Administration/Center for Food Safety and Applied Nutrition, 2004), which suggests that even low levels of faecal pollution could pose a risk of infection.

Of the five hepatotropic viruses, HAV is the major cause of self-limiting acute viral hepatitis worldwide (World Health Organisation [WHO], 2000). Recovery is usually uneventful and there is no permanent adverse effect from the infection (Beard and Lemon, 1999; Zuckerman and Zuckerman, 1999). Relapsing HA, with concomitant faecal excretion of virus, can however occur in 3–20% of cases (Hollinger and Ticehurst, 1996; Cuthbert, 2001). Enteric viruses are excreted in high numbers (10^5-10^{12} per gram) in water during recreational activities, with each person excreting up to 0.14 g of faecal material per exposure (Gerba, 2000). Maximal faecal excretion of HAV occurs two to three weeks prior to the onset of clinical symptoms (Zuckerman and Zuckerman, 1999; Cuthbert, 2001) and remains infectious for three to four weeks after the alanine aminotransferase (ALT) levels peak (Polish et al., 1999), facilitating the spread of the virus. HAV is resistant to free residual chlorine of up to 2 mg L^{-1} (Hollinger and Ticehurst, 1996; Feinstone and Gust, 2002), high temperatures (Feinstone and Gust, 1997; Koopmans et al., 2002), low relative humidity (Mbithi et al., 1991) and pH values as low as pH 1 (King et al., 2000; Feinstone and Gust, 2002). HAV has been shown to survive for months in experimentally contaminated fresh water, seawater, marine sediments, wastewater, soils, and oysters (Hollinger and Emerson, 2001) and depending on conditions, can be stable in the environment for months (CDC, 1999).

HAV is predominantly spread by the faecal-oral route with person-to-person contact being the most important route of infection (Ryder, 1999; Cuthbert, 2001). High risk populations include the elderly, immunocompromised patients, intravenous drug abusers, and individuals living in close proximity

together such as families, young children and the staff in day care centres, military personnel, and institutionalized individuals (Lemon and Stapleton, 1993; Hollinger and Ticehurst, 1996; Feinstone and Gust, 2002). Children experience asymptomatic infections in >90% of cases (Zuckerman and Zuckerman, 1999), and serve as reservoir of infection for adults who are more likely to experience clinically apparent and more severe infection (Termorshuizen et al., 2000; Hollinger and Emerson, 2001), with a fatality rate of 1.5% in persons over the age of 64 (Martin, 1992; Hollinger and Ticehurst, 1996; Feinstone and Gust, 1997; Cuthbert, 2001). Recent data indicates that faecal excretion of HAV may be prolonged in HIV-infected individuals thereby serving as additional reservoir of infection (Ida et al., 2002). Food and water have been identified as important vehicles of HAV infection worldwide (Grabow, 1997; Appleton, 2000; Cuthbert, 2001; Koopmans et al., 2002), with outbreaks linked to faecally contaminated treated and untreated drinking water (Bloch et al., 1990, Gerba and Rose, 1990; Friedman-Huffman and Rose, 1998) and recreational water sources (Taylor et al., 1995; Gammie and Wyn-Jones, 1997).

5.3.2 Exposure assessment

5.3.2.1 Surface water samples

Over a period of three years (June 1997-June 2000) weekly water samples, ± 190 L and ± 25 L, were collected from the same sites from a dam and river in Gauteng, SA, respectively. The dam water is used as source water for a water purification facility as well as by the higher socio-economic community for recreational purposes, while the river and dam water is used by the lower socio-economic communities for domestic and recreational purposes. Hepatitis A virus was detected in 27/154 (17.5%) river and 23/154 (14.9%) dam water samples by an integrated cell culture-reverse transcriptase-polymerase chain reaction (RT-PCR)-oligonucleotide probe hybridization assay as described previously (Taylor et al., 2001). In addition to HAV, the

water samples were routinely analysed for selected indicator organisms, namely total and faecal coliforms and F-RNA coliphages.

5.3.2.2 Exposure Analysis

The exposure analysis was based on: (1) the concentration of HAV in the two sources respectively; (2) the efficiency of the recovery technique used; (3) the viability of the virus; and (4) the average volume of water consumed during recreational activities or drinking purposes per individual in the different socio-economic populations. Since the higher socio-economic population does not use the surface water for drinking purposes, only possible health risk values for recreational activities were calculated. The daily exposure (N) was determined using the following equation (Teunis et al., 1997; Haas et al., 1999):

$$N = C \times 1/R \times I \times 10^{-DR} \times V_c$$

Where: C = Concentration of HAV in the water samples (viruses L^{-1})

R = Efficiency of recovery (%)

I = Fraction of the detected pathogens capable of infection (viability)

DR = Removal or inactivation efficiency of treatment processes (DR = 0 since surface water is untreated)

V_c = Daily individual consumption of water (L day^{-1})

Concentration (C_0): The integrated cell culture-RT-PCR-oligonucleotide probe hybridization assay used to detect HAV in the water samples gives qualitative and not quantitative results. In order to determine the concentration of HAV in the different water sources, a random distribution of viruses within and between samples is assumed and described by a Poisson distribution. The Poisson parameter, λ, was calculated for the dam and river water sources respectively (Table 1). The mean concentration of HAV L^{-1}

was calculated to be 7.94 X 10^{-3} in the river water and 8.53 X 10^{-4} for the dam water (Table 1).

Corrected HAV concentration (C): To calculate the corrected mean concentration of HAV in water, the efficiency of recovery value plays an important role. The calculation for corrected viral concentration per litre of water is:

$$C = C_0 \text{ X } (1/R) \text{ X } I$$

Values to calculate the corrected mean HAV concentration in the water sources investigated are summarized in Table 1 and 2.

Table 12: Results used to calculate the mean hepatitis A virus (HAV) concentration per litre during the three year investigation period

	Calculation	River	Dam
Mean volume of water analysed	Average volume of 154 water samples	24.3 L	190 L
Mean HAV detected	Fraction of positive HAV results from 154 water samples (RT-PCR)	17.5%	14.9%
Negative	1 – (mean HAV detected)	8.25 X 10^{-1}	8.51 X 10^{-1}
Poisson parameter (λ)	-ln (negative)	1.93 X 10^{-1}	1.62 X 10^{-1}
Concentration viruses (C_0)	λ / mean volume of water	7.94 X 10^{-3}	8.53 X 10^{-4}

Table 13: Summary of the model parameters used in the deterministic model to estimate probable health risk posed by hepatitis A virus (HAV)

Model parameters	River	Dam	Dimension
HAV Concentration in water (C_0)	7.94×10^{-3}	8.53×10^{-4}	Virus L^{-1}
Recovery (R)	40%	40%	
Decimal reduction by treatment (DR)	0	0	
Infectivity (I)	1	1	
Volume consumed (recreational) (V_r)	0.1	0.1	L day^{-1}
Volume consumed (drinking) (V_d)	2	2	L day^{-1}
Dose response parameter (r)	0.549	0.549	

Efficiency of recovery (EOR) method: This represents the fraction of pathogenic microorganisms recovered. Viruses present were recovered using a glass wool adsorption-elution technique (Grabow and Taylor, 1993) and secondarily concentrated by precipitation with polyethylene glycol 6000 in the presence of sodium chloride (Vilaginès et al., 1997; Taylor et al., 2001). Based on in-house efficiency of recovery studies (unpublished data), an overestimate EOR value of 40% was used in calculations.

Viability: Recovered viruses were isolated and amplified on FRhK-4R, passages 60-100 (kindly supplied by Prof. Dr B Flehmig, Hygiene-Institut der Eberhard Karls Universität, Tübingen) and the PLC/PRF/5 (ATCC CRL 8024), passages 80-100, cell lines. Cell monolayers in 25 cm^2 flasks were infected in duplicate and harvested after 21 days prior to analysis using a RT-PCR-oligonucleotide probe hybridization assay. Amplification of the nucleic acid in cell culture is considered to be an indication of the potential viability and hence infectiousness of the virus since *in vitro* amplification requires infection of a host cell and the activation of the replication cycle (Deng et al., 1994; Taylor et al., 2001). Since naked viral nucleic acids do not adsorb to glass wool (Grabow et al., 2001), and will degrade rapidly and not be able to survive in the environment (Pallin et al., 1997), it can be concluded that the viruses detected in this study are intact, viable and potentially infectious.

Consumption: For the purpose of this study, an assumed value of 100 ml was taken as the volume ingested per day for recreational exposure (one or more exposures per day) (Haas, 1983; Haas and Eisenberg, 2001), and that an individual consumed an estimated of 2 L of unboiled water per day for drinking purposes (Haas et al., 1993; Macler and Regli, 1993; Haas and Eisenberg, 2001; Vivier et al., 2001). This assumption represents an overestimate of water consumed and will not result in an underestimated risk value.

5.3.3 Hazard characterization

5.3.3.1 Risk model

For this investigation, an exponential model was applied to estimate the risk constituted by HAV in water to consumers. Since this is the first mathematically-based risk analysis done to determine the probable risk of infection constituted by HAV in surface water, many uncertainties and variables were identified, consequently, this deterministic model was chosen in an effort to minimize the various uncertainties. The model uses mean values and works on the basis of an overestimate, so as to represent the worst-case scenario. The daily risk of infection with HAV was calculated as follows:

$$P_{inf/day} = 1 - \exp(-rN)$$

where $P_{inf/day}$ = probability of becoming infected

N = number of HAV particles ingested

r = dose response parameter

The estimated annual risk of infection ($P_{inf/year}$) was calculated as follows:

$$P_{inf/year} = 1 - (1 - P_{inf/day})^{365}$$

5.3.3.2 Uncertainties

Since point estimates are used, the degree of uncertainty in the risk determination is not represented. These uncertainties include variable consumption volume, socio-economic status, population behaviour, exposure patterns, immune competency, age, etc. Sensitivity analyses demonstrate the model's response to various input parameters such as variant recovery values and consumption volumes. Virus concentrations, and therefore the risk of infection can change depending on recovery, isolation and detection techniques applied.

5.3.3.3 Dose-response parameter and probabilities

The dose-response parameter, r (0.549), used in this investigation was that reported by Haas and Eisenberg (2001). The probability of the two socio-economic populations becoming clinically ill ($P_{illness}$) from an infection was calculated by multiplying the probability of infection (P_{inf}) with the morbidity rates for each group. Depending on age, the morbidity for the high socio-economic groups can be between 11% and >70% as reported for the US (CDC, 1999). An arbitrary and overestimate value of 45% was therefore used in the model to cover all age groups within the heterogeneous higher socio-economic population. For the lower socio-economic population the morbidity value used was 10% as infection occurs predominantly in children where approximately 90% infections are asymptomatic (Feinstone and Gust, 2002). Mortality rates for HAV varies from 0.5-1.5% (CDC, 1999), depending on age, immune status and socio-economic impact, and an average of 1% was used throughout the risk model. This value was multiplied with the probability value of becoming ill from infection to calculate the risk of death (P_{mort}) from a clinical infection. The parameters used in the model are presented in Table 2.

5.4 RESULTS

Microbial indicator analysis: Somatic and F-RNA coliphages were present in all dam and river water samples analysed. In the river water faecal coliform counts ranged from 130 to 66 000 colony forming units (cfu).100 ml^{-1}, with more than 99.99% of samples exceeding 200 cfu.100 ml^{-1}. In the dam water samples faecal coliforms counts ranged from 4-3100 cfu.100 ml^{-1}, with counts <100 cfu.100 ml^{-1} in 85.7% (132/154) of the samples. In 7.8% (12/154) of dam water samples the faecal coliform counts were >100 cfu.100 ml^{-1}, and in 6.5% (10/154) of samples counts exceeded 200 cfu.100 ml^{-1}.

Corrected HAV concentration: The corrected mean concentration of HAV per litre of water was 2.13 X 10^{-3} and 1.99 X 10^{-2} for the dam and river water respectively. These values could be a gross underestimate of the actual concentration of HAV in the water as the isolation and detection of HAV in cell culture is influenced by factors such as viral strain, cell type, incubation time and incubation temperature.

Sensitivity analysis: The EOR of the glass wool adsorption-elution technique used in this study was taken as 40%. The effect of varying recovery values on the probable risk of HAV infection per day for the different socio-economic populations in the dam water is demonstrated in Figure 1. From the graph it is clear that the more efficient the recovery, the lower the P_{inf} rate as the fraction of 1/R in the calculation becomes smaller. A similar trend was observed for the river water (results not shown).

The influence of the volume of water ingested (V) during either recreational or domestic activity on the P_{inf} of the two economic groups for dam water is demonstrated in Figure 2. As can be expected, the larger the volume ingested, the higher the probability of encountering and becoming infected with HAV. A similar trend is observed for the river water (results not shown).

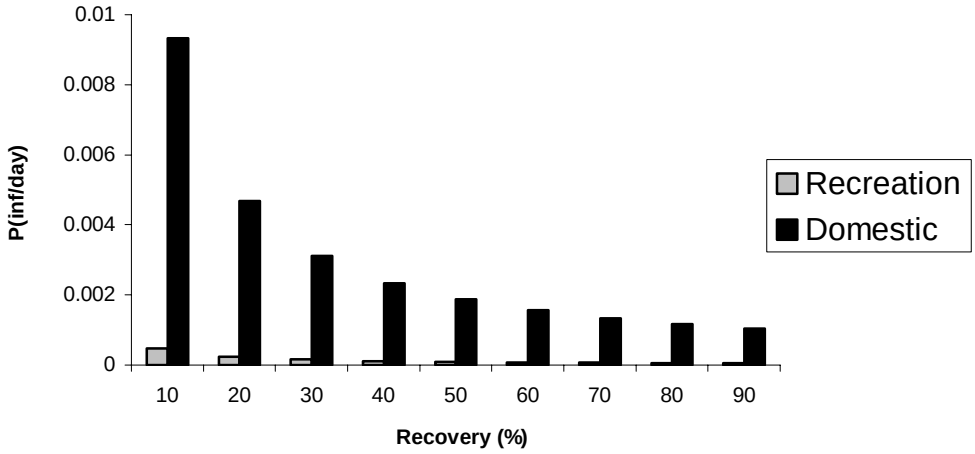


Figure 19: Effect of viral efficiency of recovery (R-values 10-90%) on the P_{inf} day^{-1} to individuals using dam water for recreational and drinking purposes

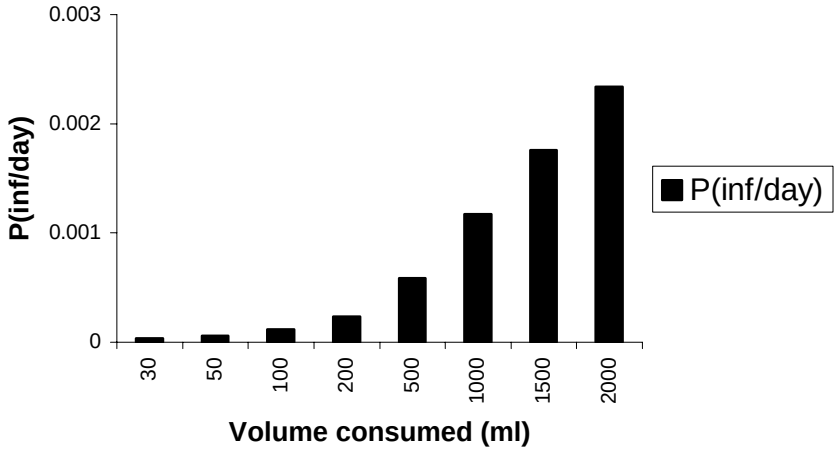


Figure 20: Influence on volume of dam water consumed on P_{inf} day^{-1} when an efficiency of recovery of 40% was used in calculations

Risk to higher socio-economic populations: The estimated daily and annual risks of HAV infection, morbidity and mortality are represented in Table 3. These results indicate that for the higher socio-economic group the daily risk of infection during recreational activity in the river water is 1.1 infection per 1 000 recreational users. For dam water recreation, the risk for infection is 1.1 in 10 000 recreational users. Avid water-sportspersons who are exposed to the

river water on a daily basis will however have a 32% annual risk of HAV infection should 100 ml be ingested per day. For recreational exposure to the dam water the annual risk of infection would be 4%.

Table 14: Calculated risk values for the higher socio-economic (HSE) and lower socio-economic (LSE) groups in South Africa during recreational and drinking use of surface water sources

Population group	River water		Dam water	
	Per day	Annual	Per day	Annual
HSE* (Recreation)				
P_{inf} ¶	1.09×10^{-3}	3.28×10^{-1}	1.17×10^{-4}	4.18×10^{-2}
$P_{illness}$ **	4.09×10^{-4}	1.48×10^{-1}	5.27×10^{-5}	1.88×10^{-2}
P_{mort} ††	4.90×10^{-6}	1.48×10^{-3}	5.27×10^{-7}	1.88×10^{-4}
†LSE (Recreation‡)				
P_{inf}	1.09×10^{-3}	3.28×10^{-1}	1.17×10^{-4}	4.18×10^{-2}
$P_{illness}$	1.09×10^{-4}	3.28×10^{-2}	1.17×10^{-5}	4.18×10^{-3}
P_{mort}	1.09×10^{-6}	3.28×10^{-4}	1.17×10^{-7}	4.18×10^{-5}
LSE (Drinking§)				
P_{inf}	2.16×10^{-2}	1	2.34×10^{-3}	5.75×10^{-1}
$P_{illness}$	2.16×10^{-3}	1×10^{-1}	2.34×10^{-4}	5.75×10^{-2}
P_{mort}	2.16×10^{-5}	1×10^{-3}	2.34×10^{-6}	5.75×10^{-4}

* Higher socio-economic population

† Lower socio-economic population

‡ 100 ml ingested per day

§ 2 litre consumed per day

¶ Probability of infection

** Probability of illness from infection

†† Probability of mortality from infection

Risk to lower socio-economic populations: The $P_{inf/day}$ to the lower socio-economic communities using the same water sources for drinking purposes

proved to be higher (Table 3). For the river water the daily risk of HAV infection was calculated to be 2%, with an annual risk of 100%. Communities using dam water for drinking (2 L day^{-1}) purposes have a daily risk of infection of approximately 2.5 in a 1 000 and an annual risk of 57%.

5.5 DISCUSSION

This investigation evaluates the potential risk of infection constituted by HAV to populations exposed to surface water sources in SA. To our knowledge this is the first study applying a mathematical model to assess the risk of HAV infection in an epidemiologically heterogeneous population exposed to contaminated surface water.

Based on the US EPA guideline of an acceptable risk of 1 waterborne infection per 10 000 consumers per year for drinking water the use of these waters for drinking purposes was unacceptable as >50% of consumers per annum were at risk of infection. However, since it is the high density lower socio-economic, predominantly immune, communities living in close proximity to these water sources that utilise the water for drinking purposes, the risk values are not cause for concern for individuals older than 10 years of age. These risk values will only be of concern for the very young (children <10 years), immunocompromised or non-immune individuals using these water sources for drinking purposes. The faecal coliform counts in these waters exceeded acceptable levels for drinking water, i.e. 0 counts.100 ml^{-1} (Department of Water Affairs and Forestry [DWAF], 1996; WHO, 1996) and persons using these waters for drinking purposes were at risk of infection (DWAF, 1996). F-RNA coliphages, used as surrogates for enteric viruses in water environments (Ashbolt et al., 2001; Grabow, 2001;Wade et al., 2003) and indicators of faecal pollution (Havelaar et al., 1993; Grabow, 1996) were also demonstrated in all of the dam and river water samples tested, implying that enteric viruses, including HAV, could have been present. The risk of

infection as determined in the model was therefore corroborated by the microbial indicator data.

If an acceptable risk level of 1 infection per 1 000 swimmers (Grabow, 1996) was applied, there was a minimal risk of infection (1-1.1 infection per 1 000 recreational users) in the higher socio-economic, predominantly non-immune, population using these surface waters for recreational activities. There was no risk of infection to adolescents and adults in the lower socio-economic communities using these waters for recreational purposes. Although the calculated annual risk of mortality appears to be high (Table 3), it must be borne in mind that the high socio-economic group, who use these waters for recreational activities, are not exposed on a daily basis. The lower socio-economic group who use the same water for drinking purposes develop immunity at a very young age, and this seemingly high annual risk of mortality would therefore only be significant for non-immune very young, elderly or immunocompromised individuals who consume 2 L of unboiled water on a daily basis.

The water samples were not tested for *E. coli* or enterococci, hence the US EPA water quality criteria for freshwater bathing waters, i.e. 126 *E. coli* cfu.100 ml^{-1} or the 33 enterococci cfu.100 ml^{-1}, which are generally applied as predictors of gastrointestinal illness (EPA, 1986; Wade et al., 2003) could not be applied to assess the risk of HAV infection. The river water did not conform to the US EPA bacterial criteria of 200 cfu.100 ml^{-1} faecal coliforms for bathing waters (Wade et al., 2003), hence on the basis of this criteria, non-immune persons using the river water for recreational purposes were at risk of infection. If these bacterial criteria were applied to the dam water, the risk of infection during recreational activity would be minimal as only 6.5% of the dam water samples exceeded the 200 cfu.100 ml^{-1} limit for faecal coliforms.

In their latest water quality guidelines (WHO, 2003), the WHO uses a volume of 20-50 ml for estimating risk of infection to recreational water users. Should these volumes, instead of 100 ml, have been applied in the model, the

calculated risk of infection would have been proportionately lower (Fig. 2). As the volume of water ingested per day could differ depending on the type of sport involved, an estimated volume of 100 ml was used to determine the risk per day for recreational water. The annual risk of HAV infection calculated for water-sportspersons exposed to these water sources on a daily basis, i.e. 32%, is supported by sero-epidemiological data. In an investigation of a cohort of South African canoeists, predominantly from the higher socio-economic group, 37% seropositivity to HAV could be attributed to canoeing (Taylor et al., 1995).

Previous investigators have estimated the risk of infection in recreational waters by determining the ratio between the pathogen and indicator organisms (López-Pila and Szewzyk, 2000; Wade et al., 2003). This could not be applied in this investigation due to the uncertainties associated with in the isolation and detection of HAV. Although the model was formulated to work on overestimates, due to the number of uncertainties and confounders identified in this investigation, the calculated risk of infection could be a gross underestimate of the actual risk constituted by HAV. Practical limitations in techniques for the recovery, isolation and detection of HAV, which clearly influence the virus concentration and subsequent calculations, were identified. Even though various primary and continuous cell cultures of primate origin support the replication of HAV, wild type (WT) HAV grows slowly or not at all in conventional cell cultures (Hollinger and Ticehurst, 1996; Taylor et al., 2001). Selected cell culture systems, namely an African green monkey kidney cell line (AGMK), and the PLC/PRF/5 and FRhK-4R cell lines, have been shown to be most susceptible for the isolation and propagation of WT HAV as well as cell culture-adapted strains of HAV (Daemer et al., 1981; Flehmig, 1981; Gust and Feinstone, 1988; Taylor et al., 2001). Primary vervet monkey kidney cells and the Vero African green monkey kidney cell line have been used to a lesser extent (Flehmig, 1981; Wheeler et al., 1986; Crance et al., 1987; Ashida et al., 1989; Stapleton et al., 1991; Bishop and Anderson, 1997; Taylor et al., 2001). Incubation periods from two weeks up to twelve months

(Crance et al., 1987), with incubation temperatures ranging between 32ºC and 37ºC, have been reported (Flehmig, 1980; Siegl et al., 1984; Crance et al., 1987; Dotzauer et al., 1994). In this study only the PLC/PRF/5 and the FRhK-4R cell lines and a single incubation temperature of 37ºC for 21 days were used prior to molecular analysis, which could have limited the number of viruses detected. In addition, it must be borne in mind that HAVs excreted later in the infection cycle are less infectious to susceptible non-human primates, e.g. marmosets, than those excreted in the first few weeks before the onset of clinical symptoms (Polish et al., 1999), which could further affect the virus infectivity and detection. Another factor that could influence the viral concentration (C) is the turbidity of the water investigated. The EOR of glass wool adsorption-elution technique for viruses has been found to be more efficient for less turbid waters (Vilaginès et al., 1997). In this study the turbidity of the water was not taken into account, thus the EOR of 40% used in the calculations could also have been an overestimate of the actual recovery of HAV from the water sources.

No risk assessment model can be universally applicable (WHO, 2003), and models need to be modified or adapted to take the micro-organism being assessed and local or national demographics and socio-cultural behaviour into consideration. A number of viruses, e.g. adenovirus, hepatitis E virus, HAV (and other picornaviruses), have been proposed as possible candidates for risk assessment models for pathogenic agents in drinking and recreational waters (Havelaar et al., 2001). As HAV represents a major health threat (Macler and Regli, 1993), and has a clearly defined clinical picture with complete recovery, it is an attractive candidate for risk assessment studies. From this investigation it is evident that this may only be applicable for countries where HAV is non-endemic and the burden of disease is clearly defined, e.g. the US (CDC, 1999). In countries where HAV is endemic and the burden of disease is not clearly defined there are too many confounders to facilitate accurate risk analysis. This study therefore only reflects the possible risk of infection

constituted by HAV in two localised surface water sources in SA and the data cannot be applied universally.

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5.7 REFERENCES

Abdool Karim, S.S. and Coutsoudis, A. (1993) Sero-epidemiology of hepatitis A in black South African children. *South African Medical Journal* **83**, 748-750.

Appleton, H. Control of food-borne viruses. (2000) *British Medical Bulletin* **56**, 172-183.

Ashbolt, N.J., Grabow, W.O.K. and Snozzi, M. (2001) Indicators of microbial water quality. In *Water Quality: Guidelines, Standards and Health: Assessment of risk and risk management for water-related infectious disease* ed. Fewtrell, L. and Bartram, J. pp.289-315. London: IWA Publishing.

Ashida, M., Hara, H., Kojima, H., Kamimura, T., Ichida, F. and Hamada, C. (1989) Propagation of hepatitis A virus in hybrid cell lines derived from marmoset liver and Vero cells. *Journal of General Virology* **70**, 2487-2494.

Beard M.R. and. Lemon S.M. (1999) Hepatitis A virus (*Picornaviridae*). In *Encyclopaedia of Virology, 2nd edition, volume 1* ed. Granoff, A. and Webster, R.G. pp.631-639. New York: Academic Press.

Bishop, N.E. and Anderson, D.A. (1997) Hepatitis A virus subviral particles: purification, accumulation, and relative infectivity of virions, provirions and procapsids. *Archives of Virology* **142**, 2147-2160.

Bloch, A.B., Stramer, S.L., Smith, J.D., Margolis, H.S., Fields, H.A., McKinley, T.W., Gerba, C.P., Maynard, J.E. and Sikes, R.K. (1990) Recovery of hepatitis A virus from a water supply responsible for a common source outbreak of hepatitis A. *American Journal of Public Health* **80**, 428-430.

Bosch, A., Lucena, F., Diez, J.M., Gajardo, R., Blasi, M. and Jofre, J. (1997) Waterborne viruses associated with hepatitis outbreak. *Water Science and Technology* **35**, 243-247.

Centers for Disease Control and Prevention [CDC]. (1999) Prevention of hepatitis A through active and passive immunisation: recommendations of the Advisory Committee on Immunisation Practices (ACIP). *Morbidity and Mortality Weekly Report (MMWR)* **48**, 1-34.

Crance, J.M., Passagot, J., Biziagos, E. and Deloince, R. (1987) Continuous production of hepatitis A virus in PLC/PRF 5 cell cultures: use of antigen for serology. *Journal of Virological Methods* **18**, 193-203.

Cuthbert, J.A. (2001) Hepatitis: Old and new. *Clinical Microbiology Reviews* 38-58.

Daemer, R.J., Feinstone, S.M., Gust, I.D. and Purcell, R.H. (1981) Propagation of human hepatitis A virus in African Green Monkey kidney cell

culture: Primary isolation and serial passage. *Infection and Immunity* **32**, 388-393.

Deng, M.I., Day, S.P. and Cliver, D.O. (1994) Detection of hepatitis A virus in environmental samples by antigen-capture PCR. *Applied and Environmental Microbiology* **60**, 1927-1933.

Department of Health. (1995) The new childhood immunisation schedule. Directorate: Epidemiology, Department of Health, South Africa. *Epidemiological Comments* **22**, 39.

Department of Water Affairs and Forestry. (1996) Domestic Use. In *South African Water Quality Guidelines 2nd edition, volume 1*. pp.87-88.

Dotzauer, A., Feinstone, S.M. and Kaplan, G. (1994) Susceptibility of nonprimate cell lines to hepatitis A virus infection. *Journal of Virology* **68**, 6064-6068.

Environment Protection Agency. (1986) *Ambient Water Quality Criteria for Bacteria - 1986*. US Environmental Protection Agency, Report No. EPA 440/5-84-002. Washington DC.

Feinstone, S.M. and Gust, I.D. (1997) Hepatitis A virus. In *Clinical Virology, 1st edition* ed. Richman, D.D., Whitley, R.J. and Hayden, F.G. pp.1049-1067. New York: Churchill Livingstone Inc.

Feinstone, S.M. and Gust, I.D. (2002) Hepatitis A virus. In *Clinical Virology 2nd edition* ed. Richman, D.D., Whitley, R.J. and Hayden, F.G. pp.1019-1039. Washington DC: ASM Press.

Fewtrell, L. (1991) Freshwater recreation: a cause for concern? *Applied Geography* **91**, 215-226.

Fewtrell, L., Godfree, A.F., Jones, F., Kay, D., Salmon, R.L. and Wyer, M.D. (1992) Health effects of white-water canoeing. *Lancet* **339**, 1587-1589.

Flehmig, B. (1980) Hepatitis A virus in cell culture: I. Propagation of different hepatitis A virus isolates in a foetal rhesus monkey kidney cell line (FRhK-4). *Medical Microbiology and Immunology* **168,** 239-48.

Flehmig, B. (1981) Hepatitis A virus in cell culture: II. Growth characteristics of hepatitis A virus in FRhK-4/R cells. *Medical Microbiology and Immunology* **170**, 73-81.

Friedman-Huffman, D. and Rose, J (1998). Emerging waterborne pathogens. *Water Quality International* **November/December**, 14-15.

Gammie, A.J. and Wyn-Jones, A.P. (1997) Does hepatitis A pose a significant health risk to recreational water users? *Water Research and Technology* **35**, 171-177.

Garin, D., Fuchs, F., Crance, J.M., Rouby, Y., Chapalain, J.C., Lamarque, D., Gounot, A.M. and Aymard, M. (1994) Exposure to enteroviruses and hepatitis A virus among divers in environmental waters in France, first biological and serological survey of a controlled cohort. *Epidemiology and Infection* **113**, 541-549.

Gerba, C.P. (2000) Assessment of enteric pathogen shedding by bathers during recreational activity and its impact on water quality. *Quantitative Microbiology* **2**, 55-68.

Gerba, C.P. and Haas, C.N. (1988) Assessment of risks associated with enteric viruses in contaminated drinking water. In *Chemical and Biological*

Characterization of Sludges, Sediments, Dredge Spoils, and Drilling Muds. pp.489-494. Philadelphia: American Society for Testing and Materials.

Gerba, C.P. and Rose, J.B. (1990) Viruses in source and drinking water. In *Drinking Water Microbiology* ed. McFeters, G.A. pp.380-396. New York: Springer-Verlag Inc.

Grabow, W.O.K. (1996) Waterborne diseases: Update on water quality assessment and control. *Water SA* **22**, 193-202.

Grabow, W.O.K. (1997) Hepatitis viruses in water: Update on risk and control. *Water SA* **23**, 379-386.

Grabow, W.O.K. (2001) Bacteriophages: Update on application as models for viruses in water. *Water SA* **27**, 251-268.

Grabow, W.O.K. and Taylor, M.B. (1993) New methods for the virological analysis of drinking water supplies. In *Proceedings (Volume 1): Biennial Conference and Exhibition of the Water Institute of Southern Africa, Elangeni Hotel, Durban, 24-27 May 1993*. pp.259-264. Johannesburg: Water Institute of Southern Africa.

Grabow, W.O.K., Taylor, M.B. and de Villiers, J.C. (2001) New methods for the detection of viruses: call for review of drinking water quality guidelines. *Water Science and Technology* **43**, 1-8.

Guidelines for Canadian Recreational Water Quality (1992) pp. 1-48. Available at:
URL: *http://www.hc-sc.gc.ca/hecs-sesc/water/recreational_water.htm* [screen 1].

Gust, I.D. and Feinstone, S.M. (1988) *Hepatitis A.* Boca Raton: CRC Press, Inc.

Haas, C.N. (1983) Estimation of risk due to low doses of microorganisms: a comparison of alternative methodologies. *American Journal of Epidemiology* **118**, 573-582.

Haas, C.N., Rose, J.B., Gerba, C.P. and Regli, R. (1993) Risk assessment of virus in drinking water. *Risk Analysis* **13**, 545-552.

Haas, C.N., Rose, J.B. and Gerba, C.P. (1999) *Quantitative Microbial Risk Assessment.* pp.86-106. New York: John Wiley and Sons, Inc.

Haas, C.N. and Eisenberg, J.N.S. (2001) Risk assessment. In *Water Quality: Guidelines, Standards and Health*. pp.161-184. London: IWA Publishing.

Havelaar, A., Blumenthal, U.J., Strauss, M., Kay, D. and Bartram, J. (2001) Guidelines: the current position. In *Water Quality: Guidelines, Standards and Health*. pp.17-42. London: IWA Publishing.

Hollinger, F.B. and Ticehurst, J.R. (1996) Hepatitis A virus. In *Fields Virology, 3rd edition* ed. Fields, B.N., Knipe, D.M., Howley, P.M., Griffin, D.E., Lamb, R.A., Martin, M.A., Roizman, B. and Straus, S.E. pp.735-782. Philadelphia: Lippincott-Raven Publishers.

Hollinger, F.B. and Emerson, S.U. (2001) Hepatitis A virus. In *Fields Virology, 4th edition* ed. Knipe, D.M., Howley, P.M., Griffin, D.E., Lamb, R.A., Martin, M.A., Roizman, B. and Straus, S.E. pp.799-840. Philadelphia: Lippincott-Raven Publishers.

Hunter, P.R. (1997) Viral hepatitis. In *Waterborne Disease: Epidemiology and Ecology*. pp.207-221. Chichester: John Wiley and Sons Ltd.

Hunter, P.R., Payment, P., Ashbolt, N. and Bartram, J. (2003) Assessment of Risk. In *Assessing Microbial Safety of Drinking Water: Improving Approaches and Methods*. pp.79-109. Geneva: OECD, WHO.

Ida, S., Tachikawa, N., Nakajima, A., Daikoku, M., Yano, M., Kikuchi, Y, Yasuoka, A., Kimura, S. and Oka, S. (2002) Influence of human immunodeficiency virus type 1 infection on acute hepatitis A virus infection. *Clinical Infectious Diseases* **34**, 379-385.

King, A.W.Q., Brown, F., Hovi, T., Knowles, N., Lemon, S.M., Minor, P.D., Palmenberg, A.C., Skern, T. and Stanway, G. (2000) Picornaviridae. In *Virus Taxonomy: Classification and Nomenclature of Viruses* ed. Van Regenmortel, M.H.V., Fauquet, C.M., Bishop, D.H.L., Carstens, E.B., Estes, M.K., Lemon, S.M., Maniloff, J., Mayo, M.A., McGeoch, D.M., Pringle, C.R. and Wickner, R.B. pp.671-673. San Diego: Academic Press.

Koopmans, M., von Bonsdorff, C., Vinjé, J., de Medici, D. and Monroe, S. (2002) Foodborne viruses. *FEMS Microbiology Reviews* **26**, 187-205.

Lee, S. and Kim, S. (2002) Detection of infectious enteroviruses and adenoviruses in tap water in urban areas in Korea. *Water Research* **36**, 248-256.

Lemon, S.M. and Stapleton, J.T. (1993) Prevention. In *Viral hepatitis: Scientific Basis and Clinical Management* ed. Zuckerman, A.J. and Thomas, H.C. pp.61-79. New York: Churchill Livingstone Inc.

López-Pila, J.M. and Szewzyk, R. (2000) Estimating the infection risk in recreational waters from faecal indicator concentration and from the ratio between pathogens and indicators. *Water Research* **34**, 4195-4200.

Macler, B.A. (1993) Acceptable risk and U.S. microbial drinking water standards. In *Safety of Water Disinfection: Balancing Chemical and Microbial Risks* ed. Craun, G.F. pp.619-623. Washington, D.C: ILSI Press.

Macler, B.A. and Regli, S. (1993) Use of microbial risk assessment in setting US drinking water standards. *International Journal of Food Microbiology* **18**, 254-256.

Mahoney, F.J., Farley, T.A., Kelso, K.Y., Wilson, S.A., Horan, J.M. and McFarland, L.M. (1992) An outbreak of hepatitis A associated with swimming in a public pool. *Journal of Infectious Diseases* **165**, 613-618.

Martin, D.J. (1992) Hepatitis A vaccination – an option for South Africa? *South African Medical Journal* **82**, 5-6.

Martin, D.J., Blackburn, N.K., Johnson, S. and McAnerney, J.M. (1994) The current epidemiology of hepatitis A infection in South Africa: implications for vaccination. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **88**, 288-291.

Mbithi, J.N., Springthorpe, V.S. and Sattar, S.A. (1991) Effect of relative humidity and air temperature on survival of hepatitis A virus on environmental surfaces. *Applied and Environmental Microbiology* **57**, 1394-1399.

Pallin, R., Wyn-Jones, A.P., Place, B.M and Lightfoot, N.F. (1997) The detection of enteroviruses in large volume concentrates of recreational waters by polymerase chain reaction. *Journal of Virological Methods* **67**, 57-67.

Phillip, R., Waitkins, S., Caul, O., Roome, A., McMahon, S. and Enticott, R. (1989) Leptospiral and hepatitis A antibodies amongst windsurfers and waterskiers in Bristol City Docks. *Public Health* **103**, 123-129.

Polish, L.B., Robertson, B.H., Khanna, B., Krawczynski, K., Spelbring, J., Olson, F. and Shapiro, C.N. (1999) Excretion of hepatitis A virus (HAV) in adults: Comparison of immunologic and molecular detection methods and relationship between HAV positivity and infectivity in tamarins. *Journal of Clinical Microbiology* **37**, 3615-3617.

Regli, S., Rose, J.B., Haas, C.N. and Gerba C.P. (1991) Modelling the risk from *Giardia* and viruses in drinking water. *Journal of American Water Works Association* **92**, 76-84.

Robertson, B.H. (2001) Viral hepatitis and primates: historical and molecular analysis of human and nonhuman primate hepatitis A, B, and the GB-related viruses. *Journal of Viral Hepatitis* **8**, 233-242.

Ryder, S.D. (1999) Viral hepatitis. In *Infectious Diseases, volume 1* ed. Armstrong, D., Cohen, J., Berkley, S.F., Carbon, C.J., Clumeck, N., Durack, D.T., Finch, R.G., Kiehn, T.E., Louria, D.B., McAdam, K.P.W.J., Norrby, S.R., Opal, S.M., Polsky, B.W., Quie, P.G., Ronald, A.R., Solberg, C.O. and Verhoef, J. pp.39.1-39.12. London: Harcourt Publishers Ltd.

Sathar, M.A., Soni, P.N., Fernandes-Costa, F.J.T.D., Wittenberg, D.F. and Simjee, A.E. (1994) Racial differences in the seroprevalence of hepatitis A virus infection in Natal/KwaZulu, South Africa. *Journal of Medical Virology* **44**, 9-12.

Schoub, B.D., Blackburn, N.K. and McAnerney, J.M. (2000) Hepatitis A virus seroprevalence in upper and lower socio-economic groups in South Africa: implications for vaccine policies. In *Viral hepatitis and liver disease:*

Proceedings of the 10th International Symposium on Viral Hepatitis and Liver disease; April 9-13, 2000 ed. Smillie, J. pp.46-48. London: International Medical Press.

Siegl, G., deChastonay, J. and Kronauer, G. (1984) Propagation and assay of hepatitis A virus *in vitro*. *Journal of Virological Methods* **9**,53-67.

Stapleton, J.T., Frederick, J. and Meyer, B. (1991) Hepatitis A virus attachment to cultured cell lines. *Journal of Infectious Diseases* **164**, 1099-1103.

Taylor, M.B. (1997) Molecular epidemiology of South African strains of hepatitis A virus: 1982-1996. *Journal of Medical Virology* **51**, 273-279.

Taylor, M.B., Becker, P.J., Janse van Rensburg, E., Harris, B.N., Bailey, I.W. and Grabow, W.O.K. (1995) A serosurvey of waterborne pathogens amongst canoeists in South Africa. *Epidemiology and Infection* **115**, 299-307.

Taylor, M.B., Cox, N., Vrey, M.A. and Grabow, W.O.K. (2001) The occurrence of hepatitis A and astroviruses in selected river and dam waters in South Africa. *Water Research* **35**, 2653-2660.

Termorshuizen, F., Dorigo-Zetsma, J.W., de Melker, H.E., Van den Hof, S. and Conyn-Van Spaendonck, M.A.E. (2000) The prevalence of antibodies to hepatitis A virus and its determinants in The Netherlands: a population-based survey. *Epidemiology and Infection* **124**, 459-466.

Teunis, P.F.M., Medema, G.J., Kruidenier, L. and Havelaar, A.H. (1997) Assessment of the risk of infection by Cryptosporidium or Giardia in drinking water from a surface water source. *Water Research* **31**, 1333-1346.

US Food and Drug Administration/Center for Food Safety and Applied Nutrition: Foodborne Pathogenic Microorganisms and Natural Toxins Handbook, Hepatitis A virus (2004) Available at:
URL: *http://vm.cfsan.fda.gov/~mow/chap31.htm* [screen 1-2].

Vilaginès, Ph., Suarez, A., Sarrette, B. and Vilaginès, R. (1997) Optimisation of the PEG reconcentration procedure for virus detection by cell culture or genomic amplification. *Water Science and Technology* **35**, 455-459.

Vivier, J.C., Ehlers, M.M., Grabow, W.O.K. and Havelaar, A.H. (2002) Assessment of the risk of infection associated with coxsackie B viruses in drinking water. *Water Science and Technology* **2**, 1-8.

Wade, T.J., Pai, N., Eisenberg, J.N.S. and Colford, J.M. Jr. (2003) Do U.S Environmental Protection Agency water quality guidelines for recreational waters prevent gastrointestinal illness? A systematic review and meta-analysis. *Environmental Health Perspectives* **111**, 1102-1109.

Ward, R.L., Bernstein, D., Young, D.E., Sherwood, J., Knowlton, D.R. and Schiff, G.A. (1986) Human rotavirus studies in volunteers: determination of infectious dose and serological response to infection. *Journal of Infectious Diseases* **154**, 871-880.

Wheeler, C.M., Fields, H.A., Schable, C.A., Meinke, W.J. and Maynard, J.E. (1986) Adsorption, purification and growth characteristics of hepatitis A virus strain HAS-15 propagated in foetal rhesus monkey kidney cells. *Journal of Clinical Microbiology* **23**, 434-440.

World Health Organization. (1996) *Guidelines for Drinking-Water Quality 2nd edition, volume 2: Health criteria and other supporting information*. pp.93-106. Geneva: WHO.

World Health Organization. (2000) WHO position paper on Hepatitis A vaccines (International notes). *WHO Weekly Epidemiological Record* **75**.

World Health Organization. (2003) *Guidelines for Safe Recreational Water Environments volume 1: Coastal and fresh waters*. pp.62. Geneva: WHO.

Zuckerman, J.N. and Zuckerman, A.J. (1999) Hepatitis viruses. In *Infectious Diseases, volume 2* ed. Armstrong, D., Cohen, J., Berkley, S.F., Carbon, C.J., Clumeck, N., Durack, D.T., D.T., Finch, R.G., Kiehn, T.E., Louria, D.B., McAdam, K.P.W.J., Norrby, S.R., Opal, S.M., Polsky, B.W., Quie, P.G., Ronald, A.R., Solberg, C.O. and Verhoef, J pp.4.1-4.12. London: Harcourt Publishers Ltd.

CHAPTER 6

DISCUSSION AND CONCLUSIONS

HAV is the leading cause of viral hepatitis worldwide (CDC, 1999; WHO, 2000). This widespread disease is endemic in many geographical regions, especially the developing world (Feinstone and Gust, 1997). Transmission occurs mainly via the faecal-oral route and prevention of HA is a global socio-economic, political and engineering problem (Lemon and Stapleton, 1993; Feinstone and Gust, 1997). HA is endemic in SA (Martin, 1992; Robertson et al., 1992) and HAV has been detected in water sources used by different socio-economic communities for recreational and domestic purposes (Taylor et al., 2001). Clinical infections are more prevalent in the higher socio-economic population, while nearly all children from the lower socio-economic communities experience predominantly asymptomatic infections and develop immunity before the age of 10 (Abdool Karim and Coutsoudis, 1993; Sathar et al., 1994). With improvement of sanitation and general living conditions for the lower socio-economic population and the current trend of urbanisation in SA, a change in the epidemiology of HA is expected (Martin. 1992).

Without efficient viral recovery procedures, virological analysis of drinking and surface water sources is futile. Many virus recovery techniques have been evaluated, i.e. glass wool adsorption-elution (Menut et al., 1993; Vilaginès et al., 1993; Environment Agency Report NC/99/40, 2000), glass powder (Gajardo et al., 1991; Menut et al., 1993), ultrafiltration using positively charged membranes (Gilgen et al., 1997) and flat membranes (Soule et al., 2000). HAV, however, has been used only in a limited number of studies as a model virus to evaluate the EOR of these techniques (Divizia et al., 1989b; Gajardo et al., 1991; Tsai et al., 1993; Soule et al., 2000). Two variations of the glass wool adsorption-elution technique were assessed during this study.

The first was a standard glass wool column, packed with 10 g of glass wool at a density of 0.5 g/cm^3 (glass wool dry w/v basis), as this was the density described in literature for optimal viral recovery. In the second glass wool column investigated, 15 g of glass wool was packed at the same density, but with three gauze metal grids inserted at 5 g intervals to ensure optimal dispersement of water when passed through the column. From the results it was evident that the modified glass wool column had a higher EOR than the standard glass wool column, but that the placement of the grids definitely influenced the final EOR. The increased quantity of glass wool incorporated into the modified method could also have, contributed towards the improved EOR. The EOR of ultrafiltration using commercial flat membranes was also investigated and proved to be the most efficient recovery technique of the three methods assessed in this study. Even though this method gave a constant EOR value of 20%, it was surprisingly low when compared to the EOR results of similar studies using poliovirus, where up to 100% recovery was reported (Divizia et al., 1989b; Soule et al., 2000). Since this was the first study comparing the EOR of different techniques using HAV as a model, it was concluded that the washing procedure to recover the viruses from the membrane may not have been optimal and clumping of the virus could have occurred. This should be investigated further. Although the ultrafiltration technique may result in the highest EOR of all the methods investigated, it was evident that this method was the least cost efficient and most impractical technique for the routine virological analysis of water sources. It was concluded that the modified glass wool technique, with the placement of grids in the positions as described in Figure 12 (column 1), would be the most efficient technique for the recovery of HAV from drinking and surface waters.

During this study, it was evident that >50% of HAV was lost during the secondary PEG/NaCl concentration technique. These results were unexpected, as an EOR of >80% as described by Vilaginès et al. (1997a), was anticipated. This phenomenon would have to be investigated further in order to establish the reason or to identify an alternative method for the secondary

concentration of HAV. The results indicate that detection of virus in the routine monitoring of the virological quality of water samples could be a major underestimate. Since recovery efficiency plays an important role in risk analysis of viruses, the estimation of risk of infection to a community could be misinterpreted. Another secondary concentration procedure, using commercially available Centricon® Plus-80 Biomax-100 filter devices was investigated as an alternative secondary concentration technique. The two techniques were compared to each other in conjunction with various viral RNA extraction methods, to determine the most effective combination of techniques for the effective isolation, and consequent detection, of viral nucleic acid by molecular techniques. The High Pure Viral Nucleic Acid kit proved to be the best viral nucleic acid extraction kit for the isolation of HAV RNA in combination with either of the two secondary viral concentration procedures included in this investigation. For practical purposes and cost effectiveness, the use of the PEG/NaCl secondary concentration procedure in combination with either the High Pure Viral Nucleic Acid, the QIAamp® Viral RNA Mini or QIAamp® UltraSens™ extraction kits is recommended. Once again the results could not be applied universally to all viruses since viral properties and structure differ, influencing their behaviour during recovery procedures. Consequently, for an efficient viral surveillance system, viral recovery and concentration techniques for individual viruses would need to be optimised, but this would once again be impractical for the routine surveillance of water sources for viruses.

Unlike other picornaviruses, WT HAV cannot readily be isolated in conventional cell cultures (Beard and Lemon, 1999; Hollinger and Emerson, 2001), and propagation does not necessarily result in CPE, e.g. cell culture-adapted strain MBB (Deinhardt et al., 1981; Taylor, 1985). The correct combination of cell type (Gust and Feinstone, 1988; Hollinger and Emerson, 2001), incubation temperature and time (Dotzauer et al., 1994), possibly integrated with passaging (Hollinger and Emerson, 2001), would have to be found before infection and subsequent amplification could take place. For this

reason a range of human (PLC/PRF/5) and non-human (BGM and FRhK-4R) primate cell lines were studied for their susceptibility to WT HAV and pHM-175 under different conditions. These cell cultures were investigated in combination with two incubation temperatures (32ºC and 37ºC), with and without blind passaging, for up to six weeks p.i. From the results it was clear that the FRhK-4R cell line, infected and incubated for 21 days at 32ºC and 37ºC without serial passage was the most suitable combination of conditions for propagation of the WT HAV strain used. The BGM cell line also supported replication of the WT HAV at 32ºC (Table 4). These results could not be applied universally to all WT HAV strains, since different strains would require different combinations of cell cultures types and propagation conditions. For routine surveillance of water sources, this would however be impractical. Various HAAg detection techniques, not relying on the formation of CPE visualisation, were investigated for the detection and quantification of HAV in cell cultures. IIF has been applied successfully for the visualisation of both pHM-175 and WT HAV in cell cultures and does not rely on plaque-formation. However, the equipment used for visualisation is expensive and interpretation of results is operator dependant, which could result in false positives or negatives. The IPFA technique, using either DAB or 3AEC as chromogen, was effectively applied for the visualisation of the WT HAV infected foci and plaques, which are small and usually not readily visible even if stained by crystal violet. This technique is however labour intensive, reagents are expensive and results are not as easily visualised as for IIF. Molecular detection techniques investigated included RT-PCR and RT-PCR-oligonucleotide probe hybridisation assays. Both assays had a sensitivity limit of only 100 $TCID_{50}$. This implies that the HAV detected in virus concentrates recovered from water and infected cell culture extracts by RT-PCR is an underestimate of the true presence of HAV. An integrated cell culture-RT-PCR-oligonucleotide probe hybridisation assay was applied for the routine detection of HAV in water samples to facilitate the detection of viable, and hence potentially infectious, virus. Although this integrated cell culture-RT-PCR-oligonucleotide probe hybridisation assay increases the sensitivity of

detection of viable viruses, the limitations in the cell culture and RT-PCR techniques still result in an underestimate of the total HAV present in a sample. In addition it must be borne in mind that viruses excreted late in the infection cycle have been shown to be non-infectious (Polish et al., 1999). This further impacts on the risk assessment, resulting in a gross underestimate of the risk of infection posed by HAV to consumers and recreational water users in SA.

Over a period of three years, HAV RNA was detected in 17.5% of samples from a river and 14.9% of samples from a dam using an integrated cell culture-RT-PCR oligonucleotide probe hybridisation assay. These water sources are used by the higher socio-economic and lower socio-economic populations for recreational and drinking purposes, respectively. To assess the possible risk of infection constituted by HAV to these communities, a simple deterministic exponential model was applied. This is the first risk assessment study applying a mathematical model to assess the potential risk of HAV infection constituted to different socio-economic communities in SA exposed to contaminated surface water. This model, incorporating mean values and conservative assumptions, estimated the potential daily risk of infection for the higher socio-economic population, using the dam and river water source for recreational purposes, to be 1.17×10^{-4} and 1.09×10^{-3} respectively. The annual P_{inf} for this socio-economic group was 4.18×10^{-2} for the dam and 3.28×10^{-1} for the river water source. These results were based on the assumption that a 100 ml of water was ingested per day. None of the water sources investigated conformed to the US EPA guidelines with regard to the bacterial quality for either drinking or recreational water. Even though the results for the lower socio-economic, predominantly rural communities were 10-fold higher than for the higher socio-economic group, immunity is required by the age of 10, and results would only be of concern to urbanized individuals or the very young. Risk assessment studies would therefore have to be done for different age groups, and socio-economic populations. Once again, political and social factors could influence the epidemiology of HA in

SA. Since only two major water sources were investigated during this study, these results cannot be applied universally. Individual studies will have to be performed for individual geographical areas according to the communities involved. It must be borne in mind though that difficulties with the isolation and detection of WT HAV could result in an underestimated prevalence of HAV in the environment, consequently leading to an underestimated estimation of the potential risk involved as well. EOR values also influence the calculation of risk in this model, and therefore there is a need to optimise the recovery, isolation and detection techniques involved to accurately estimate the P_{inf} constituted by HAV in water. All these techniques have been addressed in this investigation.

The question remains whether or not the HAV strains detected in water sources used by the different socio-economic groups for domestic and recreational purposes are in fact responsible for clinical infections. To this end selected HAV isolates from water samples and clinical specimens were sequenced and compared to each other and to globally recognised reference strains. Only one of the HAV strains, from an asymptomatic paediatric patient, showed a high percentage of nt identity to the majority of strains isolated from various water sources. This suggests that water could be a reservoir for HAV infection in SA. This further suggests that selected strains circulating in the environment could be associated with asymptomatic infections, not necessitating clinical investigation and thus go undetected by health authorities.

The hypothesis of this investigation was that the HAV detected in water samples was viable, and potentially infectious, thereby posing a significant risk of infection to individuals using the contaminated water sources for domestic and recreational purposes. Results from this study could not prove this hypothesis. This finding must however be interpreted with caution, taking into account the epidemiological susceptibility of the different population groups in SA. This is the first meaningful assessment of the prevalence of

HAV in selected water sources and the associated potential health risk to the SA population using these waters for recreational and drinking purposes.

CHAPTER 7

REFERENCES

Abad FX, Pintó RM, Gajardo R, Bosch A. Viruses in mussels: public health implications and depuration. Journal of Food Protection 1997;60:677-81.

Abdool Karim SS, Coutsoudis A. Sero-epidemiology of hepatitis A in black South African children. South African Medical Journal 1993;83:748-50.

Agnès F, Crance JM, Lévêque F. Separate detection of the two complimentary RNA strands of hepatitis A virus. Journal of Virological Methods 1994;49:323-30.

Alexander JJ, Bey EM, Geddes EW, Lecatsas G. Establishment of a continuously growing cell line from primary carcinoma of the liver. South African Medical Journal 1976:2124-28.

American Public Health Association, American Water Works Association, Water Environment Federation. Detection of Enteric Viruses. In: Clesceri LS, Greenberg AE, Eaton AD, Franson MAH, editors. Standard Methods for the Examination of Water and Wastewater. 20th ed. Baltimore: United Book Press Inc.; 1998. p. 9-115 to 9-131.

Anderson DA. Cytopathology, plaque assay, and heat inactivation of hepatitis A virus strain HM-175. Journal of Medical Virology 1987;22:35-44.

Appleton H. Control of food-borne viruses. British Medical Bulletin 2000;56: 172-83.

Arauz-Ruiz P, Sundqvist L, García Z, Taylor L, Visoná K, Norder H, et al. Presumed common source outbreaks of hepatitis A in an endemic area confirmed by limited sequencing within the VP1 region. Journal of Medical Virology 2001;65:449-56.

Asher LVS, Binn LN, Mensing TL, Marchwicki RH, Vassell RA, Young GD. Pathogenesis of Hepatitis A in orally inoculated Owl monkeys (*Aotus trivirgatus*). Journal of Medical Virology 1995;47:260-8.

Ashbolt NJ, Grabow WOK, Snozzi M. Indicators of microbial water quality. In: Fewtrell, L. and Bartram, J, editors. Water Quality: Guidelines, Standards and Health: Assessment of risk and risk management for water-related infectious disease. London: IWA Publishing; 2001. p. 289-315.

Ashida M, Hara H, Kojima H, Kamimura T, Ichida F, Hamada C. Propagation of hepatitis A virus in hybrid cell lines derived from marmoset liver and Vero cells. Journal of General Virology 1989;70:2487-94.

Aszkenasy OM. A community outbreak of hepatitis A in a religious community in Indiana: failure of immune serum globulin to prevent the spread of infection. Epidemiology and Infection 2000;124:309-13.

Backer H. Water disinfection for international and wilderness travellers. Clinical Infectious Diseases 2002;34:55-64.

Beales LP, Wood DJ, Minor PD, Saldanha JA. A novel cytopathic microtitre plate assay for hepatitis A virus and anti-hepatitis A neutralizing antibodies. Journal of Virological Methods 1996;59:147-54.

Bearcroft WGC. Studies on the transmission of the agent of infectious hepatitis to Patas monkeys. Journal of Medical Microbiology 1968;1:1-21

Beard MR, Lemon SM. Hepatitis A virus (*Picornaviridae*). In: Granoff A, Webster RG, editors. Encyclopaedia of Virology. 2nd ed, vol 1. New York: Academic Press; 1999. p. 631-9.

Beuret C, Kohler D, Baumgartner A, Lüthi TM. Norwalk-like virus sequences in mineral waters: one-year monitoring of three brands. Applied and Environmental Microbiology 2002;68:1925-31.

Bidawid S, Farber JM, Sattar SA, Hayward S. Heat inactivation of hepatitis A virus in dairy foods. Journal of Food Protection 2000a;63:522-8.

Bidawid S, Farber JM, Sattar SA. Rapid concentration and detection of hepatitis A virus from lettuce and strawberries. Journal of Virological Methods 2000b;88:175-85.

Bishop NE, Hugo DL, Borovec SV, Anderson DA. Rapid and efficient purification of hepatitis A virus from cell culture. Journal of Virological Methods 1994;47:203-16.

Bishop NE, Anderson DA. Hepatitis A virus subviral particles: purification, accumulation, and relative infectivity of virions, provirions and procapsids. Archives of Virology 1997;142:2147-60.

Biziagos E, Passagot J, Crance J, Agbalika F, Laveran H, Deloince R. Concentration of hepatitis A virus. Water Research 1987;21:683-6.

Biziagos E, Passagot J, Crance J, Agbalika F, Deloince R. Long-term survival of hepatitis A virus and poliovirus type 1 in mineral water. Applied and Environmental Microbiology 1988;54:2705-10.

Blank CA, Anderson DA, Beard M, Lemon SM. Infection of polarized cultures of human intestinal epithelial cells with hepatitis A virus: Vectorial

release of progeny virions through apical cellular membranes. Journal of Virology 2000;74:6476-84.

Bloch AB, Stramer SL, Smith JD, Margolis HS, Fields HA, McKinley TW, et al. Recovery of hepatitis A virus from a water supply responsible for a common source outbreak of hepatitis A. American Journal of Public Health 1990;80:428-30.

Boccia D, Tozzi AE, Cotter B, Rizzo C, Russo T, Buttinelli G, et al. Waterborne outbreak of Norwalk-like virus gastroenteritis at a tourist resort, Italy. Emerging Infectious Diseases 2002;8:563-8.

Bosch A, Lucena F, Diez JM, Gajardo R, Blasi M, Jofre J. Waterborne viruses associated with hepatitis outbreak. Journal of American Water Works Association 1991:80-3.

Bosch A, Sánchez G, Le Guyader F, Vanaclocha H, Haugarreau L, Pintó RM. Human enteric viruses in Coquina clams associated with a large hepatitis A outbreak. Water Science and Technology 2001;43:61-5.

Bowden DS, Moaven LD, Locarnini SA. New hepatitis viruses: are there enough letters in the alphabet? Medical Journal of Australia 1996;164: 87-9.

Bower WA, Nainan OV, Han X, Margolis HS. Duration of viremia in hepatitis A infection. Journal of Infectious Diseases 2000;182:12-7.

Brown EA, Jansen RW, Lemon SM. Characterization of a simian hepatitis A virus (HAV): antigenic and genetic comparison with human HAV. Journal of Virology 1989;63:4932-7.

Brown EA, Day SP, Jansen RW, Lemon SM. The 5' nontranslated region of hepatitis A virus RNA: secondary structure and elements required for translation *in vitro*. Journal of Virology 1991;65:5828-38.

Callahan KM, Taylor DJ, Sobsey MD. Comparative survival of hepatitis A virus, poliovirus and indicator viruses in geographically diverse seawaters. Water Science and Technology 1995;31:189-93.

Centers for Disease Control and Prevention. Prevention of hepatitis A through active and passive immunisation: recommendations of the Advisory Committee on Immunisation Practices (ACIP). Morbidity and Mortality Weekly Report (MMWR) 1999;48:1-34.

Chaves RL, Graff J, Normann A, Flehmig B. Specific detection of minus strand hepatitis A virus RNA by tail-PCR following reverse transcription. Nucleic Acid Research 1994;22:1919-20.

Chernev AV, Matsievskaya EA, Perederij NG. Outbreak of viral hepatitis A in Murmansk City. EpiNorth 2001;2:1-2 Available from:
URL: http//www.epinorth.org/English/2001/1/0101c.html.

Ching KZ, Nakano T, Chapman LE, Demby A, Robertson B. Genetic characterisation of wild-type genotype VII hepatitis A virus. Journal of General Virology 2002;83:53-60.

Chudy M, Budek I, Keller-Stanislawski B, McCaustland KA, Neidhold S, Robertson BH, et al. A new cluster of hepatitis A infection in hemophiliacs traced to a contaminated plasma pool. Journal of Medical Virology 1999;57:91-9.

Cohen JL, Ticehurst JR, Purcell RH, Buckler-White A, Baroudy BM. Complete nucleotide sequence of wild-type hepatitis A virus: comparison with

different strains of hepatitis A virus and other picornaviruses. Journal of Virology 1987;61:50-9.

Conaty S, Bird P, Bell G, Kraa E, Grohmann G, McAnulty JM. Hepatitis A in New South Wales, Australia, from the consumption of oysters: the first reported outbreak. Epidemiology and Infection 2000;124:121-30.

Cooksley WGE. What did we learn from the Shanghai hepatitis A epidemic? Journal of Viral Hepatitis 2000;7 Suppl 1:1-3.

Corey L, Holmes KK. Sexual transmission of hepatitis A in homosexual men: Incidence and mechanism. New England Journal of Medicine 1980;302:435-8.

Coriell LL. Preservation, Storage and Shipment. In: Jakoby WM, Pastan IH, editors. Methods in Enzymology. Vol LVIII, Cell culture. New York: Academic Press, Inc.; 1979. p. 29-36.

Costa-Mattioli M, Ferré V, Monpoeho S, Garcia L, Colina R, Billaudel, et al. Genetic variability of hepatitis A virus in South America reveals heterogeneity and co-circulation during epidemic outbreaks. Journal of General Virology 2001a;82:2647-52.

Costa-Mattioli M, Monpoeho S, Schvoere C, Besse B, Aleman M, Billaudel S et al. Genetic analysis of hepatitis A virus outbreak in France confirms the co-circulation of sub-genotypes Ia, Ib and reveals a new genetic lineage. Journal of Medical Virology 2001b;65:233-40.

Costa-Mattioli M, Di Napoli A, Ferré V, Billaudel S, Perez-Bercoff R, Cristina J. Genetic variability of hepatitis A virus. Journal of General Virology 2003;84:3191-201.

Crance JM, Passagot J, Biziagos E, Deloince R. Continuous production of hepatitis A virus in PLC/PRF 5 cell cultures: use of antigen for serology. Journal of Virological Methods 1987;18:193-203.

Croci L, De Medici D, Scalfaro C, Fiore A, Divizia M. Donia D, et al. Determination of enteroviruses, hepatitis A virus, bacteriophages and Eschericia coli in Adriatic sea mussels. Journal of Applied Microbiology 2000;88:293-8.

Cromeans TL, Sobsey MD, Fields HA. Development of a plaque assay for a cytopathic, rapidly replicating isolate of hepatitis A virus. Journal of Medical Virology 1987;22:45-56.

Cromeans TL, Fields HA, Sobsey MD. Kinetic studies of a rapidly replicating, cytopathic hepatitis A virus. Viral Hepatitis and Liver Disease 1988:24-6.

Cromeans TL, Fields HA, Sobsey MD. Replication kinetics and cytopathic effect of hepatitis A virus. Journal of General Virology 1989;70:2051-62.

Cromeans TL, Nainan OV, Margolis HS. Detection of hepatitis A virus in oyster meat. Applied and Environmental Microbiology 1997;63:2460-3.

Cuthbert, JA. Hepatitis: Old and new. Clinical Microbiology Reviews 2001:38-58.

Daemer RJ, Feinstone SM, Gust ID, Purcell RH. Propagation of human hepatitis A virus in African Green Monkey kidney cell culture: Primary isolation and serial passage. Infection and Immunity 1981:388-93.

Dahling DR, Berg G, Berman D. BGM, a continuous cell line more sensitive than primary rhesus and African green kidney cells for the recovery of viruses from water. Health Laboratory Science 1974;11:275-82.

Dawson DJ, Sartory DP. Microbial safety of water. British Medical Bulletin 2000;56:74-83.

De Chastonay J, Siegl G. Replicative events in hepatitis A virus-infected MRC-5 cells. Virology 1987;l157:268-75.

Deinhardt F, Scheid R, Gauss-Müller V, Frösner G, Siegl G. Propagation of human hepatitis A virus in cell lines of primary human hepatocellular carcinomas. Progress in Medical Virology 1981;27:109-13.

Delpech VC, Thackway SV, Young LC, Pontivivo G, Smedley EJ, Morgan KR, et al. Outbreak of hepatitis A among illicit drug users in south-east Sydney. Medical Journal of Australia 2000;172:143.

Deng MY, Day SP, Cliver DO. Detection of hepatitis A virus in environmental samples by antigen-capture PCR. Applied and Environmental Microbiology 1994;60:1927-33.

Dentinger CM, Bower WA, Nainan OV, Cotter SM, Myers G, Dubusky LM, et al. An outbreak of hepatitis A associated with green onions. Journal of Infectious Diseases 2001;183:1273-6.

Department of Health. The new childhood immunisation schedule. Directorate: Epidemiology, Department of Health, South Africa. Epidemiological Comments 1995;22:39.

Department of Water Affairs and Forestry. South African Water Quality Guidelines. 2nd ed, vol 2. Recreational Use. Indicator Organisms: Enteric Viruses. Pretoria: Department of Water Affairs and Forestry; 1996. p. 61-64.

De Paula VS, Baptista ML, Lampe E, Niel C, Gaspar AMC. Characterisation of hepatitis A virus isolates from subgenotypes IA an IB in Rio de Janeiro, Brazil. Journal of Medical Virology 2002;66:22-7.

De Paula VS, Saback FL, Gaspar AMC, Niel C. Mixed infection of a child care provider with hepatitis A virus isolates from subgenotypes 1A and 1B revealed by heteroduplex mobility assay. Journal of Virological Methods 2003;107:223-8.

Diaz BI, Sariol CA, Normann A, Rodríguez L, Flehmig B. Genetic relatedness of Cuban HAV wild-type isolates. Journal of Medical Virology 2001;64:96-103.

Dienstag JL, Feinstone SM, Purcell RH, Hoofnagle JH, Barker LF, London WT, et al. Experimental infection of chimpanzees with hepatitis A virus. Journal of Infectious Diseases 1975;132:532-45.

Dienstag JL, Davenport FM, McCollum RW. Nonhuman primate-associated viral hepatitis type A: Serological evidence of hepatitis A virus infection. Journal of the American Medical Association 1976;236:462-4.

Divizia M, de Filippis P, di Napoli A, Venuti A, Perez-Bercoff R, Panà A. Isolation of wild type hepatitis A virus from the environment. Water Research 1989a;23:1155-60.

Divizia M, Santi AL, Panà A. Ultrafiltration: an efficient second step for hepatitis A virus and poliovirus concentration. Journal of Virological Methods 1989b;23:55-62.

Divizia M, Gnesivo C, Bonapasta RA, Morace G, Pisani G, Panà A. Virus isolation and identification by PCR in an outbreak of hepatitis A: epidemiological investigation. Water Science and Technology 1993;27:199-205.

Dotzauer A, Feinstone SM, Kaplan G. Susceptibility of nonprimate cell lines to hepatitis A virus infection. Journal of Virology 1994;68:6064-8.

Dubrou S, Kopecka H, Lopez Pila JM, Maréchal J, Prévot J. Detection of hepatitis A virus and other enteroviruses in wastewater and surface water samples by gene probe assay. Water Science and Technology 1991;24:267-72.

Environment Agency Report. Optimisation of a new method for detection of viruses in groundwater. National Groundwater and Contaminated Land Centre Project NC/99/40 2000.

Environment Protection Agency. Ambient Water Quality Criteria for Bacteria - 1986. US Environmental Protection Agency, Report No. EPA 440/5-84-002. Washington DC; 1986.

FDA/CFSAN: US Food and Drug Administration/Center for Food Safety and Applied Nutrition: Foodborne Pathogenic Microorganisms and Natural Toxins Handbook. Hepatitis A virus (2004). Available at:
URL: *http://vm.cfsan.fda.gov/~mow/chap31.htm* [screen 1-2].

Feinstone SM, Kapikian AZ, Purcell RH. Hepatitis A: Detection by immune-electronmicroscopy of a virus-like antigen associated with acute illness. Science 1973;182:1026-8.

Feinstone SM, Gust ID. Hepatitis A virus. In: Richman DD, Whitley RJ, Hayden FG, editors. Clinical Virology. 1st ed. New York: Churchill Livingstone Inc.; 1997. p. 1049-67.

Feinstone SM, Gust ID. Hepatitis A virus. In: Richman DD, Whitley RJ, Hayden FG, editors. Clinical Virology. 2nd ed. Washington DC: ASM Press; 2002. p. 1019-39.

Fewtrell L. Freshwater recreation: cause for concern? Applied Geography 1991;91: 215-26.

Fewtrell L, Godfree AF, Jones F, Kay D, Salmon RL, Wyer MD. Health effects of white-water canoeing. Lancet 1992;339:1587-9.

Flehmig B. Hepatitis A virus in cell culture: I. Propagation of different hepatitis A virus isolates in a foetal rhesus monkey kidney cell line (FRhK-4). Medical Microbiology and Immunology 1980;168:239-48.

Flehmig B. Hepatitis A virus in cell culture: II. Growth characteristics of hepatitis A virus in FRhK-4/R cells. Medical Microbiology and Immunology 1981;170:73-81.

Flehmig B, Vallbracht A, Wurster G. Hepatitis A virus in cell culture: III Propagation of hepatitis A virus in human embryo kidney cells and human embryo fibroblast strains. Medical Microbiology and Immunology 1981;170:83-9.

Friedman-Huffman D, Rose J. Emerging waterborne pathogens. Water Quality International 1998:14-5.

Frösner GG, Deinhardt F, Scheid R, Gauss-Müller V, Holmes N, Messelberger V, et al. Propagation of human hepatitis A virus in a hepatoma cell line. Infection 1979;7:303-6.

Fuhs GW. Mechanisms of adsorption and elution of viruses to solids in the natural environment. In: Berg G, editor. Methods for recovering viruses from the environment. Florida: CRC Press Inc.; 1987. p. 139-77.

Gajardo R, Díez JM, Jofre J, Bosch A. Adsorption-elution with negatively and positively charged glass powder for the concentration of hepatitis A virus from water. Journal of Virological Methods 1991;31:345-52.

Gale P. Developments in microbial risk assessment for drinking water. Journal of Applied Microbiology 2001;91:191-205.

Gammie AJ, Wyn-Jones AP. Does hepatitis A pose a significant health risk to recreational water users? Water Research and Technology 1997;35:171-7.

Gardner PS, McQuillin J. Rapid virus diagnosis: Application of immunofluorescence. London, UK: Butterworth and Co. (Publishers) Ltd.; 1974. p. 5-34.

Garin D, Fuchs F, Crance JM, Rouby Y, Chapalain JC, Lamarque D, et al. Exposure to enteroviruses and hepatitis A virus among divers in environmental waters in France, first biological and serological survey of a controlled cohort. Epidemiology and Infection 1994;113:541-9.

Garin D, Biziagos E, Crance JM, Gantzer C, Lévêque F, Deloince R, et al. Survival of infectious hepatitis A virus in mineral water and seawater. In: Buisson Y, Coursaget P, Kane M, editors. Enterically-transmitted hepatitis viruses. Tours, France: La Simarre; 1996. p. 48-9.

George VG, Hierholzer JC, Ades EW. Cell culture. In: Mahy BWJ, Kangro HO, editors. Virology Methods Manual. London: Academic Press Ltd.; 1996:3-24.

Gerba CP. Methods for recovering viruses from the water environment. In: Berg G, editor. Viral pollution of the environment. Florida: CRC Press, Inc.; 1983. p. 19-35.

Gerba CP. Recovering of viruses from sewage, effluents and water. In: Berg G, editor. Methods for recovering viruses from the environment. Florida: CRC Press Inc.; 1987. p. 2-18.

Gerba CP. Assessment of enteric pathogen shedding by bathers during recreational activity and its impact on water quality. Quantitative Microbiology 2000;2:55-68.

Gerba CP, Haas CN. Assessment of risks associated with enteric viruses in contaminated drinking water. In: Chemical and Biological Characterization of Sludges, Sediments, Dredge Spoils, and Drilling Muds. Philadelphia: American Society for Testing and Materials; 1988. p. 489-94.

Gerba CP, Rose JB. Viruses in source and drinking water. In: McFeters GA, editor. Drinking water microbiology. New York: Springer-Verlag Inc.; 1990. p. 380-96.

Gilgen M, Germann D, Lüthy J, Hübner Ph. Three-step isolation method for sensitive detection of enterovirus, rotavirus, hepatitis A virus, and small round structured viruses in water samples. International Journal of Food Microbiology 1997;37:189-99.

Gilroy NM, Tribe IG, Passaris I, Hall R, Beers MY. Hepatitis A in injecting drug users: a national problem. Medical Journal of Australia 2000;172:142-3.

Gosert R, Egger D, Bienz K. A cytopathic and cell culture adapted hepatitis A virus strain differ in cell killing but not in intracellular membrane rearrangements. Virology 2000;266:157-69.

Goyal SM. Indicators of viruses. In:Berg G, editor. Viral pollution of the environment. Boca Raton: CRC Press, Inc.; 1983. p. 211-30.

Grabow WOK. Progress in studies on the type A (infectious) hepatitis virus in water. Water SA 1976;2:20-4.

Grabow WOK. Waterborne diseases: Update on water quality assessment and control. Water SA 1996;22:193-201.

Grabow WOK. Hepatitis viruses in water: Update on risk and control. Water SA 1997;23:379-86.

Grabow WOK. Bacteriophages: Update on application as models for viruses in water. Water SA 2001;27:251-68.

Grabow WOK, Prozesky OW. Lesser bushbabies (*Galago senegalensis*) may be susceptible to the CR 326 hepatitis A virus. South African Journal of Science 1975;71:310-1.

Grabow WOK, Prozesky OW, Bradley DW, Maynard JE. Response of *Galago* bushbabies to the hepatitis A virus. South African Journal of Science 1981;77:314-8.

Grabow WOK, Gauss-Müller V, Prozesky OW, Deinhardt F. Inactivation of hepatitis A virus and indicator organisms in water by free chlorine residuals. Applied and Environmental Microbiology 1983;46:619-24.

Grabow WOK, Coubrough P, Hilner C, Bateman BW. Inactivation of hepatitis A virus, other enteric viruses and indicator organisms in water by chlorination. Water Science and Technology 1984;17:657-64.

Grabow WOK, Taylor MB. New methods for the virological analysis of drinking water supplies. Proceedings of the Biennial Conference and Exhibition of the Water Institute of Southern Africa; 1995 May 24-27; Durban, South Africa. Johannesburg: Water Institute of Southern Africa;1993.

Grabow WOK, Taylor MB, De Villiers JC. New methods for the detection of viruses: call for review of drinking water quality guidelines. Water Science and Technology 2001;43:1-8.

Graff J, Ticehurst J, Flehmig B. Detection of hepatitis A virus in sewage sludge by antigen capture polymerase chain reaction. Applied and Environmental Microbiology 1993;59:3165-70.

Grinde B, Stene-Johansen K, Sharma B, Hoel T, Jensenius M, Skaug K. Characterisation of an epidemic of hepatitis A virus involving intravenous drug abusers – infection by needle sharing? Journal of Medical Virology 1997;53:69-75.

Grist NR, Bell EJ, Follett EAC, Urquhart GED. Diagnostic methods in clinical virology. London: Blackwell Scientific Publications; 1979. p. 61-80, 84, 187-9.

Guidelines for Canadian Recreational Water Quality 1992 [cited 2004 June14]; [screen 1]. p. 1-48. Available at:
URL: *http://www.hc-sc.gc.ca/hecs-sesc/water/recreational_water.htm*

Gust ID, Feinstone SM. Hepatitis A. Boca Raton: CRC Press, Inc., 1988.

Guttman-Bass N. Cell cultures and other host systems for detecting and quantifying viruses in the environment. In: Berg G, editor. Methods for recovering viruses from the environment. Boca Raton: CRC Press, Inc.; 1987. p. 195-228.

Haas CN. Estimation of risk due to low doses of microorganisms: a comparison of alternative methodologies. American Journal of Epidemiology 1983;118:573-82.

Haas CN, Rose JB, Gerba C, Regli S. Risk assessment of virus in drinking water. Risk analysis 1993;13:545-52.

Haas CN, Rose JB, Gerba CP. Quantitative microbial risk assessment. New York: John Wiley and Sons, Inc.; 1999. p. 86-106.

Haas C, Eisenberg JNS. Risk assessment. In: Fewtrel L, Bartram J, editors. Water Quality: Guidelines, standards and health. London: IWA Publishing; 2001. p. 161-84.

Halliday ML, Kang L, Zhou T, Hu M, Pan Q, Fu T, et al. An epidemic of hepatitis A attributed to the ingestion of raw clams in Shanghai, China. Journal of Infectious Diseases 1991;164:852-9.

Ham RG, McKeehan WL. Media and Growth requirements. In: Jakoby WM, Pastan IH, editors. Methods in Enzymology. Vol LVIII, Cell culture. New York: Academic Press, Inc.; 1979. p. 44-93.

Havelaar A, Blumenthal UJ, Strauss M, Kay D, Bartram J. Guidelines: the current position. In: Water Quality: Guidelines, Standards and Health. London: IWA Publishing 2001. p. 17-42.

Hay R, Caputo J, Chen TR, Macy M, McClintock P, Reid Y. American Type Culture Collection Catalogue of Cell lines and Hybridomas. 7th ed. Maryland: American Type Culture Collection; 1992. p. 150.

Hermann JE. Methods for rapid detection and rapid identification of viruses. In: Berg G, editor. Methods for recovering viruses from the environment. Boca Raton: CRC Press, Inc.; 1987. p. 179-94.

Hierholzer JC, Killington RA. Virus Isolation and quantitation. In: Mahy BWJ, Kangro HO, editors. Virology Methods Manual. London: Academic Press Ltd.; 1996. p. 25-46.

Hollinger FB. Hepatitis A virus. In: Hollinger FB, Melnick JL, Robinson WS, editors. Viral Hepatitis: Biological and clinical features, specific diagnosis, and prophylaxis. New York: Raven Press Books, Ltd.; 1985. p. 11-8.

Hollinger FB, Khan NC, Oefinger PE, Yawn DH, Schmulen AC, Dreesman GR, et al. Posttransfusion hepatitis type A. Journal of American Medical Association 1983; 250:2313-7.

Hollinger FB, Ticehurst JR. Hepatitis A virus. In: Fields BN, Knipe DM, Howley PM, et al. editors. Fields Virology. 3rd ed. Philadelphia: Lippincott-Raven Publishers; 1996. p. 735-82.

Hollinger FB, Emerson SU. Hepatitis A virus. In: Knipe DM, Howley PM, et al. editors. Fields Virology. 4th ed. Philadelphia: Lippincott-Raven Publishers; 2001. p. 799-840.

Hornei B, Kämmerer R, Moubayed P, Frings W, Gauss-Müller V, Dotzauer A. Experimental hepatitis A virus infection in guinea pigs. Journal of Medical Virology 2001;64:402-9.

Huang PW, Laborde D, Land VR, Matson DO, Smith AW, Jiang X. Concentration and detection of caliciviruses in water samples by reverse transcriptase-PCR. Applied and Environmental Microbiology 2000;66:4383-8.

Hunter PR. Viral hepatitis. In: Waterborne Disease: Epidemiology and Ecology. Chichester: John Wiley and Sons Ltd.; 1997a. p. 207-21.

Hunter PR. Viral gastroenteritis. In: Waterborne Disease: Epidemiology and Ecology. Chichester: John Wiley and Sons Ltd.; 1997b. p. 222-31.

Hunter PR, Colford JM, LeChevallier MW, Binder S, Berger PS. CDC-Panel on waterborne diseases. Emerging Infectious Diseases [serial online] 2001 June [cited 2001 July 4];7:[3 screens]. Available from:
URL: *wysiwyg://63/http://www.cdc.gov/ncidod/eid/vol7no3_supp/hunter.htm*

Hunter PR, Payment P, Ashbolt N, Bartram J. Assessment of Risk. In: Assessing Microbial Safety of Drinking Water: Improving Approaches and Methods. Geneva: OECD, WHO; 2003. p. 79-109.

Hurst CJ. Recovering viruses from sewage sludges and from solids in water. In: Berg G, editor. Methods for recovering viruses from the environment. Boca Raton: CRC Press, Inc.; 1987. p. 25-51.

Hutin YJF, Pool V, Cramer EH, Nainan OV, Weth J, Williams IT et al. A multistate, foodborne outbreak of hepatitis A. New England Journal of Medicine 1999;340:595-602.

Ida S, Tachikawa N, Nakajima A, Daikoku M, Yano M, Kikuchi Y, et al. Influence of human immunodeficiency virus type 1 infection on acute hepatitis A virus infection. Clinical Infectious Diseases 2002;34:379-85.

Inoue K, Yoshiba M, Yotsuyanagi H, Otsuka T, Sekiyama K, Fujita R. Chronic hepatitis A with persistent viral replication. Journal of Medical Virology 1996;50:322-4.

Jacobs RJ, Grover SF, Meyerhoff AS, Paivanas TA. Cost effectiveness of vaccinating food service workers against hepatitis A infection. Journal of Food Protection 2000;63:768-74.

Jansen RW, Siegl G, Lemon SM. Molecular epidemiology of human hepatitis A virus defined by an antigen-capture polymerase chain reaction method. Proceedings of the National Academy of Science USA 1990;87:2867-71.

Jaykus L, De Leon R, Sobsey M. A virion concentration method for detection of human enteric viruses in oysters by PCR and oligoprobe hybridisation. Applied and Environmental Microbiology 1996;62:2074-80.

Jothikumar N, Cliver DO, Mariam TW. Immunomagnetic capture PCR for rapid concentration and detection of hepatitis A virus from environmental samples. Applied and Environmental Microbiology 1998;64:504-8.

Kaul A, Chaves RL, Eissele R, Matzdorff P, Normann A, Flehmig B. Analysis of a foodborne hepatitis A epidemic. Biotest Bulletin 2000;6:217-24.

Kedda M, Kew MC, Cohn RJ, Field SP, Schwyzer R, Song E, et al. An outbreak of hepatitis A among South African patients with haemophilia: evidence implicating contaminated factor VIII concentrate as source. Hepatology 1995;22:1363-7.

King AWQ, Brown F, Hovi T, Knowles N, Lemon SM, Minor PD et al. Picornaviridae. In: Van Regenmortel MHV, Fauquet CM, Bishop DHL, Carstens EB, Estes MK, Lemon SM et al., editors. Virus Taxonomy:

Classification and nomenclature of viruses. San Diego: Academic Press; 2000. p. 671-3.

Kingsley DH, Meade GK, Rischards GP. Detection of both hepatitis A virus and Norwalk-like viruses in imported clams associated with food-borne illness. Applied and Environmental Microbiology 2002;68:3914-8.

Klinger MHF, Kämmerer R, Hornei B, Gauss-Müller V. Perinuclear accumulation of hepatitis A proteins, RNA, and particles and ultrastructural alterations in infected cells. Archives of Virology 2001;146:2291-307.

Koff R. Hepatitis A. Lancet 1998;341:1643-9.

Koopmans M, von Bonsdorff C, Vinjé J, de Medici D, Monroe S. Foodborne viruses. FEMS Microbiology Reviews 2002;26:187-205.

Kukavica-Ibrulj I, Darveau A, Fliss I. Immunofluorescent detection and quantification of hepatitis A virus in sewage treatment effluent and on agri-food surfaces using scanning confocal microscopy. Journal of Virological Methods 2003;108:9-17.

LaBelle RL, Gerba CP. Influence of pH, salinity and organic matter on the adsorption of enteric viruses to estuarine sediments. Applied and Environmental Microbiology 1979;38:93-101.

Lappalainen M, Chen RW, Maunula L, Von Bonsdorff C, Pluysnin A, Vaheri A. Molecular epidemiology of viral pathogens and tracing of transmission routes: hepatitis-, calici- and hantaviruses. Journal of Clinical Virology 2001;21:177-85.

Lee S, Chan C, Yu M, Wang Y, Chang F, Lo K, et al. Safety and immunogenicity of inactivated hepatitis A vaccine in patients with chronic liver disease. Journal of Medical Virology 1997;52:215-8.

Lee S, Kim S. Detection of infectious enteroviruses and adenoviruses in tap water in urban areas in Korea. Water Research 2002;36:248-56.

Lees DN, Hensilwood K, Doré WJ. Development of a method for detection of enteroviruses in shellfish by PCR with poliovirus as a model. Applied and Environmental Microbiology 1994;60:2999-3005.

Legeay O, Caudrelier Y, Cordevant C, Rigottier-Gois L, Lange M. Simplified procedure for detection of enteric pathogenic viruses in shellfish. Journal of Virological Methods 2000;90:1-14.

Lemon SM. Inactivated hepatitis A virus vaccines. Hepatology 1992;15:1194-7.

Lemon SM, Binn LN, Marchwicki RH. Radioimmunofocus assay for quantitation of hepatitis A in cell cultures. Journal of Clinical Microbiology 1983;17:834-9

Lemon SM, Stapleton JT. Prevention. In: Zuckerman AJ, Thomas HC, editors. Viral hepatitis: Scientific basis and clinical management. New York: Churchill Livingstone Inc. 1993. p. 61-79.

Levy JA, Fraenkel-Conrat H, Owens RA. Nonenveloped viruses with positive-sense RNA genomes. Virology. 3rd ed. New Jersey: Prentice Hall, Inc.; 1994. p. 31-8.

Lewis GD, Molloy SL, Greening GE, Dawson J. Influence of environmental factors on virus detection by RT-PCR and cell culture. Journal of Applied Microbiology 2000;88:633-4.

López-Pila JM, Szewzyk R. Estimating the infection risk in recreational waters from faecal indicator concentration and from the ratio between pathogens and indicators. Water Research 2000;34:4195-200.

Lu L, Ching KZ, De Paula VS, Nakano T, Siegl G, Weitz M, et al. Characterization of the complete genomic sequence of genotype II hepatitis A virus (CF53/Berne isolate). Journal of General Virology 2004;85:2943-52.

Macler BA. Acceptable risk and U.S. microbial drinking water standards. In: Craun GF, editor. Safety of Water Disinfection: Balancing Chemical and Microbial Risks. Washington, D.C: ILSI Press; 1993. p. 619-23.

Macler BA, Regli S. Use of microbial risk assessment in setting US drinking water standards. International Journal of Food Microbiology 1993;18:254-6.

Mahoney FJ, Farley TA, Kelso KY, Wilson SA, Horan JM, McFarland LM. An outbreak of hepatitis A associated with swimming in a public pool. Journal of Infectious Diseases 1992;165:613-8.

Martin DJ. Hepatitis A vaccination – an option for South Africa? South African Medical Journal 1992;82:5-6.

Martin DJ, Blackburn NK, Johnson S, McAnerney JM. The current epidemiology of hepatitis A infection in South Africa: implications for vaccination. Transactions of the Royal Society of Tropical Medicine and Hygiene 1994;88:288-91.

Mbayed VA, Sookoian S, Alfonso V, Campos RH. Genetic characterisation of hepatitis A virus isolates from Buenos Aires, Argentina. Journal of Medical Virology 2002;68:168-74.

Mbithi JN, Springthorpe VS, Sattar SA. Effect of relative humidity and air temperature on survival of hepatitis A virus on environmental surfaces. Applied and Environmental Microbiology 1991;57:1394-9.

Melnick JL. History and epidemiology of hepatitis A virus. Journal of Infectious Diseases 1995;171 Suppl 1:S2-8.

Menut C, Beril C, Schwartzbrod L. Poliovirus recovery from tap water after concentration over glass powder and glass wool. Water Science and Technology 1993;27:291-4.

Metcalf TG, Jiang X, Estes MK, Melnick JL. Nucleic acid probes and molecular hybridisation for the detection of viruses in environmental samples. Progress in Medical Virology 1988;35:186-214.

Mignotte B, Maul A, Schwartzbrod L. Comparative study of techniques used to recover viruses from residual urban sludge. Journal of Virological Methods 1999;78:71-80.

Mims C, Wakelin D, Playfair J, Williams R, Riott I. Gastrointestinal tract infections. In: Crowe L, editor. Medical Microbiology. 2nd ed. London: Mosby International Ltd.; 1998. p. 253-86.

Minor PD. Growth, assay and purification of Picornaviruses. In: Mahy BWJ, editor. Virology: a practical approach. Washington: IRL Press; 1985. p. 25-42.

Minor P. Picornaviridae. In: Francki RIB, Fauquet CM, Knudson DL, Brown F, editors. Classification and Nomenclature of viruses. Wien:Springer-Verlag; 1991. p. 320-6.

Mix TW. Mechanisms of adsorption and elution of viruses to and from surfaces. In: Berg G, editor. Methods for recovering viruses from the environment. Florida: CRC Press Inc.; 1987. p. 127-38.

Morace G, Pisani G, Divizia M, Pina A. Detection of hepatitis A virus in concentrated river water by polymerase chain reaction. Zentralblatt fur Hygiene 1993;193:521-7.

Mosley JW. Transmission of viral diseases by drinking water. In: Berg G, editor. Transmission of viruses by the water route. New York: Interscience; 1967. p. 5-23.

Mullendore JL, Sobsey MD, Shieh YC. Improved method for the recovery of hepatitis A virus from oysters. Journal of Virological Methods 2001;94:25-35.

Mushahwar IK. Recently discovered blood-borne viruses: Are they hepatitis viruses or merely endosymbionts? Journal of Medical Virology 2000;62:399-404.

Nadala ECB Jr., Loh PC. Immunofluorescence and immunoperoxidase assays for the titration of infectious hepatitis A virus (HAV). Journal of Virological Methods 1990;28:117-24.

Nainan OV, Margolis HS, Robertson BH, Balayan M, Brinton MA. Sequence analysis of a new hepatitis A virus infecting cynomolgus macaques (*Macaca fascicularis*). Journal of General Virology 1991;72:1685-9.

Nasser AM, Metcalf TG. An A-ELISA to detect hepatitis A virus in estuarine samples. Applied and Environmental Microbiology 1987;53:1192-5.

Nasser A, Weinberg D, Dinoor N, Fattal B, Adin A. Removal of hepatitis A virus (HAV), poliovirus and MS2 coliphage by coagulation and high rate filtration. Water Science and Technology 1995;31:63-8.

Page NA, Steele AD. Antigenic and genetic characterisation of serotype G2 human rotavirus strains from South Africa from 1984 to 1998. Journal of Medical Virology 2004;72:320-7.

Pallin R, Wyn-Jones AP, Place BM, Lightfoot NF. The detection of enteroviruses in large volume concentrates of recreational waters by polymerase chain reaction. Journal of Virological Methods 1997;67:57-67.

Panà A, Divizia M, de Filippis P, di Napoli A. Isolation of hepatitis A virus from polluted river water on Frp/3 cells. Lancet 1987;8571:1328.

Passagot J, Crance JM, Deloince R, Laveran H, Beytout D, Fontanges R. Hepatitis A virus concentration on cellulose membranes. Water Research 1985;19:1167-70.

Paul AV, Tada H, Von der Helm K, Wissel T, Kiehn R, Wimmer E et al. The entire nucleotide sequence of the genome of human hepatitis A virus (isolate MBB). Virus Research 1987;8:153-71.

Phillip R, Waitkins S, Caul O, Roome A, McMahon S, Enticott R. Leptospiral and hepatitis A antibodies amongst windsurfers and waterskiers in Bristol City Docks. Public Health 1989;103:123-9.

Pina S, Buti M, Jardí R, Clemente-Casares P, Jofre J, Girones R. Genetic analysis of hepatitis A virus strains recovered from the environment and from patients with acute hepatitis. Journal of General Virology 2001;82:2955-63.

Polish LB, Robertson BH, Khanna B, Krawczynski K, Spelbring J, Olson F, et al. Excretion of hepatitis A virus (HAV) in adults: Comparison of immunologic and molecular detection methods and relationship between HAV positivity and infectivity in tamarins. Journal of Clinical Microbiology 1999;37:3615-7.

ProMed. Hepatitis A- UK (England). ProMed mail: *promed@promed.isid.harvard.edu* 2 October 2004

Racaniello VR. Picornaviridae: The viruses and their replication. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, et al., editors. Fundamental Virology. 4th ed. Philadelphia: Lippincott Williams & Wilkins; 2001. p. 529-66.

Ramsey CN, Upton PA. Hepatitis A and frozen raspberries. Lancet 1989;1:43-4.

Rao VC. Reconcentration of viruses from primary eluates. In: Berg G, editor. Methods for recovering viruses from the environment. Boca Raton: CRC Press, Inc.; 1987. p 109-26.

Regli S, Rose JB, Haas CN, Gerba CP. Modelling the risk from *Giardia* and viruses in drinking water. Journal of American Water Works Association 1991;92:76-84.

Richards GP, Watson MA. Immunochemiluminescent focus assay for the quantitation of hepatitis A virus and rotavirus in cell cultures. Journal of Virological Methods 2001;94:69-80.

Robertson BH. Viral hepatitis and primates: historical and molecular analysis of human and nonhuman primate hepatitis A, B, and the GB-related viruses. Journal of Viral Hepatitis 2001;8:233-42.

Robertson BH, Brown VK, Bradley DW. Nucleic acid sequence of the VP1 region of attenuated MS-1 hepatitis A virus. Virus Research 1987;8:309-16.

Robertson BH, Brown VK, Khanna B. Altered hepatitis A VP1 protein resulting from cell culture propagation of virus. Virus Research 1989;13:207-12.

Robertson BH, Khanna B, Nainan OV, Margolis HS. Epidemiological patterns of wild-type hepatitis A virus determined by genetic variation. Journal for Infectious diseases 1991;163:286-92.

Robertson BH, Jansen RW, Khanna B, Totsuka A, Nainan OV, Siegl G, et al. Genetic relatedness of hepatitis A virus strains recovered from different geographical regions. Journal of General Virology 1992;73:1365-77.

Robertson BH, Averhoff F, Cromeans TL, Han X, Khoprasert B, Nainan OV et al. Genetic relatedness of hepatitis A virus isolates during a community-wide outbreak. Journal of Medical Virology 2000;62:144-50.

Rosenblum LS, Mirkin IR, Allen DT, Stafford S, Hadler SC. A multistage outbreak of hepatitis A traced to commercially distributed lettuce. American Journal of Public Health 1990;80:1075-80.

Ryder SD. Viral Hepatitis. In: Armstrong D, Cohen J, Berkley SF, Carbon CJ, Clumeck N, Durack DT, et al., editors. Infectious Diseases. London: Harcourt Publishers Ltd.; 1999. p. 39.1-12.

Samuel D, Megson B, Strang M, Appleton H. A microtitre plate method for isolation and typing of poliovirus using Blue-Cell ELISA. Journal of Virological Methods 2000;90:125-33.

Sánchez G, Pintó RM, Vanaclocha H, Bosch A. Molecular characterisation of hepatitis A virus isolates from a transcontinental shellfish-borne outbreak. Journal of Clinical Microbiology 2002;40:4148-55.

Sanger F, Niklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences USA 1977;74:5463-67.

Sathar MA, Soni PN, Fernandes-Costa FJTD, Wittenberg DF, Simjee AE. Racial differences in the seroprevalence of hepatitis A virus infection in Natal/KwaZulu, South Africa. Journal of Medical Virology 1994;44:9-12.

Schoub BD, Blackburn NK, McAnerney JM. Hepatitis A virus seroprevalence in upper and lower socio-economic groups in South Africa: implications for vaccine policies. In: Smillie J, editor. Viral hepatitis and liver disease. Proceedings of the 10thInternational Symposium on Viral Hepatitis and Liver disease; 2000 April 9-13; South Africa. London: International Medical Press; 2000. p. 46-8.

Schwab KJ, De Leon R, Sobsey MD. Development of PCR methods for enteric virus detection in water. Water Science and Technology 1993;27:211-8.

Schwab KJ, Neill FH, Fankhauser RL, Daniels NA, Monroe SS, Bergmire-Sweat DA, et al. Development of methods to detect “Norwalk-like viruses” (NLVs) and hepatitis A virus in delicatessen foods: application to a food-borne NLV outbreak. Applied and Environmental Microbiology 2000;66:213-8.

Schwab KJ, Neill FH, Le Guyader F, Estes MK, Atmar RL. Development of a reverse transcription-PCR-DNA enzyme immunoassay for detection of "Norwalk-like" viruses and hepatitis A virus in stool and shellfish. Applied Environmental Microbiology 2001;67:742-9.

Seeberg S, Brandberg A, Hermodsson S, Larsson P, Lundgren S. Hospital outbreak of hepatitis A secondary to blood exchange in a baby. Lancet 1981;1:1155-6.

Senouci S, Maul A, Schwartzbrod L. Comparison study on three protocols used to concentrate poliovirus type 1 from drinking water. Zentralblatt Fur Hygiene Und Umweltmedizin 1996;198:307-17

Shieh YC, Baric RS, Sobsey MD, Ticehurst J, Miele A, De Leon R, Walter R. Detection of hepatitis A virus and other enteroviruses in water by ssRNA probes. Journal of Virological Methods 1991;31:119-36.

Shieh YC, Wait D, Tai L, Sobsey MD. Methods to remove inhibitors in sewage and other faecal wastes for enterovirus detection by polymerase chain reaction. Journal of Virological Methods 1995;54:51-66.

Siegl G, deChastonay J, Kronauer G. Propagation and assay of hepatitis A virus *in vitro*. Journal of Virological Methods 1984;9:53-67.

Sobsey MD. Methods for recovering viruses from shellfish, seawater and sediments. In: Berg G, editor. Methods for recovering viruses from the environment. Boca Raton: CRC Press, Inc.; 1987. p. 77-108.

Soule H, Genoulaz O, Gratacap-Cavallier B, Chevallier P, Liu J, Seigneuri J. Ultrafiltration and reverse transcription-polymerase chain reaction: An

efficient process for poliovirus, rotavirus and hepatitis A virus detection in water. Water Research 2000;34:1063-7.

Stapleton JT, Frederick J, Meyer B. Hepatitis A virus attachment to cultured cell lines. Journal of Infectious Diseases 1991;164:1099-103.

Tassopoulos NC, Papaevangelou GJ, Ticehurst JR, Purcell RH. Faecal excretion of Greek strains of hepatitis A virus in patients with hepatitis A and in experimentally infected chimpanzees. The Journal of Infectious Diseases 1986;154:231-7.

Taylor MB. Studies on the propagation in cell culture and the infectivity for baboons of hepatitis A virus [dissertation]. Pretoria: Univ. of Pretoria; 1985.

Taylor MB. Molecular epidemiology of South African strains of hepatitis A virus: 1982-1996. Journal of Medical Virology 1997;51:273-9.

Taylor MB, Prozesky OW. Experimental infection of the Cape baboon (*Papio ursinus*) with hepatitis A virus. Suid-Afrikaanse Tydskrif vir Wetenskap 1985;81:597-600.

Taylor MB, Becker PJ, Janse van Rensburg E, Harris BN, Bailey IW, Grabow WOK. A serosurvey of waterborne pathogens amongst canoeists in South Africa. Epidemiology and Infection 1995;115:299-307.

Taylor MB, Cox N, Vrey MA, Grabow WOK. The occurrence of hepatitis A and astroviruses in selected river and dam waters in South Africa. Water Research 2001;35:2653-60.

Tedeschi V, Purcell RH, Emerson SU. Partial characterization of hepatitis A viruses from three intermediate passage levels of a series resulting in

adaptation to growth in cell culture and attenuation of virulence. Journal of Medical Virology 1993;39:16-22.

Termorshuizen F, Dorigo-Zetsma JW, De Melker HE, Van den Hof S, Conyn-Van Spaendonck MAE. The prevalence of antibodies to hepatitis A virus and its determinants in The Netherlands: a population-based survey. Epidemiology and Infection 2000;124:459-66.

Teunis PFM, Medema GJ, Kruidenier L, Havelaar AH. Assessment of the risk of infection by Cryptosporidium or Giardia in drinking water from a surface water source. Water Research 1997;31:1333-46.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acid Research 1997;25:4876-82.

Tsai Y, Sobsey MD, Sangermano LR, Palmer C. Simple method of concentrating enteroviruses and hepatitis A virus from sewage and ocean water for rapid detection by reverse transcriptase polymerase chain reaction. Applied and Environmental Microbiology 1993;59:3488-91.

Tsarev SA, Emerson SU, Balayan MS, Ticehurst J, Purcell RH. Simian hepatitis A virus (HAV) strain AGM-27: comparison of genome structure and growth in cell culture with other HAV strains. Journal of General Virology 1991;72:1677-83.

Usuba O, Schulman JL, Deatly AM, Bona CA, Moran TM. New methods for titration of virus infectivity by immunostaining. Viral Immunology 1990;3:237-41.

Vilaginès Ph, Sarrette B, Husson G, Vilaginès R. Glass wool for virus concentration from water at ambient pH levels. Water Science and Technology 1993;27:299-306.

Vilaginès Ph, Suarez A, Sarrette B, Vilaginès R. Optimisation of the PEG reconcentration procedure for virus detection by cell culture or genomic amplification. Water Science and Technology 1997a;35:455-9.

Vilaginès Ph, Sarrette B, Champsaur H, Hughes B, Dubrou S, Joret J-C, et al. Round robin investigation of glass wool method for poliovirus recovery from drinking water and sea water. Water Science and Technology 1997b;35:445-9.

Vitral CL, Yoshida CFT, Gaspar AMC. Developments in hepatitis A and C viruses experimental infection studies in non-human primates. Infectious Disease Review 2000;1:253-60.

Vivier JC, Ehlers MM, Grabow WOK, Havelaar AH. Assessment of the risk of infection associated with coxsackie B viruses in drinking water. Water Science and Technology 2002;2:1-8.

Wade TJ, Pai N, Eisenberg JNS, Colford JM Jr. Do U.S Environmental Protection Agency water quality guidelines for recreational waters prevent gastrointestinal illness? A systematic review and meta-analysis. Environmental Health Perspectives 2003;111:1102-9.

Ward R, Krugman S, Giles JP, Jacobs AM, Bodansky O. Infectious hepatitis: Studies of its natural history and prevention. New England Journal of Medicine 1958;258:407-16.

Ward RL, Bernstein D, Young DE, Sherwood J, Knowlton DR, Schiff GA. Human rotavirus studies in volunteers: determination of infectious dose and

serological response to infection. Journal of Infectious Diseases 1986;154:871-80.

Watson JC, Fleming DW, Borella AJ, Olcott ES, Conrad RE, Baron RC. Vertical transmission of hepatitis A resulting in an outbreak in a neonatal intensive care unit. Journal of Infectious Diseases 1993;167:567-71.

Weitz M, Siegl G. Structure and molecular virology. In: Zuckerman AJ, Thomas HC, editors. Viral hepatitis: Scientific basis and clinical management. New York: Churchill Livingstone Inc.; 1993. p. 61-79.

Weltman AC, Bennett NM, Ackman DA, Misage JH, Campana JJ, Fine LS, et al. An outbreak of hepatitis A associated with a bakery, New York, 1994: The 1968 'West Branch, Michigan' outbreak repeated. Epidemiology and Infection 1996;117:333-41.

Wheeler CM, Fields HA, Schable CA, Meinke WJ, Maynard JE. Adsorption, purification and growth characteristics of hepatitis A virus strain HAS-15 propagated in foetal rhesus monkey kidney cells. Journal of Clinical Microbiology 1986;23:434-40.

Widell A, Hansson BG, Nordenfelt E, Öberg B. Enhancement of hepatitis A propagation in tissue culture with 5,6-Dichloro-1-β-D ribofuranosylbenzi-midazole. Journal of Medical Virology 1988;24:369-76.

World Health Organization. Guidelines for Drinking-Water Quality 2nd edition, volume 2: Health criteria and other supporting information. Geneva: WHO; 1996. p. 93-106.

World Health Organization. WHO position paper on Hepatitis A vaccines (international notes). WHO Weekly Epidemiological Record 2000;75.

World Health Organization. Guidelines for Safe Recreational Water Environments volume 1: Coastal and fresh waters. Geneva: WHO; 2003. p. 62.

Yap KL, Lam SK. Infectivity titration of the fast replicating and cytopathic hepatitis A virus strain HM175A.2 by an *in situ* enzyme immunoassay. Journal of Virological Methods 1994;47:217-26.

Yotsuyanagi H, Koike K, Yasuda K, Moriya K, Shintani Y, Fujie H, et al. Prolonged faecal excretion of hepatitis A virus in adult patients with hepatitis A as determined by polymerase chain reaction. Hepatology 1996;24:10-3.

Zachoval R, Deinhardt F. Natural history and experimental models. In: Zuckerman AJ, Thomas HC, editors. Viral hepatitis: Scientific basis and clinical management. New York: Churchill Livingstone Inc.; 1993. p. 35-53.

Zuckerman JN, Zuckerman AJ. Hepatitis Viruses. In: Armstrong D, Cohen J, Berkley SF, Carbon CJ, Clumeck N, Durack DT et al., editors. Infectious Diseases. Vol 2. London: Harcourt Publishers Ltd.; 1999. p. 4.1-12.

APPENDIX A

CALCULATIONS FOR $TCID_{50}$ ACCORDING TO THE KÄRBER FORMULA

Titres are conveniently calculated by the simple and easy Kärber formula (Grist et al., 1979) to determine the 50% endpoints. $TCID_{50}$ = 50% tissue culture infectious dose.

Log TCD_{50} = L – d(S – 0.5)

where L = negative log lowest dilution

d = difference between log dilution steps, and

S = sum of proportions of positive tests

Example: *Virus dilution*	*Proportion of infected cultures*
10^{-1}	4/4 = 1
10^{-2}	3/4 = 0.75
10^{-3}	2/4 = 0.5
10^{-4}	2/4 = 0.5
10^{-5}	1/4 = 0.25
10^{-6}	0/4 = 0

Log TCD_{50} = L – d(S – 0.5)

= -1 – 1(3 - 0.5)

= -1 – 2.5

= - 3.5

The Kärber formula gives a negative log term: $10^{-3.5}$ = 1/3162.28 = the $TCID_{50}$ endpoint dilution. The $TCID_{50}$ titre is more correctly expressed as $10^{.35}$ = 3.5 log units = 3162.28 $TCID_{50}$ per inoculum volume.

APPENDIX B

SEQUENCING PROTOCOL

B.1 PREPARATION OF SEQUENCING GEL

The gel was poured between two glass plates of dimensions 345 mm X 450 mm X 5 mm. Plates were pre-treated and cleaned by wiping with acetone [$(CH_3)_2CO$] and methanol (CH_3OH) for the removal of contaminating residues. The surface of the slotted plate was treated with Gel Slick solution (Bioproducts, Rockland, ME) to prevent the gel from adhering to the plate. Plates were separated by two spacers and assembled into a compact unit with clamps holding the sides together. Before pouring the gel, a 1.5 ml aliquot of gel was made up to seal the open bottom end of the unit. The larger volume (75.323 ml) was gently poured into the gel space after the sealer has set. The sequencing gel was allowed to set overnight. The gel was preheated for 1 h at 60 V before sequencing products were loaded. For this the glass plates containing the cast gel was placed in an upright position into the sequencing apparatus (Hybaid Ltd., Middlesex, UK) and connected to a power source (Consort E734 electrophoresis power supply, CONSORT nv, Belgium).

B.2 DEVELOPMENT OF SEQUENCING GEL

The gel was separated from the glass plate pretreated with the Slick, adsorbed onto a sheet of blotting paper (3 MM Chromatography paper, Whatman International Ltd., Maidstone, UK) and fixed with absolute ethanol (Merck) containing 10% acetic acid (Merck) and then rinsed with tap water. Excessive moisture was gently blotted away and the paper containing the intact gel rolled off the glass plate and transferred to the gel dryer (Drygel Sr Slab Gel Dryer,

Hoefer Scientific Instruments, San Francisco, CA). Gels were vacuum-dried for 1-2 h at 80ºC with condensation system (Refrigerated Condensation trap RT400, Savant Instruments Inc., Farmingdale, NY) and a high vacuum pump (VP 190 Two Stage, Savant Instruments Inc.) to combine the gel and blotting paper into a single membrane. This membrane was exposed to X-ray film for 12-24 h at room temperature and visualised by development of the film.

APPENDIX C

ABSTRACTS OF PRESENTATIONS AND PUBLICATIONS

C.1 NATIONAL PRESENTATIONS

1. **JME Venter,** WOK Grabow, MB Taylor. Comparison of methods for the isolation and detection of hepatitis A virus from water samples [Poster]. Faculty Day, Faculty of Health Sciences, University of Pretoria. 21-22 August 2001: Pretoria, South Africa.

Hepatitis A virus (HAV) is a highly infectious virus and the cause of the most common form of acute viral hepatitis worldwide. HAV is predominantly transmitted by the faecal-oral route and there is conclusive evidence of waterborne transmission. Although HAV has been detected in dam and river water used for domestic and recreational purposes, the contribution of faecally contaminated water as a source of HAV infection in South Africa is unknown. The aim of this investigation was to reassess and compare methods for the isolation and detection of HAV from water. Wild type (WT) HAVs cannot readily be propagated in conventional cell cultures and no cytopathogenic effect is evident. In order to select the most susceptible cell line for routine isolation of HAV from water, cell cultures of both human (PLC/PRF/5) and non-human (FRhK-4R, Vero, BGM) primate origin were infected with both WT and cell culture-adapted strains of HAV. At pre-selected intervals cell cultures were monitored by immunofluorescence (IF) and a reverse transcriptase-polymerase chain reaction (RT-PCR)-oligonucleotide probe assay for viral amplification. The FRhK-4R was found to be the most susceptible to infection with both the WT and cell culture-adapted strains of

HAV used. HAV RNA was detected in infected cell cultures earlier and more frequently by RT-PCR-oligonucleotide probe assay than by IF antigen detection technique. Incubation for a minimum of 21 days was needed before amplification of the WT HAV could be detected and serial passaging of the infected cell cultures every seven days enhanced detection of the virus. These results provide valuable guidelines for the routine detection of HAV in water.

2. **JME Venter,** WOK Grabow, MB Taylor. Comparison of methods for the isolation and detection of hepatitis A virus from water samples [Presentation]. "Microbial Diversity". 12th Biennial Congress of the South African Society of Microbiology, University of the Free State. 2-5 April 2002: Bloemfontein, South Africa.

Hepatitis A virus (HAV) is a highly infectious virus and the most common cause of acute viral hepatitis worldwide. HAV is predominantly transmitted via the faecal-oral route and there is conclusive evidence of waterborne transmission. Although HAV has been detected in dam and river water used for domestic and recreational purposes in South Africa (SA), the contribution of faecally contaminated water the overall burden of HAV infection is unknown. Wild type (WT) HAV cannot readily be propagated in conventional cell cultures and a cytopathic effect is rarely evident. Therefore alternate techniques are required for the detection of HAV directly in water samples or for the detection of viral amplification in cell cultures.

The aim of this investigation was to assess and compare methods for the isolation and detection of HAV from water. In order to select the most susceptible cell line for routine isolation of HAV from water, cell cultures of both human (PLC/PRF/5) and non-human (FRhK-4R, Vero, BGM) primate origin were infected with both WT and cell culture-adapted strains of HAV. At pre-selected intervals cell cultures were monitored for viral amplification by immunofluorescence (IF) and a reverse transcriptase-polymerase chain reaction (RT-PCR)- oligonucleotide probe assay. HAV RNA was detected as early as 7 days post-infection (p.i.), by RT-PCR-oligonucleotide probe assay, in cell cultures infected with the cell culture-adapted strain pHM-175 compared to 21 days p.i. for the WT virus. In contrast, HAV antigen was only detectable, by IF, in the pHM-175 infected cell cultures 14 days p.i. Therefore incubation for a minimum of 21 days was needed before amplification of the WT HAV could be detected. Serial passaging of the infected cell cultures every seven days enhanced detection of the WT and cell culture-adapted strains of the virus. The non-human primate cell line, FRhK-4R, was found to

be the most susceptible to infection with both the WT and cell culture-adapted strains of HAV tested. These results provide valuable guidelines for the routine isolation and detection of viable HAV in water.

3. WB van Zyl, S Nadan, JC Vivier, **JME Venter**, K Riley, EMK Tlale, LR Seautlueng, WOK Grabow, MB Taylor. The prevalence of enteric viruses in patients with gastroenteritis in the Pretoria and Kalafong Academic Hospitals, South Africa [Poster]. Microbial Diversity" 12th Biennial Congress of the South African Society for Microbiology, Faculty of Health Sciences, University of the Free State. 2-5 April 2002: Bloemfontein, South Africa.

Enteric viruses are important causative agents of waterborne diseases, such as gastroenteritis, hepatitis A and E and respiratory diseases, which are a major cause of morbidity and mortality worldwide. Although unable to multiply in water, viruses have a low infectious dose of one to ten viral particles. Several studies have addressed the prevalence of gastroenteritis, hepatitis and enteroviruses in South Africa (SA). However, no single study addresses the overall presence of enteric viruses in stool specimens in one cohort study in SA.

The aim of the study was to determine the prevalence of enteric viruses in patients with gastroenteritis presenting at the Pretoria and Kalafong Academic Hospitals over a one-year period from January to December 2001. Stool specimens referred to the Department of Medical Virology Diagnostic laboratory for routine analysis for gastroenteritis viruses, were used to determine the presence of the following enteric viruses: adeno (40/41), human astro (HAstV), human calici (HuCV), entero, hepatitis A (HAV) and rotaviruses. Adeno (40/41), HAstV and rotaviruses are routinely detected using commercially available enzyme immunoassays. RNA was isolated from 10% stool suspensions and virus-specific reverse transcriptase-polymerase chain reaction (RT-PCRs) was used to detect HuCV, HAV and enteroviruses. The sensitivity of detection of HAV and enteroviruses was enhanced by probe hybridisation and nested PCR respectively.

Results obtained for 300 stool specimens analysed from January to September 2001 were as follows: enter (54.3%), rota (18%), adeno 40/41 (2.9%), astro (2.2%), calici (1.4%) and HAV (0.01%). From the results to date it is clear that enteroviruses show the highest prevalence of all viruses investigated with

rotavirus being the second most prevalent virus. This is ascribed to the fact that 92% of the stool specimens were obtained from paediatric patients where excretion of poliovirus vaccine strains is common and rota-associated gastroenteritis is the main cause of viral diarrhoea in this age group. As paediatric HuCV infection is usually mild and self-limiting, individuals infected with HuCV are seldom hospitalised, explaining the low prevalence recorded in this study. In some studies HAstV, while in others enteric adenovirus has been found to be the second most important cause of acute virus gastroenteritis in infants and young children patients. In this study however similar prevalences for HAstV and enteric adenoviruses were noted. The low prevalence of HAV detected in this study is surprising as hepatitis A is endemic in SA with subclinical infections commonly found in children. These results provide valuable new data on enteric viruses circulating in a select community with important implications for infection control procedures in paediatric wards.

4. **JME Venter**, JC Vivier, WOK Grabow, MB Taylor. Is hepatitis A virus in surface recreational water a cause for concern? [Poster Presentation]. Faculty Day, Faculty of Health Sciences, University of Pretoria. 20-21 August 2002: Pretoria, South Africa.

The potential for human disease contracted from exposure to waterborne microorganisms is of growing public concern. Many surface waters used for domestic and recreational purposes are faecally contaminated. Hepatitis A virus (HAV) is highly infectious and spread via the faecal oral route, and recreational exposure to polluted water has unequivocally been linked to hepatitis A (HA). HAV has been detected in South African (SA) surface river and dam water used by informal housing communities for domestic and irrigation purposes, and by the higher socio-economic communities for recreational activities. Although recreational exposure to faecally contaminated water has been implicated in a number of cases of HA in SA, the actual risk of infection is unknown

Over a period of three years (June 1997-May 2000) grab samples (±300 litres) of surface water were collected weekly from the same sites on the Klip River and Vaal Dam, Gauteng, SA. HAV was recovered from the water samples by a glass-wool adsorption elution technique and detected by an integrated cell culture reverse transcriptase-polymerase chain reaction-oligonucleotide probe hybridisation assay. The potential risk of infection to individuals exposed to these water sources was estimated using the @Risk 4.0 program. A deterministic model using mean values was applied. Calculations were based on the assumption that an individual ingests 100 ml of water per recreational exposure. Initial results indicate that the probability of HAV infection per day to a person using the river and/or dam water for recreational purposes is 3,07 X 10^{-5} for the river water and 7,1 X 10^{-3} for the dam water. The risk probability value for the river water is well within the acceptable risk of 1 illness per 1000 recreational water users as stated by the USA Environmental Protection Agency, but the dam water does not conform to these requirements.

5. JME Venter, WOK Grabow, MB Taylor. Detection and characterisation of hepatitis A virus in treated and untreated drinking water in South Africa [Oral Presentation]. Medical Virology Congress of South Africa, Berg-en-Dal. 18-21 May 2003: Berg-en-Dal, Kruger National Park, South Africa.

The potential risk for human disease associated with exposure to waterborne microorganisms is of growing public concern. Hepatitis A virus (HAV) has a low infectious dose (1-10 viral particles) and outbreaks of infection has been unequivocally been linked to faecally contaminated water sources. Genetic analysis and comparison of clinical and environmental HAV isolates could provide valuable information with regard to the source of the virus in sporadic and epidemic infection.

The aim of this investigation was to detect, characterise and compare HAV isolates from clinical specimens and treated and untreated drinking water samples. During the period June 1998 to June 2001, 98 HAV IgM positive serum specimens were selected from routine specimens referred from the Pretoria and Kalafong Academic Hospitals, Pretoria, for hepatitis markers. HAV was detected directly in 40 of these specimens by the reverse transcriptase-polymerase chain reaction (RT-PCR). During the same period 768 treated and 336 untreated drinking water samples, collected from six different regions in SA as well as from Namibia, were sent to the Department of Medical Virology, Pretoria, for viral analysis. HAV was recovered from the water samples by a glass-wool adsorption elution and polyethylene glycol/sodium chloride precipitation technique. Recovered HAV was detected using an integrated cell culture-RT-PCR-oligonucleotide probe hybridisation assay. Viral isolates were characterised by sequence analysis of the RT-PCR amplicons. Sequences from clinical and environmental isolates were compared to each other and to the reference strains using the ClustalX programme of PC/Gene Version 6.85. Of the HAV isolates characterised to date, the isolates from the water samples and those from clinical specimens clustered separately, but were closely related, and were typed as subgenotype IB. These

results suggest that the treated and untreated drinking water sources are a potential reservoir for human infection.

C.2 INTERNATIONAL PRESENTATIONS

1. JC Vivier, MM Ehlers, J van Heerden, **JME Venter**, MB Taylor, WOK Grabow. An assessment of candidate viruses for harmonised risk assessment of water related microbiological hazards [Oral Presentation]. 11th International Water Association (IWA) Health-Related Water Microbiology Symposium, Melbourne 7-12 April 2002: Melbourne, Australia.

The need for a fundamental change in the guidelines for the microbiological quality of drinking water has been recognised. The current guidelines are focused towards end-product monitoring of microbiological indicators of faecal pollution. By following this approach consumers are at risk before warning signals are received or acted upon. Developments on microbial risk assessment in a risk management framework in the food industry have provided an alternative risk-based approach that can be applied to protect the consumer against potential risk of viral infections from drinking water. It is practical to narrow the focus of viral risk assessment to those viruses that (a) most readily occur, (b) are most resistant to ambient stress and treatment, (c) are most significant clinically and (d) more applicable to local circumstances.

The World Health Organisation (WHO) has identified hepatitis A virus (HAV) as a candidate virus for risk assessment and hazard analysis critical control point (HACCP) investigations. It has also been suggested that human adenoviruses (HAd), as determined by the polymerase chain reaction (PCR), be used as a possible molecular index for the presence of human viruses in the environment. Studies in our laboratory have identified enteroviruses as the most commonly detected viruses in selected drinking water sources in South Africa (SA). The objective of this investigation was to assess enteroviruses (EVs), coxsackie B viruses (CBV), HAd and HAV as candidate viruses for assessing the risk of infection constituted by viruses in drinking water supplies in SA.

A total of 172 samples of drinking water (100-1000 L), collected over a one year period, were tested for enteric viruses. The microbial quality of the water samples conformed to the recommendations of WHO. Viruses were recovered using in-line glass wool filters and the concentrate inoculated, in parallel, onto monolayers of Buffalo Green Monkey kidney (BGM), human hepatoma (PLC/PRF/5), human colonic carcinoma (CaCo-2) and foetal rhesus monkey kidney (FRhK-4R) cell lines. The infected BGM, PLC/PRF/5 and CaCo-2 cultures were passaged after 7 days (d) and harvested after a further 7 d incubation. The FRhK-4R cultures, used for the amplification of HAV, were harvested after 21 d incubation.

Viral-induced cytopathic effects were not observed in any of the infected cell cultures and viruses were detected in cell cultures harvests by the appropriate qualitative PCR or reverse transcriptase-PCR (RT-PCR) assays. The risk of infection from CBVs to the consumers was determined using a probabilistic model. EVs were detected in 15%, CBVs in 12.5%, HAds in 12% and HAV in 0.5% of drinking water samples tested. The annual risk of infection for CBVs was 5.5×10^{-3}.

Of these candidate viruses, HAV and CBV are the only viruses for which documented evidence of transmission via drinking water exists. HAV and CBV are also very stable in the environment and more resistant that faecal indicator bacteria to treatment processes. HAV has however only been detected in 0.5% of selected drinking waters in SA compared to 16% reported for CBV. Although HAV infection results in a high percentage of sub-clinical infections, especially in children, HAV has the most definite clinical manifestations compared to the other viruses under discussion. An advantage of using HAV is that it has a single host, i.e. humans, and there is only one serotype.

There are, however, many disadvantages to using HAV as model for risk assessment in SA. Although HAV can be excreted by infected persons for up

to 10 weeks after the onset of symptoms, viruses excreted in the late recovery phase may not necessarily be infectious. The occurrence of HAV in selected SA drinking waters is low and does not correlate with the presence of other potentially pathogenic enteric viruses. Of the candidate viruses CBVs are more likely candidate viruses for risk analysis in SA. The risk assessment analysis indicated that the drinking water supplies concerned constitute an unacceptable risk with regard to CBV infection. The disadvantage of CBV is that there are six serotypes that can present with a wide range of clinical manifestations ranging from asymptomatic to severe systemic and sometimes fatal disease.

Due to their double-stranded DNA genome, HAds are reportedly more stable in the environment than other enteric viruses but have, to date, only been associated with swimming pool related infections, HAds were detected in 12% of drinking water samples tested but, due to the lack of dose-response data, risk assessment analysis could not be performed. EVs were detected in 26 (15%) of the samples but, because of the diversity of viruses within the EVs and different dose response data for the different virus species, risk assessment is problematical. Risk assessment analysis using data for one species would probably not be representative of the EVs as a group. HAds and EVs have a large number of serotypes (51 and 62 respectively) resulting in a wide range of clinical and asymptomatic infections. In addition, 12% of the EVs detected in the drinking water sources were vaccine strain polioviruses that do not necessarily pose a health risk. Consequently our data suggested that HAds and EVs would not appear to be model viruses for risk assessment of water related microbial hazards in the SA context.

From the data presented it is evident that, in SA, a number of potentially infectious viruses are present in selected drinking water sources which meet international specifications for the indicator quality of drinking water supplies. With available epidemiological data in SA it is difficult to determine a burden of disease for any of these candidate viruses, especially in the lower socio-

economic communities. Of the viruses assessed, CBVs, therefore, appear to be the most suitable viruses for use in SA for determining the potential risk of infection from drinking water supplies.

2. **JME Venter**, JC Vivier, WOK Grabow, MB Taylor. Hepatitis A virus in surface recreational water in South Africa: what is the risk? [Oral presentation]. IWA International Symposium on Health-Related Water Microbiology, Cape Town 14-19 September 2003: Cape Town, South Africa.

The potential risk for human disease associated with exposure to waterborne microorganisms is of growing public concern. Enteric viruses present in domestic and industrial wastewater discharged into surface waters pose a potential risk of infection to communities using the contaminated water sources for domestic and recreational purposes. Recreational exposure to polluted water has unequivocally been linked to hepatitis A (HA). Hepatitis A virus (HAV), transmitted by the faecal oral route, is highly infectious, with an infectious dose of 1-10 viral particles. HAV is shed maximally in faeces two to three weeks prior to the appearance of jaundice and faeces can remain infectious for up to four weeks after alanine transferase enzyme levels peak. This prolonged excretion promotes the spread of the virus during recreational activities when faecally polluted water is ingested. Risk of infection increases with increased immersion in contaminated water. Recreational exposure to faecally contaminated water has been implicated in isolated cases of HA in South Africa (SA). However, in SA there is no clear definition of an acceptable risk of infection, but according to the South African water quality guidelines of 1996 for domestic and recreational use, enteric virus titres of 1-10 $TCID_{50}/10\ell$ sample pose a meaningful risk of infection to consumers. HAV has been detected in river and dam water used by South African rural and informal housing communities for domestic and irrigation purposes, and by the higher socio-economic communities for recreational activities, but actual risk of infection to persons using these water sources for recreational purposes is unknown. The aim of this investigation was to assess the risk of human infection constituted by HAV to persons exposed to these water sources during recreational activities.

Over a period of three years (June 1997-May 2000) surface water samples (±300 litres) were collected weekly from the same sites on a river and dam, in SA, which are used for domestic purposes by lower socio-economic

communities and recreational purposes by the higher socio-economic community. HAV was recovered from the water samples by a glass-wool adsorption elution technique and viruses further concentrated with the use of a polyethylene glycol/sodium chloride precipitation technique. Recovered viruses were isolated and amplified in a foetal rhesus monkey kidney cell line (FRhK-4R). HAV was detected by reverse transcriptase-polymerase chain reaction and results confirmed by an oligonucleotide probe hybridisation assay. The potential risk of infection to individuals exposed to these water sources was estimated using the @Risk 4.0 program. A deterministic model using mean values was applied. Exposure assessment was based on the following parameters and assumptions: i) the assumption that an individual ingests 100 ml of water per recreational exposure; ii) the concentration of HAV in the dam and river water; iii) the observation that as the viruses were detected in cell culture they were viable and therefore potentially infectious; iv) that the glass-wool adsorption procedure has an estimated efficiency of recovery of 30%, and v) an infective dose of one virus.

Results indicate that the probability of HAV infection per day to a person using the river and/or dam water for recreational purposes is 3.07×10^{-5} for the river water and 7.1×10^{-3} for the dam water. The risk probability value for the river water is well within the acceptable risk of 1 illness per 1000 recreational water users as stated by the USA Environmental Protection Agency (USEPA), but the dam water does not conform to these requirements. In addition these results are considered to be a gross underestimate of the true risk involved because of shortcomings in techniques currently available for the recovery and detection of HAV from water. As HA is endemic in SA, with nearly all the lower socio-economic community having been infected and acquired immunity by 15 years of age, recreational and domestic exposure to these waters will hold a minimal risk of infection to adults, but children are potentially at risk. However, as only 50-70% of the higher socio-economic population have been infected and acquired immunity by 40 years of age, recreational exposure to these waters poses a significant risk of infection to this population group.

3. MA Vrey, MM Ehlers, WB van Zyl, MB Taylor, DN Pavlov, J van Heerden, **JME Venter**, JC Vivier, JC de Villiers, E van Zyl, WOK Grabow. Microbiological quality of ground and surface water used for domestic purposes in rural areas of South Africa [Poster]. IWA International Symposium on Health-Related Water Microbiology, Cape Town 14-19 September 2003: Cape Town, South Africa.

Many households in rural and developing areas, and in informal settlements, are without piped supplies of treated drinking water – an unfortunate situation that occurs in many parts of the world. Generally, people living under these conditions collect domestic water supplies in a variety of containers from sources such as rivers, streams, springs, dug-wells, boreholes, impoundments, dams etc. Since sanitation in these communities tends to be as poor as safe water supply, the water sources utilised are, as a rule, exposed to pollution by human and animal wastes. As can be expected, this often leads to devastating health implications with far-reaching socio-economic consequences. South Africa is no exception to these problems. Unfortunately the country has no public health services or research programmes for generating meaningful details on waterborne and water-related diseases. However, there is sound evidence that people living under such conditions have a high incidence of diarrhoea, particularly among children. Certain parts of the country have been suffering from a severe epidemic of cholera for some years now. Details of a serious dysentery outbreak were used to assess the impact of waterborne diseases in terms of health implications and related economic consequences for the people concerned as well as the country as a whole. This report summarises results of a survey of the microbiological quality of typical water sources used for domestic purposes by people who do no have piped supplies of treated drinking water in their homes. The sources chosen for this study were selected rivers, springs and boreholes. Representative sampling sites were selected in four provinces of the country – Eastern Cape (EC), Free State (FS), KwaZulu Natal (KZN) and Limpopo (LP)

Samples (96), 24 from each province, were analysed from April 2001 o March 2002. They were collected and transported at 4-10°C to the laboratory in Pretoria according to procedures recommended by the World Health Organisation (WHO). Analysis of all samples commenced within 24 h of collection according to established laboratory procedures as recommended by international authorities such as the WHO and the International Organisation for Standardisation (ISO): heterotrophic plate counts (HPC), total and faecal coliforms, somatic coliphages and enteric viruses. Somatic coliphages were detected by quantitative presence-absence tests on 500 ml samples. Enteric viruses were recovered from 20 L samples by means of a glass wool adsorption-elution procedure. Recovered viruses were inoculated into appropriate cell cultures, to replicate their nucleic acid for increased sensitivity, and molecular techniques were then used to detect the nucleic acid of adeno-, astro-, entero-, hepatitis A and rotaviruses. Table 1 summarises the results expressed in terms of the range of averages for individual sampling points.

Table 1: Microbiological results from the survey

Sample type (provinces)	CFU/ml HPC	CFU/100 ml Total coliforms	Faecal coliforms	% samples positive for Somatic phages	Enteric viruses
Boreholes (all provinces)	41 - 788	3 - 8, 102	1 - 2, 330	8 - 50	42 - 55
Rivers (EC, FS, LP)	948 - 3, 050	357 – 16, 904	86 – 2, 330	75 - 83	33 - 75
Spring (KZN)	2139	127	10	25	42

The highest percentage of enteric viruses isolated from samples was, generally, enteroviruses followed by adeno-, rota-, astro-, and hepatitis A viruses (HAV) – details of the types of viruses are given in Table 2.

Table 2: Virus types by province

Province (source)	% Samples positive for:				
	Entero-virus	Rotavirus	Adenovirus	Astrovirus	HAV
EC (borehole)	25	17	8	8	0
FS (borehole)	17	17	8	0	0
FS (river)	17	0	8	25	8
LP (borehole)	17	0	25	0	0
LP (river)	17	17	17	8	8

Other than the generally high incidence of enteroviruses followed by the remaining viruses, as mentioned above, there was no meaningful trend in the relative incidence of viruses in carious water sources or provinces other than (a) the incidence of rotaviruses appeared to be exceptionally high in the EC and FS boreholes and the LP river while (b) the incidence of adenoviruses appeared to be exceptionally low in all EC and FS sources but exceptionally high in all LP sources. A notable feature of the results was that, on average, somatic coliphages were less often detected than enteric viruses in water from the boreholes and the spring. Somatic coliphages were detected more often than enteric viruses in the EC and FS rivers but not in the LP River. These results were important as in sewage the numbers of somatic coliphages generally outnumber those of enteric viruses by a wide margin. Although the sensitivity of methods used for the detection of somatic coliphages and enteric viruses may have been different, it had to be taken into account that the presence-absence test on 500 ml samples used for somatic coliphages was relatively sensitive and the test for enteric viruses on 20 L samples was relatively insensitive. The results did, therefore, suggest that enteric viruses survived more successfully in groundwater environments than somatic coliphages. This implied that somatic coliphages were not reliable indicators for the absence of enteric viruses from groundwater or for the survival of enteric viruses in groundwater environments. More detailed analyses would

be required to verify these conclusions. The apparent variation in the incidence of different viruses in different water sources and provinces may have been due to epidemiological fluctuation in the incidence of the viruses in the communities concerned. The results would not appear to suggest a relative survival advantage or disadvantage for any of the enteric viruses.

The results of this study revealed that the microbiological quality of water from many of the sources used for domestic purposes exceeded the following limits for drinking water recommended by the South African Bureau of Standards (SABS) based on internationally accepted principles: HPC of < 100 cfu/ml, no total or faecal coliforms per 100 ml, no somatic coliphages per 10 ml and no enteric viruses per 100 ml. This implied that the water sources generally used for domestic purposes without treatment or disinfection may have constituted a potential health risk. The high percentage of samples (33-75%), in which a restricted spectrum of viruses was detected by a relatively insensitive procedure from 20 L instead of 100 ml samples as specified by the SABS, is of particular concern. Since these viruses were able to infect in cell cultures and replicate their nucleic acids in the host cells, they must be considered as at least potentially infectious, particularly sine many enteric viruses readily infect humans but fail to infect cell cultures. The presence of these enteric viruses would, therefore, seem to strongly indicate a potential health risk. The results are being processed by more detailed risk assessment procedures aimed at obtaining a meaningful indication of the health risk and burden of disease constituted by the water sources concerned. These details are required to determine the potential economic benefits of investing in an appropriate infrastructure of piped supplies of safe drinking water and efficient sanitation. In situations where this is not possible, the results may justify the introduction of intervention strategies aimed at improving the quality of drinking water stored in homes without piped supplies of safe drinking water.

4. MB Taylor, S Nadan, WB van Zyl, **JME Venter**, D Pavlov, N Potgieter, JM Barnes. Application of integrated-cell culture RT-PCRs to determine the occurrence of enteric viruses in irrigation water and associated minimally processed foods [Poster]. IWA International Symposium on Health-Related Water Microbiology, Cape Town 14-19 September 2003: Cape Town, South Africa.

Despite an increase in the prevalence of food- and waterborne disease, the global importance of food safety is not fully appreciated. Contact with human faeces places virtually any food at risk of viral contamination. Minimally processed foods (MPFs), such as fruit, salads and vegetables, which are consumed raw or lightly cooked are of particular concern and diffuse widespread outbreaks due to low-level viral contamination are difficult to trace. One of the major routes of contamination is faecally polluted irrigation or post-harvest washing water. As viral pathogens may be present in irrigation water and MPFs in low numbers, the correct approach is not only to analyze the sample for enteric viruses but for organisms indicative of faecal contamination, e.g. faecal coliforms and/or *Escherichia coli,* and organisms indicative of viruses, namely bacteriophages. In this investigation surface and borehole water used for irrigation, and samples of MPFs irrigated with water from a number of these sites, were analyzed for human enteric viruses and indicator organisms.

On three separate occasions, water samples (2-5 L) were taken at two sites on a river in the western Cape, one before and one after pollution from diffuse effluents from a dense settlement. Over a period of four months four consecutive samples of water (2 L) were taken from three boreholes and one river in a rural community in the Limpopo province. Using standard methods the water samples were tested for faecal coliforms and/or *E. coli* and somatic and F-RNA coliphages. Enteric viruses were recovered from the water samples in a final volume of 6 ml phosphate buffered saline containing 1% bovine serum albumin using a sodium chloride (NaCl)/polyethylene glycol (PEG) precipitation method. Samples of irrigated MPFs, namely tomatoes,

onions and cabbages, were collected at the same sites as the water samples from the rural community. Approximately 100 g MPF was washed overnight at 4°C in 50 ml PBS. Bacteriological and phage analysis was done directly on the PBS washing while viruses were recovered from the PBS in a final volume of 6 ml by NaCl/PEG precipitation. Recovered virus was inoculated onto two flasks of PLC/PRF/5 cell cultures, one of which was treated and incubated with trypsin (10µg/mL) containing medium, and one flask of CaCo-2 cells, also treated with trypsin. Cytopathogenic viruses were identified by staining and examination of infected cover slips for virus specific inclusion bodies. Viral RNA was extracted from 1 ml of both harvested cell culture and water concentrate using a QIAamp UltraSens Virus kit. Human astroviruses (HAstVs) and hepatitis A virus (HAV) were detected by reverse transcriptase-polymerase chain reaction (RT-PCR)-oligonucleotide probe hybridisation assays. Human rotaviruses (HRV) and enteroviruses were detected by nested RT-PCRs and human calicivirus (HuCV) by RT-PCR.

Enteroviruses, namely vaccine strains of polioviruses and untyped enteroviruses, adenoviruses, HAstVs and HRVs were isolated from and/or detected in water samples drawn from the river below the overcrowded settlement in the western Cape. These data suggest that the river in question is heavily polluted with human faecal material and correlate with bacteriological data showing gross faecal contamination, namely *E. coli* counts of 9 400 to >2 000 000 per 100 ml. Although bacteriological analysis indicated the presence of faecal contamination and pathogenic bacteria such as *Salmonella* and *E. coli*, no HAV, HAstV or HuCVs were detected in any of the irrigation or associated MPF samples from the rural area. HRV was however detected in one of the irrigation water samples drawn in July, and this correlated with a high faecal coliform count (1 090 per 100 ml) and the presence of somatic coliphages. HRVs were also detected on one MPF sample, namely tomatoes. The absence of viruses in the majority of samples from the rural area could be ascribed to the small sample volume used and/or limitations in the cell culture and molecular techniques used. The occurrence of human enteric viruses and faecal coliform and/or *E. coli* counts of >1000 per 100 ml in a number of the

irrigation water samples analysed in this investigation indicates that there is a likelihood of contamination of associated MPFs which could lead to the spread of human enteric pathogens. As these water sources are also used by the communities for domestic purposes the presence of enteroviruses, human adenoviruses, HRVs and HAstVs suggests that this water poses a potential health risk, but more data are required to quantify the risk.

C.3 PUBLICATION

JME Venter, J van Heerden, JC Vivier, WOK Grabow, MB Taylor. Hepatitis A virus in surface water in South Africa: What are the risks?

Submitted to Journal of Applied Microbiology on 25 June 2004

Aims: To assess the potential risk of infection constituted by HAV to persons using surface dam and river water for domestic and recreational purposes.

Methods and Results: This investigation estimates the potential risk using a deterministic exponential risk assessment model which works with mean values and conservative assumptions. HAV was detected in 17.5% of river and 14.9% of dam water samples tested. The probability of infection (P_{inf}) to the higher socio-economic population using the river water for recreational purposes was 1.09 X 10^{-3} per day and 3.28 X 10^{-1} per annum if 100 ml was ingested per day. For recreation in the dam water the P_{inf} value was 1.17 X 10^{-4} per day and 4.18 X 10^{-2} per annum. For the lower socio-economic population, risk values for drinking purposes (2 L day^{-1}) were ten-fold greater. The number of indicator organisms in these sources exceeded drinking and recreational water quality guidelines.

Conclusion: These surface waters did not conform to the US EPA acceptable risk of 1 infection per 10 000 consumers per year for drinking water. Neither of the water sources conformed to US EPA bacterial quality guidelines for recreational water indicating possible health risks to recreational water users.

Significance and impact of study: This is the first risk assessment study addressing the risk of infection constituted by HAV in surface water to different socio-economic populations in South Africa.

Keywords: hepatitis A virus, surface water, risk assessment, drinking water, recreational water