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OPTIMISATION AND ASSESSMENT OF REAL-TIME PCR TECHNIQUES FOR THE DETECTION OF SELECTED FOOD- AND WATERBORNE VIRUSES

by

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DECLARATION

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

Signature:.....

Date:.....

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SUMMARY

The transmission of human pathogens by faecally contaminated fruit and vegetables is well established, but the burden of disease caused by foodborne pathogens is unknown. Fresh produce can be contaminated through the use of polluted irrigation water or by the handling of the produce by infected individuals either pre- or post harvest. There is very little known regarding the extent of viral contamination of irrigation water and fresh produce in South Africa. Noroviruses (NoV) and hepatitis A virus (HAV) are recognized as leading causes of foodborne viral disease. These viruses are transmitted predominantly via the faecal–oral route, primarily person-to-person by direct contact with an infected person, or indirectly by ingestion of contaminated food and water. The detection of enteric viruses in food or water is problematical and complex as many foodborne viruses, including HAV and NoV, cannot be readily isolated in cell culture.

The aim of this investigation was to develop and optimise simple and efficient methods for the concentration and detection of NoV GII and HAV in irrigation water and fresh produce. These methods would then be applied to field samples of irrigation water and fresh produce to try and establish a link between viral contamination detected in irrigation water and that on associated irrigated fresh produce. The efficiency of different commercial real-time reverse transcriptase-polymerase chain reaction amplification kits for the real-time detection of HAV, NoV GI and NoV GII was assessed, and standard curves for the quantitative detection of these viruses were constructed using the most appropriate kit. Using two types of fresh produce, three different elution buffers, each at two pHs, with two different elution times were compared to establish which buffer was the most efficient for the extraction of viruses from the fresh produce. The tris-glycine beef extract buffer (pH 9.5) with an elution time of 20 minutes most efficient for the extraction of the selected enteric viruses from fresh produce. From April 2008 to November 2009, 86 irrigation water and 72 fresh produce samples were collected from commercial and subsistence farms, street vendors and commercial outlets. All the irrigation water and fresh produce samples were analysed for HAV, NoV GI and NoV GII. Overall, 16.3 % (13/86) and 12.5 % (9/72) of irrigation water and fresh produce samples tested positive for one or more human pathogenic viruses, namely NoV GII and HAV, respectively. Nucleotide sequence and phylogenetic analysis of the HAV and NoV GII strains identified clinically relevant viruses in the irrigation water and on the fresh produce. A direct link between contaminated irrigation water and contamination of fresh produce could not be established, but irrigation water was identified as a possible source of contamination of the fresh produce. The results also suggested that food handlers contributed significantly to the viral contamination of the fresh produce. This study highlights the potential health risk posed by fresh produce to consumers in South Africa and highlights the need for further in depth studies to quantify the risk to consumers. This study represents new data on the occurrence of enteric viruses in food and water in South Africa and is crucial for the development of effective intervention and control strategies for food safety in South Africa.

PRESENTATIONS AND PUBLICATIONS

Publications

Bosch A, Sánchez G, Abbaszadegan M, Carducci A, Guix S, Le Guyader FS, **Netshikweta R**, Pintó RM, van der Poel WHM, Rutjes S, Sano D, Taylor MB, van Zyl WB, Rodríguez-Lázaro D, Kovač K, Sellwood J. Analytical methods for virus detection in water and food. Food Analytical Methods 2011:4:4-12.

Kiulia NM, **Netshikweta R**, Page NA, van Zyl WB, Kiraithe MM, Nyachieo A, Mwenda JM, Taylor MB. The detection of enteric viruses in selected urban and rural river water and sewage in Kenya, with special reference to rotaviruses. Journal of Applied Microbiology 2010;109:818-828.

International presentations

Kiulia NM*, **Netshikweta R,** Page NA, van Zyl W, Kiraithe MM, Mwenda JM, Taylor MB. Detection of rotavirus in surface water in Kenya: A pilot study [Poster]. "Realizing the Impact of Rotavirus Vaccines" 9th International Rotavirus Symposium 2-3 August 2010 Sandton Sun Convention Centre, Johannesburg, South Africa (*presenter)

Kiulia NM*, **Netshikweta R,** Page NA, van Zyl W, Kiraithe MM, Mwenda JM, Taylor MB. Detection of rotavirus in surface water in Kenya: A pilot study [Poster]. 6th African Rotavirus Symposium 4 August 2010 National Institute for Communicable Diseases, Johannesburg, South Africa (*presenter)

Netshikweta R, van Zyl WB*, Mans J, Wolfaardt M, Taylor MB. The application of real-time RT-PCR for the detection of selected enteric viruses on fresh produce in South Africa [Poster] IUFoST 2010 15th World Congress of Food Science & Technology 22-26 August 2010: Cape Town International Convention Centre, South Africa (*presenter)

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National Presentations

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Netshikweta R*, van Zyl WB, Mans J, Taylor MB. Optimisation and application of real-time RT-PCR techniques for the detection of selected food and waterborne viruses [Poster]. Faculty Day, Faculty of Health Sciences, University of Pretoria 18-19 August 2009: HW Snyman Building, Pretoria (*presenter; *awarded the third prize in the category Junior Researcher: Basic Sciences [Posters]*)

Mans J*, **Netshikweta R,** Magwalivha M, van Zyl WB, Taylor MB. Molecular detection and characterisation of noroviruses occurring in selected surface waters in South Africa [Presentation]. Faculty Day, Faculty of Health Sciences, University of Pretoria 18-19 August 2009: HW Snyman Building, Pretoria (*presenter)

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ABBREVIATIONS AND SYMBOLS

AdV	adenovirus
AstV	astrovirus
bp	base pair
BSA	bovine serum albumin
°C	degree Celsius
CDC	Centers for Diseases Control and Prevention
CEN	European Committee of Standardisation
CPE	cytopathic effect
C_{pro}	cysteine protease
CsCl	calcium chloride
DNA	deoxyribonucleic acid
EIA	enzyme immunoassys
EM	electron microscopy
EOR	efficiency of recovery
EPA	Environmental Protection Agency
g	gram
G	genogroup
GBEB	glycine beef extract buffer
HAV	hepatitis A virus
IC	internal control
ICTV	International Committee on Taxonomy of Viruses
IEM	Immune Electron Microscopy
Ig	immunoglobulin
IRES	internal ribosome entry sites
Kb	kilobase
kDa	kilo-Dalton
M	Molar
mg	milligram
Min(s)	minute(s)
Mr	molecular mass

NaCl	sodium chloride
NCR	noncoding region
nm	nanometre
NoV	norovirus
NTPase	nucleotide triphosphatase
PCR	Polymerase chain reaction
ORF	open reading frame
PBS	phosphate buffered saline
PEG	polyethylene glycol
ppm	parts per million
QA	quality assurance
QC	quality control
RdRp	RNA dependent RNA polymerase
RIA	radio-immunoassays
RNA	ribonucleic acid
rt	real-time
RT-PCR	reverse transcriptase polymerase chain reaction
RV	rotavirus
SA	South Africa
SaV	sapovirus
TGBE	tris-glycine beef extract
Tris	tris (hydromethyl) amino methane
VpG	viral protein-genome linked
US	United States of America
UV	ultra-violet

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CHAPTER 1

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

A variety of agents, namely bacteria, viruses, parasites, toxins, metals and prions can cause food- and waterborne illness (Acheson, 2001; Parashar and Monroe, 2001). Despite the progress seen in recent times in medical care and food technology, food- and waterborne diseases remain an important public health problem in both developing and industrialised countries (Leggitt and Jaykus, 2000; Egli et al., 2002; Widdowson and Vinjé, 2008). Except for the obvious epidemic cases, food and waterborne diseases frequently go unrecognized, and the impact of food and waterborne diseases on human health and economy is underestimated (Egli et al., 2002). It is estimated that foodborne gastroenteritis accounts for 76 million cases and 325,000 hospitalisations per year in the United States of America (US) (Bresee et al., 2002). Food and waterborne outbreaks of microbial and viral origin appear to be both common and underreported (Hedberg and Osterhorm, 1993; Centers for Disease Control and Prevention [CDC], 2010). Factors such as changing lifestyles and demographics, frequent travel, the globalisation of the food industry and dwindling water supplies have contributed to the increase in food- and waterborne infections (Koopmans et al., 2002; Newell et al., 2010). The interaction of water, food and human health is complex, as human health can be affected by the ingestion of contaminated water either directly or indirectly through contaminated food (Helmer, 1999). Several studies have shown that fresh produce can be contaminated with pathogenic microorganisms such as bacteria, fungi, protozoa, and viruses when irrigated with contaminated water (Baez and Coutino, 1984; Steele and Odumeru, 2004; Carter, 2005; Cheong et al., 2009a; Wei and Kniel, 2010). Food sources can also be contaminated during harvesting, processing and preparation or serving

by infected food handlers (Koopmans and Duizer, 2004; Barrabeig et al., 2010, Tuan Zainazor et al., 2010), The likelihood of fresh produce becoming contaminated during irrigation is in turn dependant on the growing location of fresh produce, the nature of the produce surface and the type of irrigation applied (Gerba and Choi, 2006). Therefore, the quality of irrigation water influences the safety of produce, since contaminated water can either contaminate or spread contamination within produce. In addition, due to an increase in demand for fresh water sources, river water, and even wastewater, is commonly used for irrigation in countries where water supplies are limited (Hamilton et al., 2006).

Although viruses were suspected to be the cause of outbreaks of nonbacterial foodborne illness for more than 75 years, very little is known or found in the historical records about viral foodborne illness (Atmar and Estes 2006). Food was first recognized as a vehicle of enteric virus infection in 1914 after an outbreak of poliomyelitis associated with raw milk (Gerba, 2006). In 1929 Zahorsky proposed the name “winter vomiting disease” to describe an epidemic of nonbacterial gastroenteritis, because of the increase seen during the winter months (Koopmans et al, 2002; Atmar and Estes, 2006). Although a specific viral agent could not be isolated *in vitro,* foodborne outbreaks of nonbacterial gastroenteritis were recognised. The first recognition of hepatitis A virus (HAV) to be transmitted via shellfish was in the mid 1950s in Sweden, and subsequently in the US (Gerba, 2006).

Waterborne diseases have plagued mankind for a long time. The first description of attempts to treat drinking water are generally attributed to Greek and Sankrit writings dating back to 4000 BC (Barry and Hughes, 2008). Among the scientists to first believe in water treatment was Hippocrates who invented the bag filter (or Hippocratic sleeve) in an attempt to make water more healthy for the human body (Barry and Hughes, 2008). Enteric viruses were first isolated from water in the early 1930s and 1940s and focused on poliovirus and other enteric virus in faeces and wastewater (Rzeżutka and

Cook, 2004). The viruses were recovered from water by using a gauze pad and identified using cell culture methods (Rzeżutka and Cook, 2004). More than 150 years have passed since the acceptance of the healthful effects of clean water, but 1.1 billion people still lack access to clean water and 2.6 billion people lack access to adequate sanitation globally (Grabow, 2007; Barry and Hughes, 2008).

Outbreaks and sporadic occurrences of food- and waterborne virus infections are documented throughout the world (Carter, 2005). It has been estimated that in the US 67% of foodborne illness was caused by viruses and the rest due to bacteria (30%) and parasites (3%) (Carter, 2005). Fresh produce was responsible for the largest number of cases of illness and seafood for the highest number of outbreaks. Although the reported numbers of food and waterborne viral diseases are considerable and increasingly recognized, based on the present data, the global burden of food and waterborne viral disease is currently unknown (Kasowski et al., 2002; Koopmans et al., 2002; Leclerc et al., 2002; Grabow, 2007; Bosch et al., 2008; Newell et al., 2010). In addition, the number of reports indicate that the diseases are far more prevalent than reported outbreaks (Leclerc et al., 2002). This is due to lack of data on environmental epidemiology, technical limitations in pathogen identification, difficulties in determining the source of infection and occurrence of unappreciated infection (Bosch et al., 2008). Person-to-person and food- and waterborne routes of transmission may overlap which further impacts on the estimation of food and waterborne illness (Koopmans and Duizer, 2004). Data from 10,000 outbreaks in different countries indicated that 10% were foodborne, 2% were waterborne and the rest due to person-to-person transmission (Newell et al., 2010). Moreover, factors such as susceptibility to infection by the malnourished and the immunocompromised, and the influence of geographic location, socio-economic circumstances and seasonality in a global warming situation further complicates the overall picture (Bosch et al., 2008).

While outbreaks of viral foodborne disease remain important in public health, their aetiology is not identified in the majority of cases (Parashar and Monroe, 2001). Considering the low infectious dose of enteric viruses, contaminated fresh produce may constitute a risk to consumers (Newell et al., 2010). To ensure human health safety, rapid detection methods for pathogens in food and water is essential (Fratamico and Bayles, 2005; Mattison and Bidawid, 2009). Even though the health impact of contaminated food and waterborne viral diseases is well recognized, tests for most of these viruses remain restricted to selected laboratories due to technical difficulties. Traditionally, the detection of viruses has been done in cell culture (Fratamico and Bayles, 2005), which poses numerous problems as the sensitivity is low, the method is tedious, labour- and cost-intensive (Gilgen et al., 1997; Meleg et al., 2006; Cliver, 2008). More importantly, no appropriate cell culture host exists for the epidemiologically important viruses such as HAV and the norovirus (NoV) (Leggitt and Jaykus, 2000; Pintó and Bosch, 2008; Rodríguez et al., 2009).

Recent advances in the development of molecular-based assays and nano-technology have facilitated the sensitive detection of a number of pathogens, including food- and waterborne viruses (Egli et al., 2002; Atmar, 2006; Wyn-Jones, 2007; Rodríguez et al., 2009). Molecular techniques such as the polymerase chain reaction (PCR) and reverse transcriptase (RT)-PCR offer the best alternative sensitive and specific methods for the detection of enteric viruses from environmental samples (Meleg et al., 2006). Using these assays even non-culturable species can be detected (Egli et al., 2006). Unfortunately, these assays are often in nested format, which pose a contamination hazard for the carryover of amplification products. In addition, they still require time-consuming sample handling and post-PCR handling (Rutjes et al., 2005). The inception of real-time (*rt*) PCR/RT-PCR assays has facilitated the quantification of viruses, which is essential in estimating the public health risk constituted by low levels of viruses in environmental samples (Rutjes et al., 2005). Real-time RT-PCR is frequently used for the detection of viruses in clinical samples, but has less frequently been applied for environmental

samples (Rutjes et al., 2005). This assay does not require post-PCR handling, and therefore can be implemented in routine viral detection in environmental samples (Nijhuis et al., 2002, Fuhrman et al., 2005). In addition, the molecular characterisation of enteric viruses detected in food and water sources and clinical specimens provides an important epidemiological tool for source tracking in the event of a foodborne outbreaks (Lappalainen et al., 2001; Koopmans et al., 2002; Verhoef et al., 2010). Despite the unlimited potential application of molecular techniques or PCR, many of the assays still need improvement with respect to robustness, reliability and quality control before they can be applied for routine analysis (Egli et al., 2002; Mattison and Bidawid, 2009; Bosch et al., 2011).

1.2 FOOD AND WATERBORNE VIRUSES

1.2.1 Introduction

More than 100 human enteric viruses, including NoV, astrovirus (AstV), rotavirus (RV), adenoviruses (AdVs), enteroviruses and HAV may be present in the gastrointestinal tract (Wyn-Jones and Sellwood, 2001; Koopmans and Duizer, 2004; Carter, 2005; Grabow, 2007). These viruses, which belong primarily to the families Adenoviridae, Astroviridae, Caliciviridae, Picornaviridae and Reoviridae (Griffin et al., 2003; Carter, 2005; Newell et al., 2010), may infect persons by ingestion after which they are shed in high numbers in the faeces (Koopmans and Duizer, 2004; Carter, 2005; Schwab, 2007). These enteric viruses are associated with a variety of diseases in humans, from ocular and respiratory infections to gastroenteritis, hepatitis, poliomyelitis, myocarditis, and aseptic meningitis (Griffin et al., 2003; Koopmans and Duizer, 2004). Noroviruses and HAV are associated with gastroenteritis and hepatitis, respectively (Koopmans et al., 2002; Koopmans and Duizer, 2004).

Noroviruses and HAV are the most commonly detected viruses associated with food- and waterborne diseases (Table 1.1) (Kasowski et al., 2002, Koopmans et al., 2002, Koopmans and Duizer, 2004; Hedberg et al., 2006; Bosch et al., 2011). In the US, 67% of foodborne illness was attributed to viruses, with 40% of foodborne viral infections attributable to NoVs, 5% to HAV, 1% to AstVs, 1% to RVs (Carter, 2005).

Table 1.1: Likelihood of food or waterborne transmission of enteric viruses according to the type of illness associated with infection. (adapted from Koopmans and Duizer, 2004)

Likelihood of water- and foodborne transmission	**Gastroenteritis**	**Hepatitis**	**Other**
Common	Norovirus	Hepatitis A virus	
Uncommon	Enteric adenovirus Rotavirus Sapovirus Astrovirus Coronavirus Aichivirus	Hepatitis E virus	Enterovirus

These viruses are excreted in high titres in faeces of infected individuals, resulting in their accumulation in community sewage which may contaminate drinking water sources, recreational and natural waters (lakes and rivers) (Meleg et al., 2006), which may in turn contaminate food sources (Steele and Odumeru, 2004; Berger et al., 2010; Newell et al., 2010; Okoh et al., 2010; Wei and Kniel, 2010). These enteric viruses are able to persist in the environment due to their extreme resistance to unfavorable conditions (heat, disinfection and pH changes) (Koopmans et al., 2002; Carter, 2005) and water quality and food safety, and therefore human health, may be significantly

affected by their presence in contaminated water (Wyn-Jones and Sellwood, 2001) and food (Koopmans and Duizer, 2004; Newell et al., 2010).

1.2.2 Norovirus

1.2.2.1 Virology

1.2.2.1.1 Viral morphology

While morphologically typical caliciviruses were first recognized in stool samples by Madeley and Cosgrove (Madeley and Cosgrove, 1975, Madeley et al., 1977; Atmar and Estes, 2001), NoVs were first described in 1972 (Kapikian et al., 1972). The virion is 27-40 nm in diameter, nonenveloped with icosahedral symmetry (Koopmans et al., 2005, Martínez et al., 2006). The name calicivirus is derived from the Latin *calyx*, meaning cup or goblet, and refers to the cup-shaped depressions on the surface of the particle (Figure 1.1) (Richards, 2005).

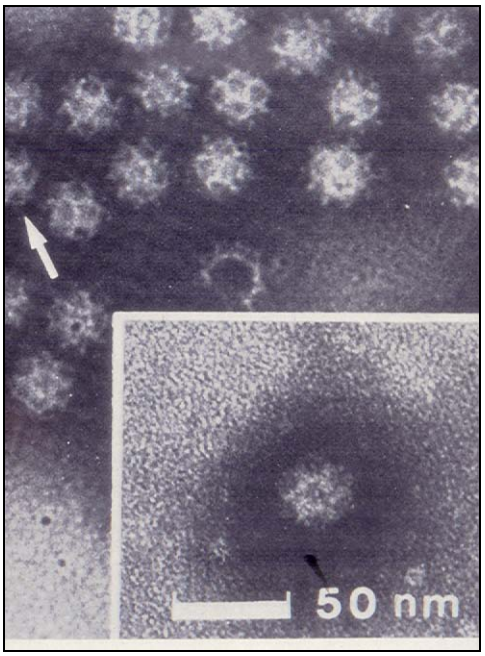

Figure 1.1: Electron micrograph of calicivirus showing cup-shaped depressions calyces (source unknown)

The major structural protein folds into 90 dimers that form a shell domain from which arch-like capsomers protrude (Atmar and Estes, 2001) with the characteristic feature of 32 cup-shaped depressions at each of the icosahedral five fold and three fold axes (Atmar and Estes, 2001). In some negatively stained virus preparations, the cup-shaped depressions appear distinct and well defined, particularly in sapoviruses [SaVs], leading to the characteristic "Star of David" appearance (Atmar and Estes, 2001). In some instances the cup-shaped depressions of the virion are less well-defined with an amorphous structure and a ragged outer edge hence the name "small round structured viruses" which was previously used to describe NoVs (Clark and Lambden, 2001; Desselberger and Gray, 2003)

1.2.2.1.2 Genomic organisation

The NoV genome is a single-stranded, positive-sense, poly A tailed RNA genome of approximately 7.5 – 7.7 Kb (Koopmans et al., 2005; Martínez et al., 2006; Atmar, 2010). The genome consists of three open reading frames (ORF) 1, 2, and 3 (Scipioni et al., 2008)(Figure 1.2).

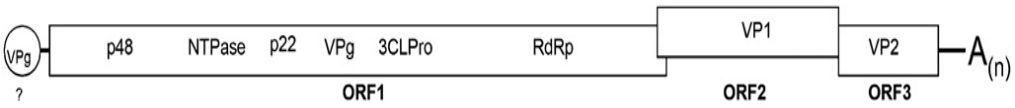

Figure 1.2: Genomic organisation of norovirus (Scipioni et al., 2008).

Open reading frame 1 at the 5' end, the largest, encodes a polyprotein precursor for several non-structural proteins that includes nucleotide triphosphatase (NTPase), proteinase and RNA dependent RNA polymerase (RdRp). Open reading frame 2 encodes the major capsid protein (VP1), and ORF3, which is located at the 3' end of the genome is the smallest, encoding a minor structural protein (VP2) ~20 kDa, and is involved in expression and stability of VP1 capsid protein (Scipioni et al., 2008). The region from the C-terminal end of ORF1 to the N-terminal of ORF2 is the most highly conserved region of NoV strains (Scipioni et al., 2008).

1.2.2.1.3 Biochemical and physical characteristics

The physiochemical properties have not been fully established for all members of the family (Koopmans et al., 2005). Noroviruses have a molecular mass (Mr) of ~15 x 10^6, a buoyant density of 1.33-1.41 g/cm^3 and sedimentation value of S_{20W} 170-187 *S* (Atmar and Estes, 2001; Koopmans et al., 2005; Green, 2007). Norovirus is resistant to low pH (pH 2.7 for 3 hours at room temperature) and heat treatment (30 min at 60 ºC) (Green, 2007). The virus can still remain infectious after 30 min in the presence of 0.5-1 mg of free chlorine per liter, meaning that the virus is relatively resistant to chlorine. The virus can be inactivated at higher concentrations of >2 mg free chlorine per liter (Koopmans and Duizer, 2004). Norovirus is more resistant to chlorine inactivation than poliovirus and simian RV (Green, 2007).

1.2.2.1.4 Classification

Noroviruses, previously known as Norwalk-like viruses, belong to the family Caliciviridae (Koopmans et al., 2005; Zheng et al., 2006; Green, 2007; Said et al., 2008). Before the cloning of the viral genome, the classification of caliciviruses was based on the morphology and structure of the viral capsid (Moreno-Espinosa et al., 2004). The current classification and nomenclature of the caliciviruses was proposed by the International Committee on Taxonomy of Viruses (ICTV) in 1998 and it has been further updated by Zheng and co-workers (Zheng et al., 2006; Scipioni et al., 2008). There are four genera within the family Caliciviridae: Norovirus and SaV which are associated with human infections, and Lagovirus and Vesivirus, which cause veterinary infections (Chiba et al., 2000, Moreno-Espinosa et al., 2004, Koopmans et al., 2005; Martínez et al., 2006, Zheng et al., 2006; Scipioni et al., 2008). An additional three genera have been proposed for inclusion within the family Caliciviridae: i) Becovirus or Nabovirus, to accommodate a unique bovine enteric calicivirus (Farkas et al., 2008; Scipioni et al., 2008) (Figure 1.1), ii) Valovirus to accommodate the St-Valérien-like viruses isolated from pigs (L'Homme et al., 2009), and iii) Recovirus for the novel calicivirus which was isolated from the stools of rhesus macaques (Farkas et al., 2008).

Based on sequence analysis of the complete capsid gene (VP1), NoVs are classified into 5 genogroups (G), GI-GV (Figure 1.3), and genogroups are further divided into genotypes or clusters (Zheng et al., 2006; Scipioni et al., 2008). Norovirus GI, GII, and GIV primarily infect humans (Green, 2007; Scipioni et al., 2008; Atmar, 2010). Norovirus GII includes both human and porcine strains, GIII and GV include bovine and murine strains with the lion, feline and canine NoVs in GIV (Scipioni et al., 2008; Atmar 2010).

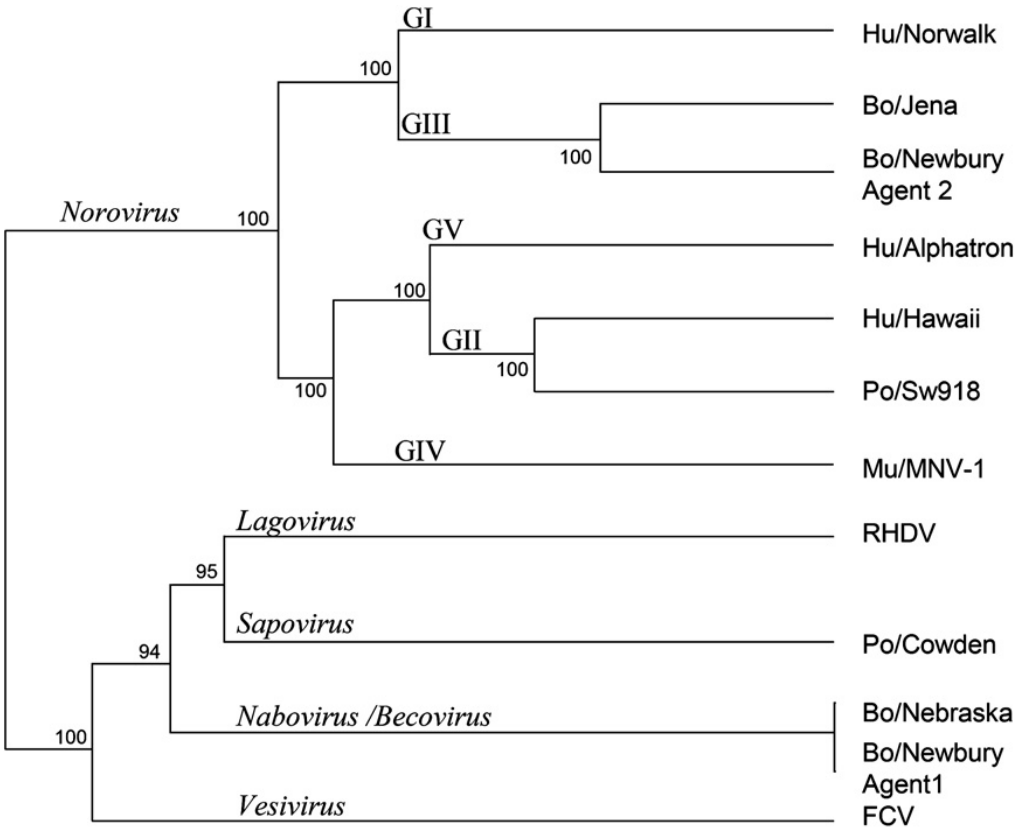

Figure 1.3: Phylogenetic tree of Caliciviridae (Scipioni et al., 2008).

Norovirus GI, GII, and GIII have been further divided into 8, 17, and 2 genotypes, respectively, whereas GIV and GV have two and one genotypes, respectively (Zheng et al., 2006; Scipioni et al., 2008; Atmar, 2010). Despite this diversity, in recent years only a few strains, primarily those of GII

genotype 4 (GII.4), are responsible for a majority of the cases of NoV- related foodborne outbreaks (Newell et al., 2010).

1.2.2.2 Pathogenesis

Norovirus is transmitted through the faecal oral-route. Since it is acid stable, it passes through the stomach and their replication is thought to occurring in the small intestine (Lopman et al., 2002). Though little is known about the mechanism by which NoV causes diarrhoea, lesions on the small intestinal mucosa were noted in a study using a healthy volunteers infected with virus (Lopman et al., 2002). The mucosal lining becomes inflamed and absorptive epithelial cells develop an abnormal appearance (Lopman et al., 2002). Blunting of the villi, shortening of the microvilli, dilation of the endoplasmic reticulum, swelling of the mitochondria, and intracellular oedema are microscopically also observed (Lopman et al., 2002; Glass et al., 2009). The small intestine returned back to normal histological appearance within two weeks in these healthy volunteers (Lopman et al., 2002).

1.2.2.3 Clinical features

Norovirus is a major cause of nonbacterial acute gastroenteritis, affects all age groups (Green, 2007; Patel et al., 2009) and is the most common cause of diarrhoea in adults and the second most common cause in children (Glass et al., 2009). The clinical symptoms of NoV associated acute gastroenteritis have been described based on the volunteer studies and description of outbreaks of acute gastroenteritis (Moreno-Espinosa et al., 2004). After exposure to the NoV, the incubation period is usually 24 to 48 hours (Moreno-Espinosa et al., 2004). Following the incubation period, the infected person may develop nausea, vomiting, non-bloody diarrhoea and abdominal cramps, malaise and low-grade fever (Moreno-Espinosa et al., 2004; Patel et al., 2009). Subclinical infection however, is common (Atmar, 2010). As this condition was first identified as “winter vomiting disease”, patients may experience vomiting alone and has been reported more frequently with GII.4 strains (Atmar, 2010). The symptoms usually last 3 to 4 days but can be longer (4-6

days) in children <11 years of age in hospitalised outbreaks (Patel et al., 2009). In healthy individuals virus can be shed in low titres up to 8 weeks after infection and for more than a year in immunocompromised individuals (Glass et al., 2009). Norovirus diarrhoea sometimes requires parenteral fluid therapy or hospitalisation (Koopmans et al 2002; Glass et al., 2009). Severe dehydration by NoV may still occur, and in rare cases this can also be fatal (CDC, 2007).

1.2.2.4 Laboratory diagnosis

Prior to the development of molecular-based assays and the cloning of NoV the laboratory diagnosis was dependent on electron microscopy (EM) for the detection of the viruses (Lopman et al., 2002; Glass et al., 2009; Atmar, 2010). Electron microscopy has a relative low sensitivity, requiring the presence of a minimum of ~10^5-10^6 particles per ml of stool sample, a skilled electron microscopist and expensive equipment (Parashar and Monroe, 2001; Richards, 2005). Unfortunately some enteric viruses, including NoV, are not shed to a very high maximum titre and detection is dependent upon collection of a stool specimen 2-3 days after the onset of illness (Koopmans et al., 2002; Schmid et al., 2004). Electron microscopy can therefore not be used for the detection of lower concentration of virus particles present in contaminated food, water and environmental samples (Seymour and Appleton, 2001). Despite numerous attempts by several groups of investigators, NoVs have never been isolated in cell culture (Koopmans et al., 2002; Widdowson and Vinjé, 2008; Bosch et al., 2011).

In the 1980s and 1990s numerous immunological-based methods such as immune EM (IEM), radio-immunoassays (RIA), enzyme immunoassays (EIA) and western blot were developed for diagnosis of NoV (Moreno-Espinosa et al., 2004). For these assays the reagents were from previously infected individuals. The source of virus antigen was stool of acutely infected persons, and the convalescent sera obtained from infected persons was used as hyperimmune sera. The problem was that immune responses are

predominately type-specific, and that assays using these reagents had limited applicability (Atmar and Estes, 2001). These restrictions limited the general availability of this diagnostic assays (Atmar and Estes, 2001). More recently commercial immunochromatographic and EIAs were developed for the detection of NoV antigen in stool samples (Burton-Macleod et al., 2004; Okitsu-Negishi et al., 2006; Atmar, 2010). These assays have been shown to be effective for the detection of selected NoV GI and NoV GII genotypes in stool specimens (Okitsu-Negishi et al., 2006; Khamrin et al., 2008), and false negative results were noted using the NoV-immunochromatography assay (Khamrin et al., 2008). This latter observation was ascribed to the fact that the detection limit of the NoV-immunochromatography assay was 10^7 and 10^8 copies of NoV GII.4 and NoV GII.3, respectively (Khamrin et al., 2008).

In the early 1990s, a breakthrough occurred with the cloning and sequencing of Norwalk virus and Southampton virus. This knowledge of the genomic organization of NoV allowed the development of sensitive molecular-based detection and characterisation methods based on RT-PCR amplification and nucleotide sequencing of the RT-PCR amplicons (Atmar and Estes, 2001; Patel et al., 2009; Atmar, 2010). Molecular-based assays are considered to be the "gold-standard" for the routine diagnosis of NoVs (Rolfe et al., 2007), and currently the most common and widely used assay for the diagnosis of NoV infection is RT-PCR (Atmar and Estes, 2006; Patel et al., 2009). More sensitive qualitative and quantitative *rt* RT-PCR singleplex and multiplex assays have also been developed and applied for the detection of NoVs in large numbers of stool samples (Ludwig et al., 2008; Patel et al., 2009; Stals et al., 2009) and sera (Takanashi et al., 2009) This technology has facilitated investigations regarding the genetic relatedness of viral strains (Malasao et al., 2008; Mladenova et al., 2008; Xu et al., 2009; Mans et al., 2010) and the identification of the potential source of infections (Patel et al., 2009). Microarray techniques for the detection of NoVs have also been developed but with the disadvantage of having to continually having to update the detection primers (Jääskeläinen and Maunula, 2006).

1.2.2.5 Epidemiology

Noroviruses are transmitted primarily via the faecal-oral route (Patel et al., 2009; Atmar, 2010), either directly from person-to-person or indirectly via contaminated water and food (Atmar and Estes, 2006; Souza et al., 2006). Transmission via vomitus, either by aerosol transmission or contact with contaminated surfaces, is thought to be responsible for outbreaks in closed settings such as hotels, hospitals, child-care centres etc. (Patel et al., 2009). Recreational exposure via faecally polluted water while canoeing, rafting, or swimming, or through direct contact with ill participants during other recreational activities can result in infection (Atmar and Estes, 2006). Infected handlers are play important role in the transmission of NoVs from ready-to-eat food (Souza et al., 2006). Noroviruses are highly contagious, with a low infectious dose (Atmar and Estes, 2006; Teunis et al., 2008) and high attack (>30%) and secondary attack rates (Atmar and Estes, 2006; Atmar, 2010). The multiple overlapping routes of infection makes it difficult to identify the source and route of infection thus hindering prevention and control measures (Atmar and Estes, 2006).

Norovirus infection occurs worldwide throughout the year with a higher incidence in winter, giving rise to its former name “winter vomiting disease” (Koopmans et al., 2002, Carter, 2005; Patel et al., 2009; Atmar, 2010). Between 1995 and 2000 more than 85% of the nonbacterial gastroenteritis outbreaks in Europe were due to NoV (Baert et al., 2009). Noroviruses are the most important cause nonbacterial acute gastroenteritis and are reportedly responsible for 47-96% of outbreaks and 5-36% of sporadic cases of acute gastroenteritis (Atmar, 2010). All age groups are affected, and after RVs, NoVs are the second most common cause of gastroenteritis in hospitalised paediatric patients (Matson and Szücs, 2003; Moreno-Espinosa et al., 2004; Atmar and Estes, 2006; Koopmans, 2008; Atmar, 2010). Immunity to NoV appears to be short-lived (Glass et al., 2009; Patel et al., 2009). The occurrence of NoVs in South Africa (SA) was first reported in 1993 when two outbreaks of acute gastroenteritis were associated with Norwalk (NoV GI.1)

and Hawaii (NoV GII.1) viruses, respectively (Taylor et al., 1993). In 1995, Mexico virus (NoV GII.3) was identified as a cause of sporadic gastroenteritis in paediatric patients in the Pretoria area (Wolfaardt et al., 1995a, 1997). In the same region (Pretoria), seroprevalence studies showed that 57% of children were exposed to NoVs by two years of age and by 40 years of age 67% of the population had been exposed to NoVs (Taylor et al., 1996). Two years later a NoV antibody prevalence of 94-96% was reported for urban and rural South Africans (Smit at al., 1999). Ten years later Mans et al. (2010) reported a NoV prevalence of ~14% in hospitalised paediatric patients with gastroenteritis in the Pretoria region, with NoV GII.4 being the most frequently detected strain.

Worldwide many different types of NoV are circulating in the general population (Koopmans and Duizer, 2004), with individual strains emerging and becoming more prominent (Blanton et al., 2006; Glass et al., 2009). For the last 15 years NoV GII infections have been more common than NoV GI infections (Koopmans, 2008; Patel et al., 2009; Atmar, 2010). In addition NoV GI has been more commonly associated with traveller's diarrhoea and shellfish-associated disease while NoV GII is associated with sporadic cases and outbreaks of gastroenteritis (Atmar and Estes, 2009). In recent years (1995-1996 and 2003-2003), NoV GII.4 strains and variants are reportedly the epidemic strains in the US, Ireland, England, The Netherlands, Germany, Japan, New Zealand and Australia (Lopman et al., 2004; Blanton et al., 2006). Co-infections or mixed infections with more than one NoV strain can occur (Kageyama et al., 2004) which facilitates recombination and the emergence of new virus strains (Atmar and Estes, 2006)

Understanding the mechanism of transmission, survivability and pathogenic potential of these viruses can assist greatly in developing the guidelines for the prevention and control of the spread of this virus (Thornton et al., 2004). Control depends on hygienic measures, particularly those aimed at reducing the spread by the faecal-oral route. These include proper management of food,

water supplies, sewage, and individual measures such as hand washing and correct disposal of contaminated material (Moreno-Espinosa et al., 2004; Green, 2007).

1.2.3 Hepatitis A virus

Hepatitis viruses can be divided into enterically transmitted viruses (HAV and hepatitis E virus), and parenterally transmitted hepatitis viruses (hepatitis B, C and D viruses) (Koopmans et al., 2002; Kumar et al., 2010). Although the aetiological agent was identified only in 1972, epidemic hepatitis has been recognised since ancient times (Cuthbert, 2001; Carter, 2005; Hollinger and Emerson, 2007; Dotzauer, 2008; Pintó et al., 2010).

1.2.3.1 Virology

1.2.3.1.1 Viral morphology

Hepatitis A virus is a small, 27-28nm in diameter, non-enveloped single-stranded RNA virus with icosahedral symmetry (Stanway et al., 2005; Nainan et al., 2006; Dotzauer, 2008).

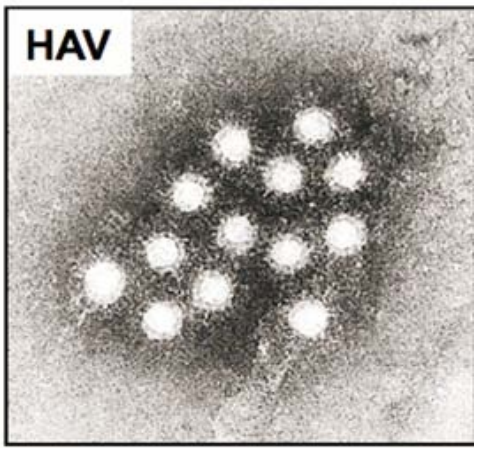

Figure 1.4: Transmission electron micrograph of hepatitis A virus particles aggregated by antibodies (Kumar et al., 2010).

The capsid contains 60 copies each of the major proteins VP1 (1D), VP2 (1B) and VP3 (1C) (Cuthbert, 2001; Dotzauer, 2008). The HAV capsid structure

differs from that of other picornaviruses as: i) the canyon surrounding the 5-fold symmetry axes is missing (Dotzauer, 2008), ii) the VP4 (1A) protein is small and appears not to be myristylated at the N-terminus (Stanway et al., 2005), and iii) although essential for virion formation (Cuthbert, 2001), the VP4 (1A) protein appears not to be part of the mature virion (Cuthbert, 2001; Stanway et al., 2005).

1.2.3.1.2 Genomic organisation

Hepatitis A virus has a positive-sense, single-stranded RNA genome 7.5 kb in length and the organisation of the genome is similar to that of other picornaviruses (Cuthbert, 2001). The genome consists of a 5' noncoding region (NCR) of ~740 nucleotides followed by a coding region of ~2,225 nucleotides, and a 3' NCR of ~60 nucleotides with a poly(A) tail (Cuthbert, 2001; Dotzauer, 2008; Kumar et al., 2010) (Figure 1.5).

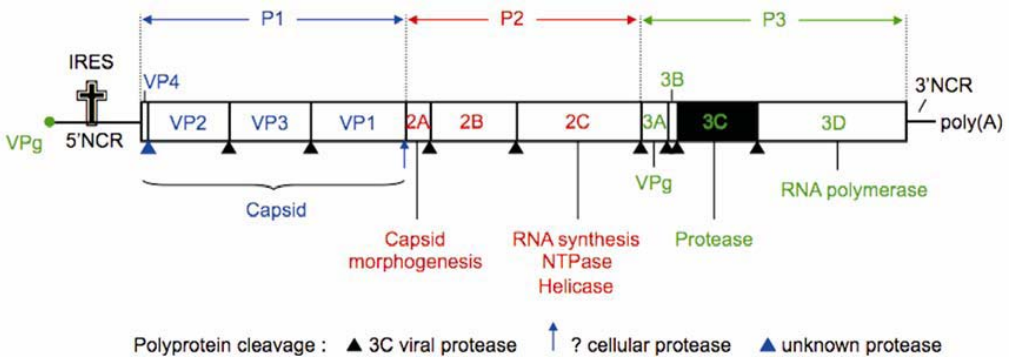

Figure 1.5: Diagram of the hepatitis A virus genome with coding regions in blocks (Kumar et al., 2010).

The genome contains a single large open ORF encoding a polyprotein for the major capsid proteins (VP1, VP2, VP3), and putative VP4 at the amino N-terminal third which is also known as the P1 or 2A region (Martin and Lemon, 2006; Dotzauer, 2008; Song et al., 2009). The remainder of the polyprotein comprises a series of non-structural proteins required for HAV RNA replication: 2B, 2C (segment P2), 3A, 3B (a small protein, also known as VPg, that is covalently linked to the 5´end of genomic RNA and that probably serve

as the protein primer for RNA synthesis), $3C_{pro}$ (a cysteine protease responsible for most post-translational cleavage events within the polyprotein), and $3D_{pol}$ (the viral RNA-dependent, RNA polymerase) (segment P3) (Martin and Lemon, 2006). The 5' NCR contains an extensive secondary structure, an internal ribosome entry site (IRES), required for cap-independent translation of polyprotein, and covalently linked to viral protein VPg (Nainan et al., 2006; Hollinger and Emerson, 2007).

1.2.3.1.3 Biochemical and physical characteristics

Like other picornaviruses, HAV has a Mr of 8 x 10^6, a buoyant density of 1.33-1.45g/cm^3 in CsCl (Stanway et al., 2005) and sedimentation coefficient of 160 *S* (Dotzauer, 2008). Hepatitis A virus is stable, being resistant to low pH (up to pH 1 for 2 hours at room temperature) and heating (60 ºC for 1 hour) (Koopmans et al., 2002; Stanway et al., 2005; Dotzauer, 2008). The virus is resistant to chemical agents such as 20% ether and chloroform and can survive for years at -20°C or lower (Hollinger and Emerson, 2007) and can be inactivated by autoclaving, ultra-violet (UV) irradiation and formalin (Hollinger and Emerson, 2007). Hepatitis A virus, especially when it is associated with organic material, appears to be resistant to free chlorine (Koopmans et al., 2002). The virus can be inactivated by a 30 minute exposure to 10-15 parts per million (ppm) residual chlorine, 15 min exposure to 2.0-2.5 mg/ℓ free residual chlorine, or 3-5 min exposure at 20°C to chlorine containing compounds, e.g. sodium hypochlorite, at a 3-10 mg/ℓ concentration (Hollinger and Emerson, 2007). Surfaces can be disinfected with a 1:100 solution of sodium hypochlorite (Koopmans et al., 2002) and quaternary ammonium compounds (Spradling et al., 2009). Hepatitis A virus can also be inactivated by appropriate concentrations of glutaraldehyde (2%), iodine (3mg/ℓ for 5 minutes) and potassium permanganate (30 mg/ℓ for 5 min)(Spradling et al., 2009).

1.2.3.1.4 Classification

Hepatitis A virus is classified within the family Picornaviridae, which includes the genera Enterovirus, Rhinovirus, Cardiovirus, Aphthovirus, Hepatovirus, Parechovirus, Erbovirus, Kobuvirus and Teschovirus (Stanway et al., 2005). Due to their similarity to enteroviruses, HAV was initially classified in the genus Enterovirus as enterovirus type 72 (Richards, 2005; Hollinger and Emerson, 2007). Due to the unique properties of HAV, namely their genetic structure, replication procedure, heat and acid stability, and behaviour in cell culture, HAV was re-classified as the type species of the genus Hepatovirus (Carter, 2005; Richards, 2005; Spradling et al., 2009; Kumar et al., 2010). Despite genetic heterogeneity at the nucleotide level, immunological evidence has demonstrated the existence of a single serotype of HAV (Nainan et al., 2006, Hollinger and Emerson, 2007; Song et al., 2009) with two phylogenetically distinct biotypes (Stanway et al., 2005). Based on the analysis of a 168 base pair (bp) region of the VP1/P2A junction, HAVs are further divided into six genotypes, with genotypes I, II and III found in humans, while genotypes IV, V and VI are of simian origin (Song et al., 2009; Pintó et al., 2010). Genotypes I, II and III contain subgenotypes (IA, IB, IIA, IIB, IIIA, and IIIB) (Song et al., 2009; Pintó et al., 2010). Genotype I is the most common human type worldwide, particularly IA, with genotype III representing the majority of the remaining human strains (Hollinger and Emerson, 2007; Pintó et al., 2010). Genotypes IIA and IIB have been rarely reported (Jothikumar et al., 2005; Sánchez et al., 2007). Clusters of strains within genotypes and subgenotypes dominate in certain geographical regions (Robertson et al., 1992; Hollinger and Emerson, 2007)

1.2.3.2 Pathogenesis

The pathogenesis of HAV is not clearly understood (Koopmans et al., 2002). Once HAV is acquired, either by direct contact with a HAV-infected person or by the ingestion of contaminated food or water, HAV enters, and presumably replicates, in the small intestine (Spradling et al., 2009; Kumar et al., 2010). Primary replication has also been detected in the oropharynx (Spradling et al.,

2009). Primary replication is followed by a viremic stage whereby HAV is transported to the liver with further replication in the hepatocytes (Cuthbert, 2001; Koopmans et al 2002; Kumar et al., 2010). The virus is then shed into the bile canaliculi from where it passes back into the intestinal tract (Dotzauer, 2008) with shedding in the faeces in high titres (Figure 1.6) (Stanway et al., 2005; Spradling et al., 2009; Pintó et al., 2010). Hepatitis A virus is not cytolytic and hepatic damage is immune-mediated (Stanway et al., 2005; Hollinger and Emerson, 2007; Spradling et al., 2009). The concentration of HAV in the stool is maximal, and the peak of infectivity is the two weeks prior to the onset of jaundice (Richards, 2005; Spradling et al., 2009). Children can shed the virus for longer periods (up to 10 weeks) than adults (Spradling et al., 2009).

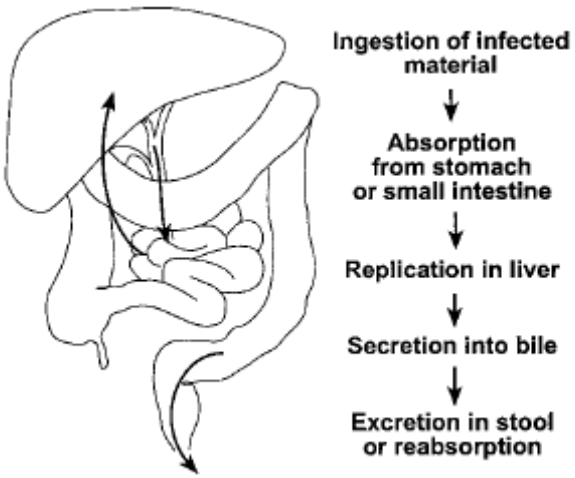

Figure 1.6: Possible ‘enterohepatic cycle” of hepatitis A virus (Cuthbert, 2001)

1.2.3.3 Clinical features

After a median incubation period of 28-30 days (15-50 days), HAV can manifest from silent infection, asymptomatic or symptomatic infection to fulminant hepatitis (Koopmans et al., 2002; Dotzauer, 2008). The likelihood of developing symptomic HAV infection is highly dependant on age. Hepatitis A in children is mostly an asymptomatic disease, while adolescents

and adults usually show symptoms of clinical hepatitis (Koopmans et al 2002, Fiore, 2004; Pintó et al., 2010). In children younger than 6 years, less than 10% with HAV infection have jaundice, while 76-97% of young adults have symptoms and 40-70% are jaundiced (Fiore, 2004).

After infection the prodromal phase is characterised by the abrupt onset of non-specific systemic symptoms such as low-grade fever, malaise, nausea, myalgias, arthralgias, loss of appetite, headache, fatigue and vomiting (Dotzauer, 2008; Kumar et al., 2010). Early specific symptoms of hepatitis include dark urine, light coloured stools, upper quadrant pain and jaundice (Koopmans et al 2002, Spradling et al., 2009; Kumar et al., 2010). Raised levels of alanine aminotransferase and aspartate aminotransferase persist for 2-3 weeks in children and up to 4 weeks in young adults (Spradling et al., 2009). Individuals with HAV infection usually recover completely within 2 to 6 months (Yuan, 1995). Although there is no evidence of chronic liver disease or persistent infection, prolonged hepatitis A may occur in 15-20% of patients and last for up to 6 months (Nainan et al., 2006; Dotzauer, 2008). In general the case-fatality rate is low (0.3%), but increases with age to 1.8% among persons aged≥ 50 years (Fiore, 2004; Dotzauer, 2008). Individuals with underlying chronic liver disease have an increased risk of death (Fiore, 2004; Dotzauer, 2008). Reported complications of HAV infection are cholestatic hepatitis A and relapsing hepatitis A (Cuthbert, 2001; Richards, 2005; Spradling et al., 2009). Extrahepatic manifestations of HAV infection, e.g. transient rash and pancreatitis (Hollinger and Emerson, 2007), are rare (Dotzauer, 2008).

1.2.3.4 Laboratory diagnosis

On the basis of clinical symptoms hepatitis A can not be distinguished from other types of viral hepatitis (Koopmans et al., 2002; Hollinger and Emerson, 2007; Dotzauer, 2008) and diagnosis is dependent on virus specific serological or nucleic acid amplification techniques (Dotzauer, 2008). The specific detection of HAV associated with acute hepatitis was first accomplished by

Feinstone and colleagues in 1973 who visualised virus-like particles using IEM on faecal extracts (Cuthbert, 2001).

The diagnosis of acute hepatitis A infection is made by the detection of immunoglobulin M (IgM) antibody to the capsid of HAV (anti-HAV IgM) in the serum of patients, and is used as the primary marker of acute HAV infection (Cuthbert, 2001; Koopmans et al., 2002, Nainan et al., 2006; Dotzauer, 2008). In most people with acute HAV infection anti-HAV IgM becomes detectable 5-10 days after the onset of symptoms and persist for up to 3-6 months (Koopmans et al., 2002; Hollinger and Emerson, 2007). A number of methods are available detecting virus-specific IgM antibodies, including RIA, EIA, immunoblotting, and dot immunogold filtration (Nainan et al., 2006; Spradling et al., 2009). The specificity and sensitivity of the anti-HAV IgM RIAs found to be 99% and 100%, respectively, with a positive predictive value of 88% (Cuthbert, 2001). Infection with HAV in the past is determined by the detection of immunoglobulin G (IgG) to HAV which confers lifelong immunity (Hollinger and Emerson, 2007; Dotzauer, 2008; Pintó et al., 2010). Commercial diagnostic assays determine total antibodies to HAV (anti-HAV), therefore the presence of total anti-HAV and the absence of anti-HAV IgM can be used to differentiate between past and current infections (Nainan et al., 2006; Hollinger and Emerson, 2007; Spradling et al., 2009). Even though a small proportion (8-20%) of vaccinated persons have a transient anti-HAV IgM response, anti-HAV IgG is produced by all successfully immunised persons (Nainan et al., 2006).

The detection of HAV antigen in stool is another possible option for the diagnosis of HAV infection (Dotzauer, 2008), but although techniques for the detection of HAV antigen in faeces and serum are available, they are not recommended for routine diagnostics (Spradling et al., 2009). Molecular-based nucleic acid detection techniques can be applied to detect HAV in samples of different origin (Spradling et al., 2009), but are also not generally used for diagnostic purposes (Koopmans et al., 2002). Assays such as RT-

PCR and *rt* RT-PCR, which are currently the most sensitive assays and widely used assays to detect HAV RNA, are used in epidemiological studies to identify infection sources and transmission patterns (Hollinger and Emerson, 2007). Nucleic acid sequence analysis of selected genomic regions can be used for the determination of the genetic relatedness of isolates (Hollinger and Emerson, 2007) and tracing the origin of outbreaks (Pintó et al., 2010).

Hepatitis A virus has successfully been propagated in different types of cell cultures of both human and non-human origin, including primary and secondary African green monkey kidney cells and foetal rhesus monkey kidney cells (Nainan et al., 2006). However HAV requires an extensive adaption period, and once adapted, it produces a persistent infection in the infected cell culture with no cytopathic effect (CPE) or apparent cell damage (Nainan et al., 2006). Due to the absence of a CPE, immunological- or molecular-based assays are required to detect HAV antigen or RNA in the infected cell cultures (Nainan et al., 2006). For the quantification of HAV in cell culture the commonly used methods include radio-immunofocus assays, fluorescent focus assays, *in situ* RIA, and *in situ* hybridisation (Nainan et al., 2006). Clinical isolates of HAV grow slowly in cell culture making viral isolation an unreliable and expensive assay which is not practical for diagnostic purposes (Spradling et al., 2009).

1.2.3.5 Epidemiology

The prevalence of hepatitis A varies from one country to another and depends largely on the level of socioeconomic development, and the hygiene and sanitary standards of each geographical area (Alavian, 2005; Nainan et al., 2006). The principal mode of transmission of HAV is person-to-person via the faecal-oral route through close contact with an infected person (Hollinger and Emerson, 2007; Dotzauer, 2008; Spradling et al., 2009). Outbreaks are common in crowded situations such as institutions, schools, prisons and in the military forces where susceptible individuals are housed together which allows common source epidemics to evolve rapidly (Koopmans et al., 2002;

Spradling et al., 2009). Hepatitis A virus is less commonly transmitted through blood transfusion and blood products, the sharing of needles by drug abusers, sexual contact - particularly between male homosexuals, travel from areas of low endemnicity to countries where HAV is endemic (Collier et al., 2006; Nainan et al., 2006; Dotzauer, 2008; Spradling et al., 2009). Contaminated food and water are also a source of HAV transmission (Cuthbert, 2001; Nainan et al., 2006). Outbreaks of HAV associated with food, particularly raw produce, contaminated before reaching the food service establishments are been increasingly recognised (Koopmans et al., 2002).

Three basic epidemiological patterns of hepatitis A are recognised (Cuthbert, 2001, Spradling et al., 2009). Hepatitis A infection is still endemic in densely populated lower socio-economic communities such as those found in much of the developing world. The majority of persons are infected in early childhood (by 10 years of age) and virtually all (90%) adults are immune (Nainan et al., 2006; Spradling et al., 2009). Epidemics are therefore uncommon in areas of high endemicity in Africa, Asia, Central America and Southern America (Koopmans et al., 2002; Alavian, 2005). In areas of intermediate endemicity, in some countries in Asia and Europe, the overall incidence and average age of reported cases often increases because more (50-60%) older individuals are susceptible and develop symptomatic infection (Koopmans et al., 2002; Fiore, 2004; Spradling et al., 2009). Developed areas such as North America and northern Europe are considered to be areas of low endemicity. Since sanitation and hygiene is good in these countries, the rate of infection in children is generally low (Alavian, 2005) resulting in a large number of susceptible of adults (Koopmans et al., 2002; Spradling et al., 2009). A fourth pattern of HAV infection is emerging in areas with improvements in sanitation and access to clean water. An epidemiological shift from intermediate to low prevalence is noted, with a consequent increase in the susceptibility of the older children and adults with a resultant increase in morbidity and outbreak potential (Spradling et al., 2009; Pintó et al., 2010). In SA hepatitis A is endemic with epidemiological features of both developing (high endemicity)

and developed (low endemicity) countries (Venter et al., 2007). In the lower socio-economic, predominately black, communities nearly 100% of the children are immune by the age of 10 years (Martin et al., 1994) while in the higher, predominately white, communities 50-70% of the adults are immune to HAV infection (Venter et al., 2007). A change in the epidemic vulnerability of the SA population can, however, be expected with the current trends in urbanisation (Taylor et al., 2001; Venter et al., 2007).

Effective measures for the prevention and control of HAV are available. Physical prevention can be effected by good personal hygiene and the provision of adequate sanitation and potable water (Dotzauer, 2008). Individual measures such as washing hands and correct disposal of potentially contaminated material also play an important role in the prevention of HAV infection (Koopmans et al., 2002). Immunological protection is available through active or passive immunisation (Spradling et al., 2009). Effective inactivated vaccines are available for active immunisation and the provision of long-term immunity against HAV (Nainan et al., 2006; Dotzauer, 2008; Pintó et al., 2010). Passive immunisation with pooled immunoglobulin, which provides immunity for up to 5 months, is still used for post-exposure prophylaxis (Dotzauer, 2008; Spradling et al., 2009)

1.3 ROLE OF FOOD AND WATER IN THE TRANSMISSION NoVs AND HAV

The transmission of viruses by faecal contaminated food and water is a well recognised public health problem worldwide (Gerba, 2006; Grabow 2007) and foodborne viruses are recognised as a major cause of illness in humans (Richards, 2005; Guévremont et al., 2006). The socio-economic impact and burden of food- and waterborne disease is considerable (Grabow, 2007; Widdowson and Vinjé, 2008). Food or water contaminated with viruses may conform to acceptable bacterial standards (van Heerden et al., 2005a; Verhoef et al., 2009), and look, smell and taste normal (Koopmans and Duizer, 2004).

Unlike bacteria, viruses are strict intracellular parasites and they can not replicate in food and water (Koopmans and Duizer, 2004; Papafragkou et al., 2008). This means that the concentration of virus in contaminated food or water will not increase during processing but rather decrease (Koopmans et al., 2002). Infection via contaminated food or water depends on viral stability, the amount of virus shed by infected persons, the method of processing of water or food, the dose needed to induce infection, and the susceptibility of the host (Koopmans et al., 2002). Since many enteric viruses are extremely infectious, only a small amount of virus is required to infect a human host. Currently, the infectious dose for NoV and HAV is estimated to be around 10-100 infectious viral particles (Brassard et al., 2005; Guévremont et al., 2006).

1.3.1 Water sources

Water plays a major role in the dissemination of enteric viruses and contaminated water is an important source of human infection but the true burden of disease is underestimated due to underreporting (Grabow, 2007; Mena, 2007). Waterborne illnesses can be acquired in a number of ways including: i) the ingestion of or exposure to treated water used for drinking or recreational purposes, ii) the use of untreated water for domestic, recreational or agricultural purposes, and iii) during food processing or other industrial activities (Grabow, 2007). Irrigation water has been identified as a particularly important source of virus contamination and fresh produce-associated outbreaks due to viruses are more often the result of the use of contaminated irrigation water (European Commission, 2002; Koopmans and Duizer, 2004). Contamination can also occur when foods, particularly salad vegetables and fruit, are rinsed in viral-contaminated potable water in the household (Carter, 2005).

Waterborne outbreaks of hepatitis A have been reported (Morse et al., 1972; Bloch et al., 1990; Bosch et al., 1991), but since the introduction of effective drinking water treatment waterborne outbreaks of hepatitis A are less common

(Fiore, 2004; Pintó and Saiz, 2007). In contrast waterborne outbreaks of gastroenteritis cause significant morbidity and mortality worldwide (Schwab, 2007). Since the first confirmed report of a waterborne outbreak NoV gastroenteritis (Beller et al., 1997) NoVs have been identified in outbreaks from drinking water in Finland (Maunula et al., 2005), New Zealand (Hewitt et al., 2007), Belgium (Baert et al., 2009), China (He et al., 2010), The Netherlands (ter Waarbeek et al., 2010) and the US (Parshionikar et al., 2003). Contaminated recreational water was implicated in outbreaks of NoV-associated gastroenteritis in children in The Netherlands (Hoebe et al., 2004) and the US (CDC, 2004).

1.3.2 Food sources

A number of different types of food, including shellfish, cold foods and fresh produce, have been implicated in foodborne outbreaks of viral disease (Fiore, 2004; Goyal, 2006; Richards, 2006; Rutjes et al., 2006; Tuan Zainazor et al., 2010). Soft fruits and vegetables are being increasingly identified as sources of foodborne viral illness (Croci et al., 2008). In addition, consumer trends, such as the consumption of raw vegetables and undercooking to retain the natural taste and to preserve heat-labile nutrients, can increase the risk of foodborne transmission (Carter, 2005; Croci et al., 2008; Tuan Zainazor et al., 2010). Outbreaks of viral origin through consumption of contaminated fresh frozen produce has also been documented (Tuan Zainazor et al., 2010). Food products can be contaminated with enteric viruses at any point during cultivation, harvesting, processing, distribution or preparation (Fiore, 2004; Rutjes et al., 2006; Hollinger and Emerson, 2007; Wei and Kniel, 2010).

The first foodborne outbreak of infectious hepatitis was described in Sweden in 1955 where 629 cases were associated with the consumption of raw oysters (Pintó et al., 2010). The largest outbreak of HAV, affecting almost 3 000 000 individuals, occurred in Shanghai, China in 1988, and was associated with consumption of clams harvested from a sewage-polluted area (Cuthbert, 2001;

Pintó et al., 2010). Shellfish has been implicated in a number of other outbreaks of hepatitis A (Sánchez et al., 2002; Pintó et al., 2010), and both HAV and NoV were detected in clams associated with foodborne illness (Kingsley et al., 2002) In the US outbreaks of hepatitis A have been associated with consumption of green onions (Dentinger et al., 2001; Wheeler et al., 2005), strawberries (Hutin et al., 1999) and lettuce (Rosenblum et al., 1990; Cuthbert, 2001). Epidemiological investigations from outbreaks due to contaminated green onions and strawberries showed that these items required extensive handling during harvesting which may have been the source of contamination (Fiore, 2004). The ingestion of raw blueberries were identified as the cause of an outbreak of hepatitis A in New Zealand (Calder et al., 2003) and raspberries have been linked to outbreaks of hepatitis A (Croci et al., 2008). In addition to shellfish and fresh produce contaminated raw beef has been implicated in an outbreak of hepatitis A (Robesyn et al., 2009). Noroviruses are the most common cause of foodborne illness (Croci et al., 2008) and outbreaks of NoV gastroenteritis, associated with consumption of contaminated food, have been well documented (Tuan Zainazor et al., 2010). Noroviruses have been implicated in 43% of all foodborne outbreaks in England, 67% in Sweden, 80% in The Netherlands and 28% in Japan (Boccia et al., 2002; Tuan Zainazor et al., 2010). Numerous NoV outbreaks associated with contaminated shellfish have been reported in the US and Europe (Richards, 2006). Frozen raspberries have been implicated in a number of outbreaks of NoV-associated gastroenteritis (Pönkä et al., 1999; Le Guyader et al., 2004; Cotterelle et al., 2005; Falkenhorst et al., 2005), including an international outbreak (Butot et al., 2008).

1.4 RECOVERY OF VIRUSES FROM FOOD AND WATER

Even after the introduction of molecular-based techniques the detection of viruses in food and water has always been problematic as enteric viruses in environmental samples are often present in low numbers. The analysis of food and water is therefore a three-stage process: i) sample preparation and

viral recovery, ii) viral detection and iii) viral characterization (Mattison and Bidawid, 2009). In order to achieve a suitable analytical sensitivity for detection of viral contamination in water and fresh produce, sensitive recovery methods are therefore necessary before the detection system can be applied which includes the inoculation onto cultured cells and/or the use of molecular methods (Environmental Protection Agency (EPA), 2000; Guévremont et al., 2006; Wyn-Jones, 2007; Jones et al., 2009; Mattison and Bidawid, 2009).

1.4.1 Recovery of viruses from food

Methods for the recovery of viruses from foods generally involve two critical steps: i) to concentrate low level of viral particles, and ii) to eliminate any inhibitory substances that can interfere with analytical process (Mattison and Bidawid, 2009). Although a number of different methods have been described for the concentration of viruses from food the most appropriate will be determined by the nature and type of food sample to be analysed (Baert et al., 2008). None of the concentration methods have been standardised and each has advantages and drawbacks as there is no one method which fulfill all the criteria for all viruses and food matrixes (Rzeżutka and Cook, 2009). In addition there is no standard sampling procedure for the viral analysis of the different food matrices (Bosch et al., 2011).

The concept of a “statistically representative sample” is an important question to be addressed when analysing food for viruses. It is important that the sample should be representative of the entire batch or crop, but this is not clearly defined in the literature (Bosch et al., 2011). There are no guidelines or specifications indicating: i) the size or weight of a specific item of food; ii) the number of food items per batch or crop; iii) whether the food item must be whole or chopped; or iv) how many and which leaves for leafy vegetables need to be analysed. In addition the sampling procedure may be influenced by “local knowledge” regarding seasonality, possible levels of contamination and the costs involved. A number of methods use 25 g of food sample (Dubois et

al., 2006; Guévremont et al., 2006; Cheong et al., 2009b; Plante et al., 2011,) while others use 40 g (Schwab et al., 2000) or 50 g (Baert et al., 2008).

The extraction or recovery and concentration of viruses from the food matrix is challenging as this involves the liberation and concentration of low level of viruses from food matrix. A high efficiency of virus extraction is critical as this will allow the detection of viruses in food samples even when a detection method has a low sensitivity (Rzeżutka and Cook, 2009). Factors that need to be considered in the viral recovery and concentration process include: i) the sample weight and size of food item; ii) the chemical composition of the extraction solution or buffer (salt content, pH and molarity) to promote the release of the virus from the food matrix; and iii) inhibitory substances that might be introduced from the extraction solution (Rzeżutka and Cook, 2009). The recovery and concentration methods described in the literature include elution from the food matrix followed by: i) concentration with polyethylene glycol (PEG) precipitation, ii) adsorption and elution from charged filters, iii) concentration with ultracentrifugation; or iv) concentration with ultrafiltration (Mattison and Bidawid, 2009). An important feature of all these concentration procedures is that the viruses must be eluted from the food matrix into a liquid eluant for further concentration.

It is well known that plants contain high levels of PCR inhibitors which may be released into the rinsing or elution buffer and if not removed may cause inhibition in the subsequent detection assay (Plante et al., 2011). The success of the elution procedure for the removal of viruses from the surface of the fresh produce is dependent on the eluant chosen. Several elution buffers, namely distilled water, phosphate buffered saline (PBS), 3% beef extract, glycine beef extract, tris-glycine beef extract (TGBE) have been assessed for removal of viruses from fruit and vegetable surfaces (Dubois et al., 2006; Kim et al., 2008, Cheong et al., 2009b). Recovery studies have shown that improved recovery efficiency was achieved when protein containing eluting alkaline buffers, e.g. 1-3% beef extract at pH 9.5, were used (Dubois et al.,

2007; Kim et al., 2008). Bovine serum albumin (BSA) and Tween 80 has been added to a number of these buffers to improve the detection of viruses rinsed from the vegetable surfaces (Rutjes et al., 2005; de Paula et al., 2010; Plante et al., 2011). Most laboratories are restricted to their in-house developed recovery methods for fresh produce since limited attention has been spent on the evaluation and standardisation of elution methods (Stals et al., 2011).

1.4.2 Recovery of viruses from water

Viral recovery from water samples is particularly problematic because of the variability in the efficiency of recovery (EOR) for the different enteric viruses, the influence of the water quality and the time involved with these methods (Wyn-Jones, 2007). Since the levels of enteric viruses in contaminated water samples are expected to be low for most water samples (ground-, surface- and drinking water) a suitable viral concentration method needs to be selected for the success of viral monitoring. Several methods, involving different strategies, for the recovery of viruses from large volumes of water have been applied. The majority of methods are based on the ionic charge of the virus, e.g. adsorption-elution techniques. Other strategies include entrapment, e.g. ultrafiltration and immuno-affinity column, ultracentrifugation and many other methods such as flocculation (Fong and Lipp, 2005; Wyn-Jones, 2007).

1.4.2.1 Concentration based on ionic charge*: Adsorption-elution method*

The adsorption-elution methods involve manipulation of the charge on the virus surface, using pH changes to maximise their adsorption to charged filters (Fong and Lipp, 2005). The adsorption-elution method concentrates viral particles in water by adsorption to and subsequent elution with buffers from the charged membrane or filters (Fong and Lipp, 2005; Wyn-Jones, 2007). The principle involved is that viruses are brought into contact with a solid matrix to which the virus will adsorb under specific condition of pH and ionic strength (Wyn-Jones and Sellwood 2001; Wyn-Jones 2007). The virus is then released from the adsorbent by elution into a smaller volume of elution buffer

(e.g. beef extract-glycine buffer [GBEB]) at a high pH (~pH 9.5), so as to displace the virus from the adsorbent (EPA, 2000; Wyn-Jones and Sellwood, 2001; Wyn-Jones, 2007). The choice of the adsorbent matrix is, however, determined by the nature of the water sample (Wyn-Jones and Sellwood 2001). This is one of the most commonly used techniques for the recovery of viruses since water volumes can be increased without loss of efficiency and can therefore can also be used for drinking water (Fong and Lipp, 2005).

1.4.2.1.1 Glass wool adsorption-elution

The glass wool adsorption-elution technique, first applied by Menut et al. (1993) and Vilaginès et al. (1993) for the recovery of enteroviruses and RV SA11 from tap water, has been widely applied for the successful recovery of a wide range of enteric viruses from untreated surface and treated water samples (Wolfaardt et al., 1995b; Gantzer et al., 1997; Grabow et al., 1999; Powell et al., 2000; Taylor et al., 2001; Gassilloud et al., 2003; van Heerden et al., 2003, 2005a, 2005b; Vivier et al., 2004; van Zyl et al., 2006; Venter et al., 2007; Lambertini et al., 2008; Wyn-Jones et al., 2011). Depending on the quality of water, a wide range of the EORs have been reported for the glass wool columns. An EOR of 80-90% was reported for the routine recovery of enteric viruses from large volumes of water (Grabow et al., 2001) while recovery efficiencies of 70% for poliovirus, 14% for coxsackie virus B5, 19% for echovirus, 21% for AdV type 41 and 29% for NoV were reported by Lambertini et al. (2008) and 0.5% for feline calicivirus by Gassilloud et al. (2003). The benefit of this method is ease of use, there is no pre-treatment of the water sample required, and the cost of the equipment is minimal which allows successful recovery of enteric viruses from water samples in the field (EPA, 2000; Wyn-Jones and Sellwood, 2001; Wyn-Jones, 2007).

1.4.2.2 Entrapment: *Ultrafiltration*

Ultrafiltration has been applied to concentrate multiple types of viruses in large volumes (~100 ℓ) of water by physical retention based on virus and pore size, with the most commonly used filter being the hollow fibre ultrafilters (Wyn-Jones, 2007). Advantages of ultrafiltration include high recovery efficiencies and viruses are not exposed to pH extremes or other unfavourable

conditions which may affect their viability (Wyn-Jones, 2007; Wu et al., 2011). Although it is possible to adsorb viruses efficiently with this protocol, factors that limits the field application include the fact that the membranes clog rapidly which implies that the volumes of water that can be processed are restricted and equipment set-up is still not readily portable (Wyn-Jones, 2007; Wu et al., 2011).

1.4.2.3 Ultracentrifugation

Ultracentrifugation is also commonly referred to as a "catch-all" method concentrating sample based on the time and applied gravitational force (Wyn-Jones, 2007). This method however, is limited to small volumes of water. Different centrifugal separation methods such as continuous flow systems are also commercially available (Wyn-Jones, 2007).

1.4.2.4 Immuno-affinity column and others

Immuno-affinity methods involve incubation of magnetic beads that are coated with specific antibodies for a target organism, in a mixture of the sample suspension (Wyn-Jones, 2007). The efficiency of the reaction relies on the specificity and affinity of the commercially available monoclonal antibodies and on the turbidity of the water sample**.** The technique has been used to isolate a number of different organisms from water samples. Although the technique is simple and fast, the technique is only useful for a small volume of water, which makes it prohibitive for routine monitoring (Wyn-Jones, 2007).

There are several other methods that can also be used in the primary concentration of water samples such as hydro-extraction with hydrogen scopic solids, iron oxide flocculation, two-phase separation and freeze-drying (Wyn-Jones, 2007).

1.4.3 Concentration of viruses from food and water

The concentration of viruses recovered from food matrixes or secondary concentration of viruses recovered from water samples is commonly needed because the virus titres in water samples and on food surfaces are usually low. The recovered enteric viruses are further concentrated and purified to reduce the final volume of the sample to one or two millilitres for isolation or detection (Wyn-Jones and Sellwood, 2001, Wu et al., 2011). A number of methods have been used to further concentrate viral particles after the primary recovery step. These procedures include PEG precipitation, organic flocculation and centrifugal ultrafiltration (Fong and Lipp, 2005; Mattison and Bidawid, 2009).

1.4.3.1 PEG precipitation

The PEG precipitation technique is a simple and well validated method that has been used successfully by a number of investigators for the concentration of multiple types of viruses from a variety of environmental sample types (Lewis and Metcalf, 1988; Vilaginès et al., 1997; Kim et al., 2008; Mattison and Bidawid, 2009). Viral precipitation with PEG is an attractive concept as it is a chemically inert, non-toxic, water-soluble synthetic polymer known to precipitate a number of proteins (Lewis and Metcalf, 1988). There are two possible mechanisms of PEG precipitation methods that have been described: i) PEG acts as an "inert solvent sponge" that sterically excludes proteins from a solvent, effectively increasing their concentration until solubility is exceeded and precipitation occurs, and ii) while the second mechanism is dependent on protein charge (Lewis and Metcalf, 1988). Concentration of viral particles with PEG can be performed at neutral pH and at high ionic concentrations without other organic material (Kim et al., 2008). The primary benefits of the PEG precipitation method are: i) the low cost, ii) it is non-destructive to the viruses, and iii) many different viruses from many different samples can be concentrated (Lewis and Metcalf, 1988; Mattison and Bidawid, 2009). In the PEG precipitation method, viral particles are precipitated by addition of 0.5 M

sodium chloride (NaCl) and 7% PEG 6000 to the primary eluate with constant stirring which is followed by centrifugation and subsequent resuspension of the virus pellet in a buffer (Fong and Lipp, 2005; Wyn-Jones, 2007). Combined with NaCl, this type of concentration method has been widely practiced for monitoring the presence of enteric viruses in food and water (Goyal, 2006; Dubois et al., 2007; Papafragkou et al., 2008; Cheong et al., 2009a). The limitation of this method is it requires extended sample processing time (Mattison and Bidawid, 2009)

1.4.3.2 Other methods

Organic flocculation: This method involves acidifying a protein-containing eluate and recovering the precipitate by centrifugation and suspending the pellet in a smaller volume (Fong and Lipp, 2005; Atmar, 2006; Goyal., 2006; Wyn-Jones, 2007).

Inorganic precipitation: The technique involves both electrostatic interactions between the negatively charged virus surface and the positively charged aluminium hydroxide. The complexes aggregate, flocculate and then settle out of solution (Bosch et al., 2006).

Centrifugal devices: Commercial devices such as Centricon® cencentrators which concentrate viruses by ultrafiltration through an anistropic membrane are available, but are expensive for virus concentration from a large number of samples.

1.5 DETECTION AND CHARACTERISATION OF VIRUSES IN FOOD AND WATER

Given the highly infectious nature of NoV and HAV rapid, sensitive and specific methods required for the early detection of low titres of viruses. This will facilitate effective management of water and food supplies and the implementation of appropriate preventive control measures. There are a wide range of methods available for the detection of viruses in food and water samples, again each with their own advantages and disadvantages.

1.5.1 Viral isolation

Viral isolation in cell culture is the "gold standard" for the detection and quantification, by plaque assay or most probable number method, of infectious enteric viruses from food and environmental samples (Mattison and Bidawid, 2009; Bosch et al., 2011). After viral inoculation of the chosen cell cultures, the cells are evaluated for the presence of morphological changes or CPE. However, it has been long recognized that cell culture for detection of enteric viruses in water and food has numerous shortcomings (Fong and Lipp, 2005). Appropriate and susceptible cell culture systems are not available for all enteric viruses (Fong and Lipp, 2005) and in the case of NoV, efforts to isolate the virus in cell culture have been unsuccessful (Duizer et al., 2004; Goyal, 2006), while HAV does not cause a CPE (Dotzauer, 2008). Concentrates and extracts from food and water samples may also be cytotoxic to the cell cultures (Goyal, 2006). Viral culture is also costly, labour intensive and time consuming, taking several days or weeks to be conclusive which makes it unsuitable for routine analysis of enteric viruses from environmental samples (Fong and Lipp, 2005; Rutjes et al., 2005; Goyal, 2006; Meleg et al., 2006). Cell cultures are however used to in conjunction with molecular methods, e.g. integrated cell culture-PCR/RT-PCR assays, for the selective detection of infectious viruses. In these assays the target nucleic acid is increased with a consequent increase in sensitivity of the immunological or molecular detection method (Goyal, 2006; Bosch et al., 2011).

1.5.2 Direct detection

Viruses recovered from water and food samples can be detected by EM, immunological- or molecular-based assays.

1.5.2.1 Electron microscopy and immunological-based detection assays

Viruses are usually present in low numbers in food and water. The direct detection of viruses in recovered virus concentrates by EM, IEM or EIA is

limited by the sensitivity of these assays as the lower detection limit of these assays is 10^5 particles or more per mℓ (Widdowson and Vinjé, 2008; Mattison and Bidawid, 2009).

1.5.2.2 Molecular-based detection assays

The detection of enteric viruses from environmental samples with molecular techniques has been done since the early 1990s (Fong and Lipp, 2005). Molecular detection assays, such as PCR and RT-PCR, are based on the detection of a highly conserved part of viral genome using a virus-specific set of primers for each virus (Rodríguez et al., 2009; Bosch et al., 2011). At present, molecular techniques such as PCR/RT-PCR offer the best alternative sensitive and specific methods for the detection of enteric viruses from environmental samples (Meleg et al., 2006). Unlike cell culture-based methods, however, the infectivity of viruses detected by molecular methods is often unknown. Using molecular-based techniques diverse groups of enteric viruses, including NoV and HAV which are difficult or cannot be detected with cell culture-based assays, can be detected (Griffin et al., 2003; Fong and Lipp, 2005). Compared to cell culture-based methods molecular-based methods offer several advantages; i) they are rapid, less laborious, less time consuming, and ii) are highly specific and sensitive (Fong and Lipp, 2005; Mattison and Bidawid, 2009; Bosch et al., 2011). Although different molecular-based methods have been described for the detection of enteric viruses in foods, there is currently no standardized methodology for the detection of enteric viruses in food matrices (Guévremont et al., 2006; Croci et al., 2008; Mattison and Bidawid, 2009; Bosch et al., 2011).

Recent modifications to the PCR assays, called *rt* PCR have greatly expanded the application of PCR analysis for the detection and quantification of viruses in the environmental samples (Mattison and Bidawid, 2009; Spradling et al., 2009; Bosch et al., 2011). In the *rt* assays amplification and detection, with a fluorescent-labelled probe, is accomplished in a single reaction mix which makes it less prone to contamination and reduces hands-on time (Rutjes et al.,

2005). Different probe types are available, each with its own mode of action (Mattison and Bidawid, 2009). As compared to conventional RT-PCR, the procedure is less time consuming, since there is no confirmation step such as gel electrophoresis and a nested PCR is usually omitted (Fong and Lipp, 2005; Rutjes et al., 2005). As these *rt* PCR/RT-PCR assays are easily affected by inhibitory substances in water and on food the assays have been adapted to include Tween or BSA to overcome the inhibition (Rutjes et al., 2005; Bosch et al., 2011). Real-time PCR/RT-PCR may provide quantitative data for the presence of enteric viral genomes in environmental samples (Fong and Lipp, 2005) which is helpful in estimating the public health risks of low levels of enteric virus in environmental samples (Rutjes et al., 2005).

As enteric viruses are present in low numbers in food and water samples effective quality assurance and quality control procedures are required to exclude false positive or false negative results (Pintó and Bosch, 2008; Lauri and Mariani, 2009; Bosch et al., 2011). False negative results may result from poor virus recovery, inefficient nucleic acid extraction and inhibitory substances in the water or on the food matrix, while false positive results could be due to cross-contamination. It is therefore important to monitor the efficiency or accuracy of the critical steps in the recovery and detection assays for the generation of reliable and comparable results which are acceptable to the regulatory authorities (Sánchez et al., 2007; Bosch et al., 2011). Due to some technical difficulties in the environmental virology, it is therefore important to ensure consistency of the efficiency of the optimised procedure. In order to ensure the quality of sample processing, it is therefore important to include sample processing controls (Pintó and Bosch, 2008; Mattison and Bidawid, 2009; Pintó et al., 2009). The processing control should have similar features to the target virus, it must not be associated or naturally present in the water or food sample. The control must be added to the sample prior to sample processing, as this should be co-extracted and co-concentrated with the target virus and detected from the same extract (Mattison and Bidawid, 2009). Feline calicivirus (Mattison et al., 2009) and mengovirus (Costafreda et al, 2006; Pintó et al., 2009) have been proposed as candidate process controls for

controlling the whole process of sample analysis. The *rt* PCR/RT-PCR is affected by the presence of inhibitory compounds which are co-extracted during the nucleic acid isolation procedure (Sánchez et al., 2007). The *rt* PCR/RT-PCR should therefore also include an internal control (IC), which will distinguish a truly negative result from false negative results due to the presence of PCR/RT-PCR inhibitors (Rutjes et al., 2005). The following controls should also be included in each of the individual real-time RT-PCR runs: i) a negative RNA extraction control to monitor for cross-contamination; ii) a negative RT-PCR control, usually sterile distilled water, to control for contamination in the real-time RT-PCR reagents, and iii) a positive real-time RT-PCR control to control for the quality of the real-time RT-PCR reagents (Atmar, 2006; Bosch et al., 2011).

1.6 MOTIVATION FOR THIS INVESTIGATION

Recent epidemiological evidence indicates that foodborne viral disease is an emerging and increasing problem worldwide (Koopmans and Duizer, 2004; Newell et al., 2010). Minimally processed fruit and vegetables can be a source of pathogenic viruses (Steele and Odumera, 2004; Berger et al., 2010), particularly NoV (Girish et al., 2002; Le Guyader et al., 2004) and HAV (Rosenblum et al., 1990; Dentinger et al., 2001; Calder et al., 2003). Faecally contaminated irrigation water has been identified as one of the sources of human pathogens on fresh produce (Koopmans and Duizer, 2004; Steele and Odumera, 2004; Berger et al., 2010). In SA fresh produce has been identified as a source of foodborne viral disease as NoVs were implicated in an outbreak of gastroenteritis associated with the consumption of salads (Taylor et al., 1993). Hepatitis A virus and other enteric viruses have been detected in surface water sources used for irrigation (Taylor et al., 2001; Barnes and Taylor, 2004; van Zyl et al., 2006) and RVs have been detected on fresh produce (van Zyl et al., 2006). However, a link between faecally polluted irrigation water and fresh produce has not been established. It was therefore necessary to develop and optimise sensitive, reliable techniques for the

recovery and accurate detection and quantification of enteric viruses in irrigation water and on fresh produce. This methodology is required for the systematic surveillance of food sources, specifically fresh produce, for viruses and to investigate outbreaks of food- and waterborne disease. This is essential to ensure the safety of fresh produce for the consumer.

1.7 HYPOTHESIS

The hypothesis for this study is that in South Africa fresh produce, irrigated with treated and untreated water, is a potential source of enteric virus infections for consumers.

1.8 AIMS OF THE INVESTIGATION

To confirm or disprove this hypothesis, the specific objectives of this study are:

1) To optimise methods for the recovery of enteric viruses from fresh produce;

2) To optimise *rt* RT-PCR methods for the qualitative detection of human NoV and HAV;

3) To determine the presence of NoV and HAV in water sources used for the irrigation of fresh produce;

4) To determine the presence of NoV and HAV on the surfaces of fresh produce;

5) To characterise, by sequence analysis, the viral strains detected in the food and water sources.

CHAPTER 2

OPTIMISATION OF REAL-TIME RT-PCR METHODS FOR THE DETECTION OF HEPATITIS A VIRUS AND NOROVIRUSES

2.1 INTRODUCTION

Unlike many bacterial pathogens, the detection of human enteric viruses in food and water samples has been hindered by a number of technical challenges. Traditionally, the detection of viruses was performed in cell culture but this method has a low sensitivity, is tedious and labour- and cost-intensive (Gilgen et al., 1997; Meleg et al., 2006; Mattison and Bidawid, 2009). The study of enteric viruses in food and water sources has been a slow evolution, primarily because most of these viruses have been difficult, if not impossible, to propagate in cell culture (Pintó and Bosch, 2008). More importantly, no appropriate cell culture host exists for the epidemiologically important viruses such as HAV and NoV (Leggit and Jaykus, 2000, Bosch et al., 2011).

Advances in the development of sensitive and specific molecular-based assays has, however, made it possible to detect low, but significant virus numbers in clinical specimens, food and water samples without prior amplification in cell culture (Lewis et al., 2000; Atmar, 2006; Gunson et al., 2006; Pintó and Bosch, 2008). The PCR and RT-PCR techniques are based on the detection of a specific region of the target viral genome that is highly conserved with broad homology within a specific group of viruses (Fong and Lipp, 2005), and is therefore considered to be the “gold-standard” for the detection of viruses in food and water (Pintó and Bosch, 2008). Viral detection using PCR/RT-PCR is less time consuming, highly sensitive and more specific than cell culture (Fong and Lipp, 2005). These significant benefits have been greatly expanded

upon by the introduction of *rt* PCR/RT-PCR (Gunson et al., 2006). In comparison to traditional gel-based PCR/RT-PCR detection, the modification of the PCR/RT-PCR to include a fluorophore-labelled probe provides equivalent specificity and sensitivity as conventional PCR combined with Southern blot analysis (Espy et al., 2006). With this modified PCR/RT-PCR or the so called *rt* PCR/RT-PCR, a fluorescent signal is generated as the amplification takes place which allows the user to monitor the amplification, detection and quantification of target nucleic acid simultaneously and accurately, thus reducing the time required for nucleic acid detection by gel visualisation (Niesters, 2004), or further confirmation of the identity of the product (Mattison and Bidawid, 2009). As viruses can be detected qualitatively or quantitatively by *rt* PCR/RT-PCR, it is possible to perform hazard risk assessment for analysis critical for further public health action or food regulation (Bosch et al., 2011). Since the amplification and detection is performed in a single tube there is no need to open the tube which reduces the likelihood of contamination in the laboratory with amplified nucleic acid (Atmar, 2006; Espy et al., 2006; Mattison and Bidawid, 2009). Real-time PCR technology has currently been the method of choice for the diagnosis of infectious diseases due to excellent sensitivity and specificity, low contamination risk, speed and ease of use. This provides the technology with a broad enough base to ensure its acceptance (Espy et al., 2006).

Real-time PCR/RT-PCR is now widely applied for the detection of viruses in foods (Bosch et al., 2011) but it is essential to demonstrate sensitivity and reproducibility of the assay since there are no standardised methods for the detection of viruses in food and water (Mattison and Bidawid, 2009). In addition, adequate quality controls need to be applied to ensure the validity of results (Bosch et al., 2011). The objective of this study was to optimise *rt* RT-PCR methods for the detection of low, but significantly important titres, of HAV and NoV in water and fresh produce, using mengovirus as a process control (Costafreda et al., 2006; da Silva et al., 2007; Pintó and Bosch, 2008). In addition standard curves for the viruses were constructed to serve as

external standard curves for subsequent experiments for the quantitative detection of viruses recovered from fresh produce and irrigation water.

2.2 MATERIALS AND METHODS

2.2.1 Virus stock

Hepatitis A virus: HAV ($TCID_{50}$ 1 x 10^8/mℓ): The cytopathic cell culture-adapted HM-175 43c variant (referred to as pHM-175) of the HM-175 strain used for this investigation was kindly provided by Prof A Bosch, Department of Microbiology, Facultat de Biologia, University of Barcelona, Barcelona, Spain. The virus was further propagated and titrated in the FRhK-4R foetal rhesus monkey cell line (Flehmig, 1980, 1981).

Norovirus GI: NoV GI (titre unknown): A NoV GI positive sewage water sample was used.

***Norovirus GII*:** NoV GII (1.1 x 10^{10} copies/mℓ): The viral stock originated from a clinical strain detected in the stool specimen from a patient after an outbreak of gastroenteritis on a cruise ship. Dr M Wolfaardt from the Enteric Virus and Environmental Research Group, Department of Medical Virology, University of Pretoria typed the virus as NoV GII.4. The virus was quantified by Prof A Bosch and co-workers, Department of Microbiology, Faculty of Biology, University of Barcelona, Barcelona, Spain.

***Mengovirus*:** Mengovirus ($TCID_{50}$ 1 x 10^7 copies/mℓ): The viral stock culture was propagated and titrated in a Vero African Green Monkey cell line from a culture kindly provided by Prof A Bosch, Department of Microbiology, Facultat de Biologia, University of Barcelona, Barcelona, Spain.

2.2.2 Primers and Probes

To ensure that the assays being optimised for this investigation were comparable to assays being applied by the European Committee of Standardisation (CEN), the primers and probes used for the *rt* RT-PCR assays

were those recommended by the CEN TC275/WG6/TAG4 Technical Committee (Table 2.1)

Table 2.1: Nucleotide sequence data of primers and probes applied in this study.

Virus	Reference	Name	Sequence (5'→3')	Amplicon size
HAV	Costafreda et al., 2006.	HAV 68-FW	TCA CCG CCG TTT GCC TAG	172 bp
		HAV 240-REV	GGA GAG CCC TGG AAG AAA G	
		HAV 150-PROBE	FAM-CCT GAA CCT GCA GGA ATT AA-MGB	
NoV GI	Svraka et al., 2007; da Silva et al.., 2007.	QNIF4-FW	CGC TGG ATG CGN TTC CAT	98 bp
		NV1LCR-REV	CCT TAG ACG CCA TCA TCA TTT AC	
		NVGG1p-PROBE	FAM-TGG ACA GGA GAY CGC RAT CT-TAMRA	
NoV GII	Kageyama et al., 2003; Loisy et al., 2005	QNIF2-FW	ATG TTC AGR TGG ATG AGR TTC TCW GA	88 bp
		COG2R-REV	TCG ACG CCA TCT TCA TTC ACA	
		QNIFS-PROBE	FAM-AGC ACG TGG GAG GGC GAT CG-TAMRA	
Mengo-virus	Pintó et al., 2009.	Mengo 110-FW	GCG GGT CCT GCC GAA AGT	99 bp
		Mengo 209-REV	GAA GTA ACA TAT AGA CAG ACG CAC AC	
		Mengo 147-PROBE	FAM-ATC ACA TTA CTG GCC GAA GC-MGB	

2.2.3 Viral nucleic acid extraction

Genomic viral nucleic acid was extracted directly from 1 ml of the virus suspension using the MagNA Pure LC Total Nucleic Acid Isolation (large volume) kit (Roche Diagnostics GmbH, Mannheim, Germany), in a MagNA Pure LC instrument (Roche), following the manufacturer's instructions. Briefly, the MagNA Pure is a bench top instrument that can extract nucleic acids from up to 32 samples in parallel. The instrument software guides the user through the setup procedure and automatically calculates the amount of reagents needed for processing. All the remaining pipetting steps of the procedure were automatically performed with aerosol-preventive, disposable reaction tips. After incubation with a chaotropic lysis buffer and proteinase K, nucleic acids were released and bound to magnetic glass particles. Unbound molecules are removed by several washing steps and nucleic acid eluted in 50 µl of elution buffer. Purified nucleic acid was aliquoted and stored at -70°C until use. Five to ten microlitres of the eluted nucleic acid was used for amplification (Figure 2.0).

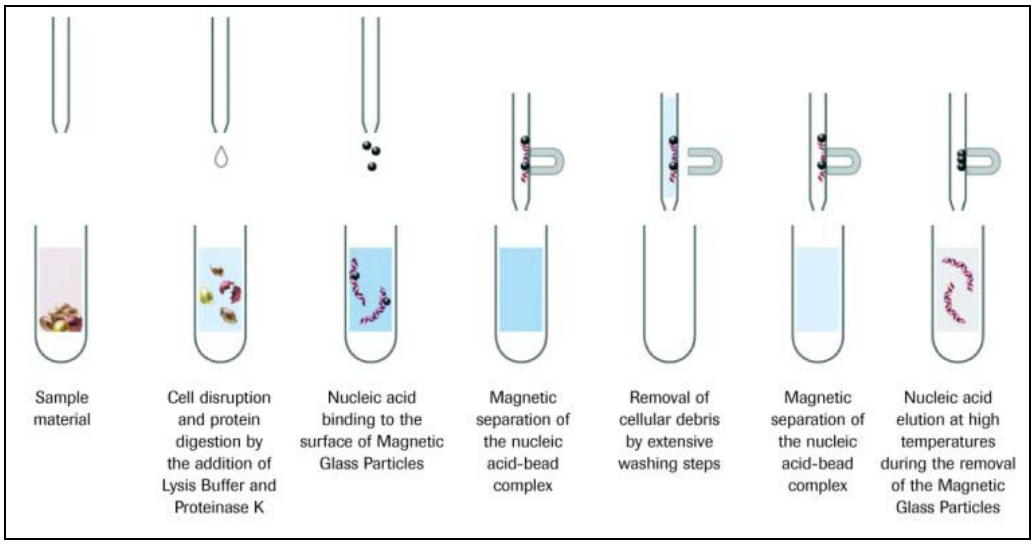

Figure 2.1: Diagram depicting the principle of the MagNA Pure magnetic bead technology for the extraction of nucleic acid (www.roche-applied-science.com).

2.2.4 Evaluation of real-time RT-PCR kits

Three different *rt* RT-PCR kits were tested for their ability to amplify and detect NoV, HAV and mengovirus using Taqman probes. Two different one-step *rt* RT-PCR systems, namely the QuantiTect Probe® RT-PCR kit (Qiagen GmbH, Hilden, Germany) and the RNA Ultrasense™ One-step qRT-PCR system (Invitrogen, Carlsbad, CA), and a two-step system using the Transcriptor First Strand cDNA Synthesis Kit and LightCycler® TaqMan® Master kit (Roche), were compared.

The sensitivity of the *rt* PCR kits was determined by testing 10-fold dilutions of HAV, NoV GI, NoV GII and mengovirus viral suspensions, thus representing a range of viral titres, in each of the assays.

2.2.5 Construction of standard curves

Stock suspensions of HAV, NoV GII, and mengovirus were prepared to a final concentration of 1 x 10^6 copies/mℓ of each virus. Total nucleic acid was extracted from 1 mℓ of the viral suspension using the MagNA Pure LC Total Nucleic Acid Isolation Kit (large volume) (Roche). Serial 10-fold dilutions of the extracted nucleic acid were done in nuclease-free water (Promega Corp, Madison, WI) and the diluted nucleic acid was amplified to obtain standard curves for each of the viruses.

Hepatitis A virus: The *rt* RT-PCR amplification of viral nucleic acid from the serial dilutions was done using the RNA Ultrasense™ One-step qRT-PCR system (Invitrogen). Five microlitres of diluted nucleic acid was added to the RNA Ultrasense™ Master Mix, containing 10 pmol each of the forward primer, 18 pmol of the reverse primer and 5 pmol of the labelled TaqMan probe. The RT-PCR was performed under the following conditions: reverse transcription at 50 °C for 45 min, DNA polymerase activation at 95 °C for 15 min and then 50 PCR cycles of amplification with denaturation at 95 °C for 15 sec, annealing at 60 °C for 1 min and extension at 65 °C for 1 min.

Norovirus GII: For the detection of NoV GII 5 µℓ RNA and 4 pmol of each of the forward primer, the reverse primer and the labelled probe were added to the Quantitect Probe Master Mix. The RT-PCR was performed under the following conditions: reverse transcription at 45 °C for 45 min, DNA polymerase activation 95 °C for 10 min and then 45 cycles of amplification with denaturation at 95 °C for 15 sec, annealing at 60 °C for 1 min and extension at 65 °C for 1 min.

Mengovirus: The *rt* RT-PCR amplification of viral nucleic acid from the serial dilutions was done using the QuantiTect Probe® RT-PCR kit (Qiagen), 5 µℓ of diluted nucleic acid was added to the Quantitect Probe Master Mix, containing 10 pmol each of the forward primer, 18 pmol of the reverse primer

and 5 pmol of the labelled TaqMan probe. The reaction parameters were the same as for HAV.

Norovirus GI: Even though a standard curve was not constructed during this study, NoV GI detection was done using the two-step assay. In the two-step assay the RT reaction was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche) and LightCycler® TaqMan® Master kit (Roche) according to manufacturer's instruction. The PCR cycling conditions included an initial DNA polymerase activation at 95 °C for 15 min, with a subsequent 45 cycles of amplification (95 °C for 45 sec, 60 °C for 1 min and 65 °C for 1 min).

The assays were performed in monoplex reactions using the cycling conditions as described by the manufacturer's and all reactions were performed on a LightCycler v1.5 (Roche). The standard curves were constructed by calculating the amount of viral copies added to the *rt* reaction for each dilution by the LightCycler 1.5 software for each virus. For HAV, in order to estimate the physical number of genomes the standard curve was adjusted by applying a factor of X60 to the infectious titre (Costafreda et al., 2006).

2.3 RESULTS

2.3.1 Evaluation of real-time RT-PCR kits

The comparative sensitivity of each of the three *rt* RT-PCR systems for each of the target viruses is presented in Table 2.2. The detection limits of *rt* RT-PCR assays, defined as the lowest dilution in the 10-fold series that amplified reliably, was 10^{-6} for HAV by the one-step RNA Ultrasense™ One-step qRT-PCR system. The same dilution of virus was also the limit of detection for mengovirus using the two step combination of the Transcriptor First Strand cDNA Synthesis Kit and LightCycler® TaqMan® Master kit (Roche). No differences were noted in the sensitivity of detection of NoV GI, with a RNA

dilution of 10^{-4} being detected by all three kits. The NoV GII could be detected by the TaqMan® Master Kit and the RNA Ultrasense™ One-step qRT-PCR system at a dilution of 10^{-2} and on the QuantiTect Probe® RT-PCR kit (Qiagen) at a dilution of 10^{-4}. The Quantitect Probe® RT-PCR Kit (Qiagen) proved to be the most sensitive kit for the detection of NoV GI, detecting a RNA dilution of 10^{-4}. The Transcriptor First Strand cDNA Synthesis Kit in combination with the Lightcycler® TaqMan® Master kit was the least sensitive for the detection of HAV, NoV GI and NoV GII and was the most sensitive for the detection of mengovirus, detecting mengovirus at a RNA dilution of 10^{-6}.

Table 2.2: Real-time amplification and detection of a 10-fold dilution series of HAV, NoV GI, NoV GII and Mengovirus.

Kits	**Viruses**	**Virus dilution**							
		10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Lightcycler® TaqMan® Master kit	NoV GI	+	+	+	+	-	-	-	-
	NoV GII	+	+	+	-	-	-	-	-
	HAV	+	+	+	+	-	-	-	-
	Mengo	+	+	+	+	+	+	+	
Quantitect Probe® RT-PCR Kit	NoV GI	+	+	+	+	-	-	-	-
	NoV GII	+	+	+	+	-	-	-	-
	HAV	+	+	+	+	+	-	-	-
	Mengo	+	+	+	+	+	+		
RNA Ultrasense™ Kit	NoV GI	+	+	+	+	-	-	-	-
	NoV GII	+	+	+	-	-	-	-	-
	HAV	+	+	+	+	+	+	+	-
	Mengo	+	+	+	+	-	-	-	-

2.3.2 Construction of standard curves

The amplification curves of the 10-fold serial dilutions for HAV, NoV GII and mengovirus are shown in Figures 2.2a, 2.3a and 2.4a, respectively. The standard curves obtained from these amplification curves for HAV, NoV GII and mengovirus are shown in Figures 2.2b, 2.3b and 2.4b, respectively. The slope for HAV was -3.962, for NoV GII -3.857 and for mengovirus -3.317. These slope of the regression lines were used to calculate the PCR efficiency according to the formula $E = (10^{1/s}-1) \times 100$, with efficiencies of 79%, 82%

and 100% being recorded for HAV, NoV GII and mengovirus, respectively (Table 2.3). A good correlation ($r \geq 0.99$) was observed between cycle number and dilution for all three different virus used.

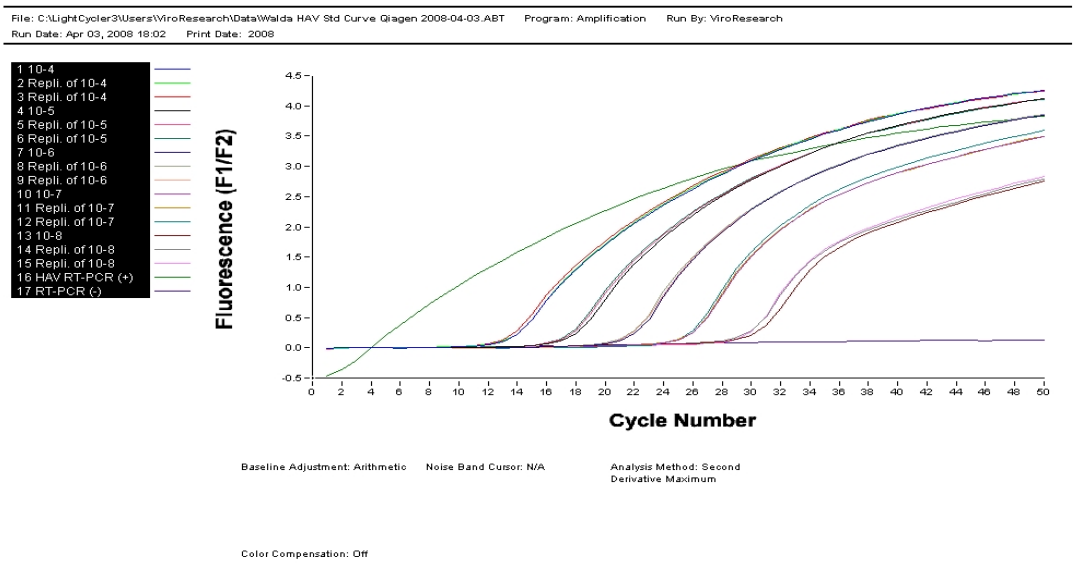

Figure 2.2a: Amplification curves of a 10-fold dilution series of a known titre of HAV.

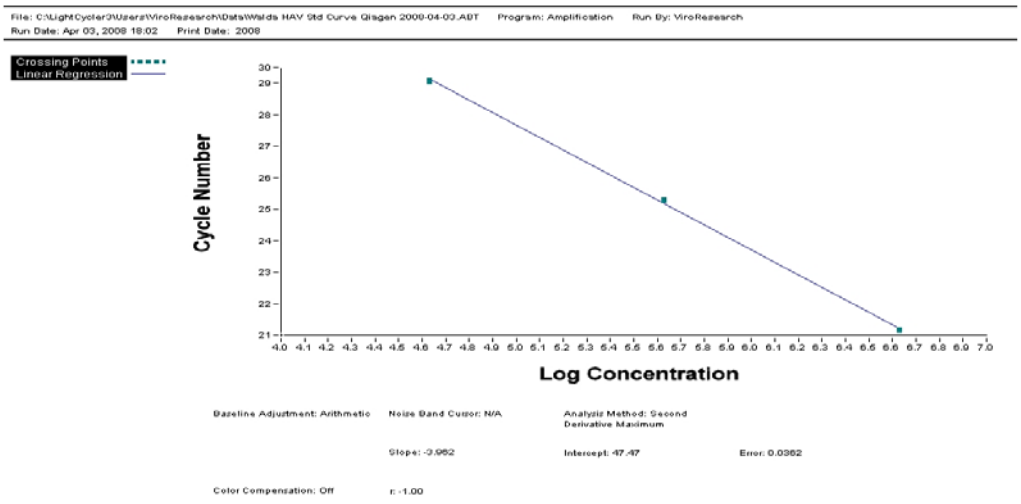

Figure 2.2b: Standard curve prepared from a 10-fold dilution series of a known titre of HAV and modified to physical genomic copy number by applying a factor of X60 to the infectious titre (Costafreda et al., 2006).

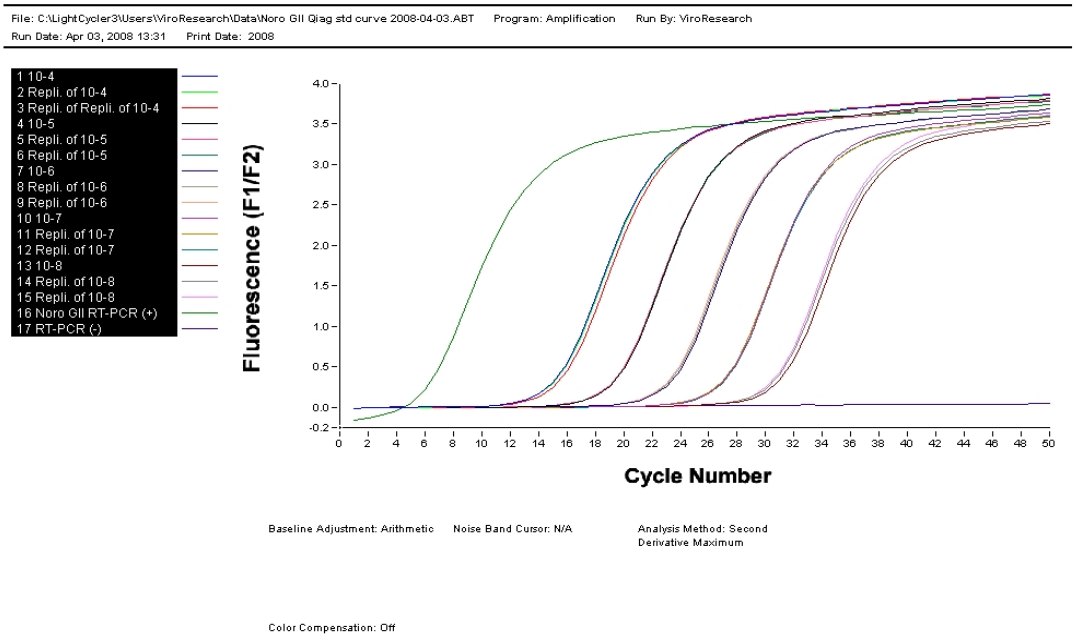

Figure 2.3a. Amplification curves of a 10-fold dilution series of a known titre of NoV GII.

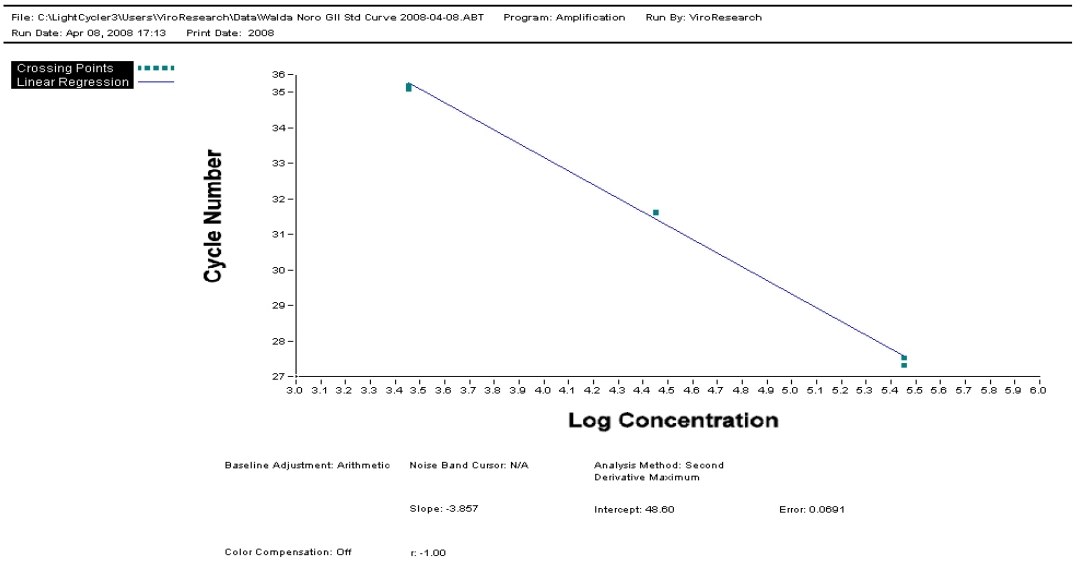

Figure 2.3b. Standard curve prepared from a 10-fold dilution series of a known titre of NoV GII.

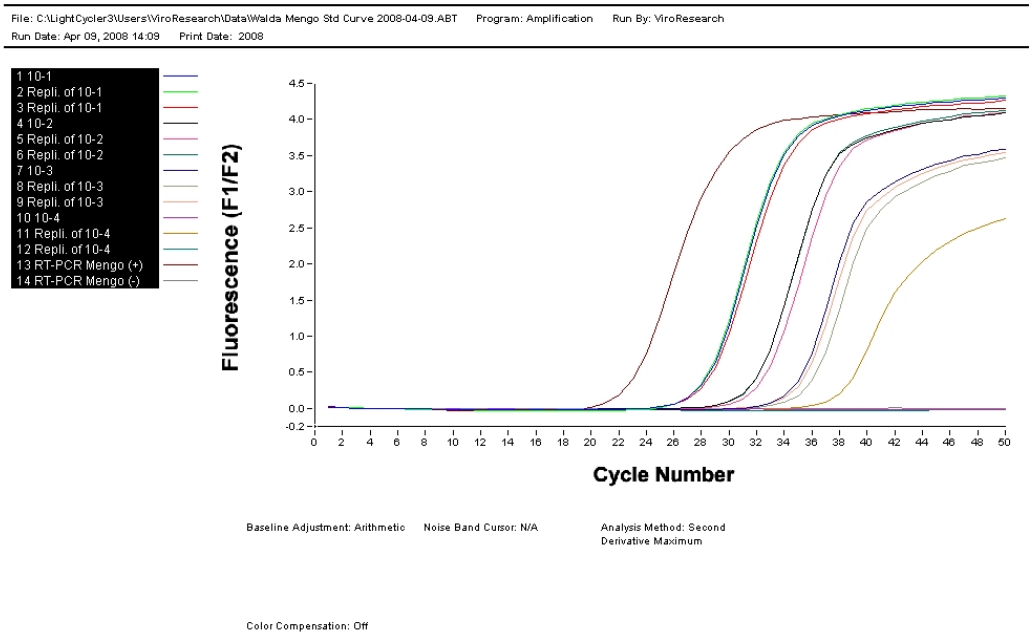

Figure 2.4a: Amplification curves of a 10-fold dilution series of a known titre of mengovirus.

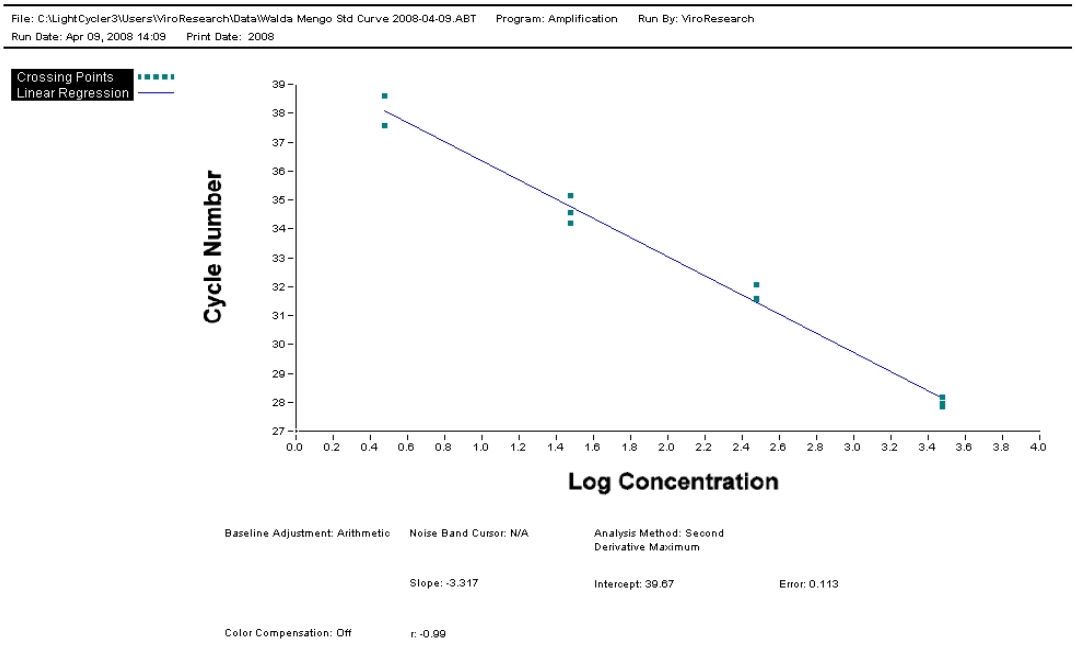

Figure 2.4b: Standard curve prepared from a 10-fold dilution series of a known titre of mengovirus.

Table 2.3: Summary of standard curve analysis

Virus	Slope	Intercept	r*	Efficiency
HAV	-3.962	47.47	-1.00	79%
NoV GII	-3.857	48.60	-1.00	82%
Mengovirus	-3.317	39.67	-0.99	100%

*r = represents the coefficient of correlation

2.4 DISCUSSION

Although many *rt* RT-PCR kits have been described, only a few papers are available presenting comparative studies of different *rt* RT-PCR assays and/or kits for the detection of HAV and NoVs (Costafreda et al., 2006; Houde et al., 2007; Butot, et al., 2010, Gregory et al., 2011). The sensitivity of detection of *rt* RT-PCR kits from three different companies, namely Qiagen, Roche and Invitrogen, were compared. Although factors such as primer sequence, primer specificity and annealing temperature can also have an influence on the performance of PCR, the primer sets used in this study have successfully been applied to the detection of HAV and NoV in shellfish (Costafreda et al., 2006; Comelli et al., 2008; Pintó et al., 2009; Uhrbrand et al., 2010) and wastewater (da Silva et al., 2007). The specificity of the primers and probes were therefore not validated in this study.

As indicated in Table 2.2, not all the kits were as efficient for the detection of all viruses tested. For instance, viral detection based on the RNA Ultrasense™ One-step qRT-PCR system was more successful for the detection of HAV, as the HAV viral nucleic acid was still detectable up to a dilution of 10^{-6}, but was less successful for the detection of NoV GII. There was no variation in the sensitivity of the detection of NoV GI for each of the three *rt* RT-PCR kits assessed. The results indicated that the most sensitive kit for the detection of NoV GII and mengovirus was the QuantiTect Probe® RT-PCR kit (Qiagen), and for HAV was the RNA Ultrasense™ One-step qRT-PCR

system (Invitrogen). Other researchers, however, have used different methodologies, either in the one-step (Butot et al., 2007; Blaise-Boisseau et al., 2010, Bianchi et al., 2011) or two-step (Jothikumar et al., 2005; Scherer et al., 2010) formats for the *rt* RT-PCR detection of enteric viruses on fresh produce.

Viral quantification is becoming increasingly important in the assessment of levels of viral contamination in food and environmental water samples. The accuracy of quantification by *rt* RT-PCR depends on the quality of the standard curve. The efficiency of the PCR reaction can be estimated from the slope of the crossing threshold against the copy number. After the evaluation of kits from different companies (Qiagen, Roche and Invitrogen), three quantification standard curves have been successfully constructed, one for HAV using the RNA Ultrasense™ One-step qRT-PCR system (Invitrogen), one for NoV GII and mengovirus using the QuantiTect Probe® RT-PCR kit (Qiagen). In this study, the good slope value for mengovirus indicated a high (100%) PCR efficiency. The possible explanations for the poor or lower efficiency in some of the standard curves or PCRs (HAV and NoV GII) might have been caused by factors, such as poor optimisation of the PCR reaction conditions and pipetting errors (http://www.bio-rad.com). Despite the low efficiency for some of the PCRs (HAV and NoV GII), all three standard curves showed a very good coefficient of correlation, ≥ 0.99 for all three viruses, indicating near perfect or perfect linearity of data points.

From this study it is evident that no single *rt* RT-PCR kit or assay can be applied universally for the viruses investigated in this study. This is one of the reasons why there is no standardised methodology for the detection a broad range of enteric viruses in vegetables and fruits. Harmonisation of molecular techniques, with the appropriate quality control and quality assurance measures, is therefore required for the routine analysis of food and water for enteric viruses.

CHAPTER 3

OPTIMISATION OF METHODS FOR THE RECOVERY OF HEPATITIS A VIRUS AND NOROVIRUSES FROM FRESH PRODUCE

3.1 INTRODUCTION

Enteric viruses are increasingly being recognized as a major cause of foodborne disease (Croci et al., 2008). Although various food types have been implicated in foodborne outbreaks, the consumption of raw or partially cooked food, namely fruit, vegetables and ready-to-eat foods contaminated with viruses are considered to be the most important causes of foodborne viral disease (Le Guyader et al., 2004; Croci et al., 2008). Epidemiological evidence suggests that NoV and HAV are the most common viral pathogens transmitted by food or fresh produce (Croci et al., 2008; Jones et al., 2009; Newell et al., 2010). Globally the microbiological quality control criteria for food still rely on indicator organisms such as faecal coliforms or *Escherichia coli*, despite increasing evidence that these criteria have limited value as predictors of enteric virus contamination (Cheong et al., 2009a; Newell et al., 2010). A major limitation in monitoring food for enteric viral contamination or for investigating foodborne outbreaks is the detection of low levels of these viruses in the various food matrices (Mattison and Bidawid, 2009). The most important step in detecting enteric viruses in food samples is to concentrate and purify the virus and to remove inhibitors before applying molecular-based detection assays such as *rt* RT-PCR (Croci et al., 2008; Mattison and Bidawid 2009). As there are differences in the morphology and hydrophobic interactions of fruit and vegetable surfaces the viral recovery procedures are specific for each food type (Rutjes et al., 2006; Croci et al., 2008). A variety of viral recovery methods have been developed and applied to elute, concentrate and purify different viruses from shellfish (Kingsley et al., 2002;

Sánchez et al., 2002; Nenonen et al., 2009; Pintó et al., 2009), fresh produce (Rutjes et al., 2006; Fino and Kniel, 2008; Kim et al., 2008; Papafragkou et al., 2008; Fumian et al., 2009; Scherer et al., 2010), berry fruits (Le Guyader et al., 2004) and ready-to-eat foods (Baert et al., 2008; Fumian et al., 2009). The aim of this study was to optimise efficient methods for the recovery of low titres of selected enteric viruses from different types of fresh produce.

3.2 MATERIALS AND METHODS

3.2.1 Virus stock

Hepatitis A virus: HAV ($TCID_{50}$ 1 x 10^8/mℓ): The cytopathic cell culture-adapted HM-175 43c variant (referred to as pHM-175) of the HM-175 strain (refer Section 2.2.1).

***Norovirus GII*:** NoV GII (1.1 x 10^{10} copies/mℓ): The viral stock originated from a clinical strain detected in the stool specimen from a patient after an outbreak of gastroenteritis on a cruise ship (refer Section 2.2.1).

***Mengovirus*:** Mengovirus ($TCID_{50}$ 1 x 10^7 copies/mℓ): The viral stock culture was propagated and titrated from a culture kindly provided by Prof A Bosch (refer Section 2.2.1).

3.2.2 Reagents and elution buffers

3.2.2.1 Tris-glycine 1% beef extract (TGBE) buffer (pH 7.2 or pH 9.5)

12.1 g Tris Base (Roche)

3.8 g Glycine (Merck, Darmstadt, Germany)

10 g Beef extract [BBL™ Becton Dickinson and Co., Sparks, MD])

The solids were dissolved in 800 mℓ molecular grade water after which the pH was adjusted to pH 7.2 and pH 9.5. The volume was made up to 1000 mℓ with molecular grade water. The buffer was sterilised by autoclaving and stored at 4°C.

3.2.2.2 Phosphate buffered saline (PBS) (pH 7.2 or pH 9.5)

The phosphate buffered saline (PBS) was prepared using phosphate buffered saline tablets (Sigma-Aldrich, Co., St Louis, MO) according to manufacturer's instructions and sterilised by autoclaving.

3.2.2.3 Glycine 0.5% beef extract (GBEB) buffer (pH7.2 or pH 9.5)

3.75 g Glycine (Merck)

5 g Beef extract (BBL™)

The solids were dissolved in 800 mℓ molecular grade water after which the pH was adjusted to pH 7.2 or pH 9.5. The volume was made up to 1000 mℓ with molecular grade water. The buffer was sterilised by autoclaving and stored at 4°C

3.2.2.4 Polyethylene glycol/sodium chloride (PEG/NaCl) precipitation solution

A 5 X PEG/NaCl solution (50% [w/v] PEG 6000, 1.5M NaCl was prepared as follows:

500 g PEG 6000 (Merck)

87 g NaCl (Merck)

The solids were dissolved in 800 mℓ molecular grade water. The volume was adjusted to 1000 mℓ with molecular grade water. The solution was sterilised by autoclaving and stored at room temperature.

3.2.3 Optimisation of viral recovery from fresh produce

3.2.3.1 Seeding of fresh produce

To simulate the isolation and purification of viral RNA from fresh produce with different surfaces, tomatoes and green onions were purchased from local commercial outlets. Each item of food was weighed and then exposed to UV light for 30 min to inactivate any contaminating pathogenic viruses before seeding (artificial contamination). A 100 µℓ aliquot of a viral suspension containing HAV, mengovirus and NoV GII, all at a final concentration of 1 X

10^6 RNA copies/mℓ was seeded onto individual items of spring onions or tomatoes. The suspension was dispersed onto as much of the surface as possible using a 1 µℓ sterile Nunc™ disposable inoculating loop (Nunc A/S, Denmark). A control sample of each food item was seeded with 100 µℓ RNase free water. After the inoculation the fresh produce was air dried for 4 hours in a biohazard dead box (Figure 3.1). Three items of each food type food were seeded for each experiment.

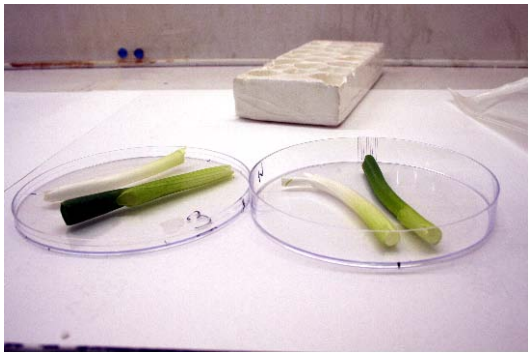

Figure 3.1: Example of fresh produce (spring onions) drying in biohazard box after being seeded with viruses.

3.2.3.2 Evaluation of elution buffers

In order to evaluate the efficiency of the recovery method, i.e. optimal elution time, buffer type and pH, the inoculated food item was placed in a hybridisation bag (Roche) with 40 mℓ of each of the elution buffers. The virus from one set of food items was eluted for 20 min with constant shaking at room temperature, while in the second set elution was done for 12-14 hours (overnight) at room temperature.

3.2.3.3 Viral concentration

After incubation, the buffer used to elute the virus from the item of fresh produce was decanted into a 50 mℓ centrifuge tube and the volume of eluate was recorded. If necessary, the pH was adjusted to pH 7.2 with concentrated HCl (Merck) after which 0.25 volumes of the 5 X PEG/NaCl precipitation solution was added to the eluate. The eluate-PEG/NaCl solution was mixed thoroughly by inversion and shaken for 60 min at 4 ºC. The solution was then

centrifuged at 10 000 x g for 30 min at 4 ºC to precipitate the virus. The supernatant was discarded and the tube was centrifuged at 10 000 x g for 5 min at 4 ºC to compact the pellet. The residual liquid was removed by careful aspiration and the pellet was resuspended in 500 µℓ of PBS (pH 7.2) (Sigma-Aldrich). The suspension was stored at -20 ºC until further processing.

3.2.4 Viral detection and quantification

3.2.4.1 Primers and probes

The nucleotide sequences of the primers and probes for the detection of HAV, NoV GII and mengovirus used have been described previously (refer: Table 2.1).

3.2.4.2 Viral nucleic acid extraction

Total nucleic acid was extracted from 1 mℓ of the recovered viral suspension using the MagNA Pure LC Total Nucleic Acid Isolation Kit (large volume) (Roche) on a MagNA Pure LC instrument (Roche) following the manufacturer's instructions (refer: Section 2.2.3). The purified nucleic acid was stored at -70 °C until use.

3.2.4.3 Real-time RT-PCR detection of viruses

Molecular detection, by *rt* RT-PCR, using the RNA Ultrasense™ One-step qRT-PCR system for HAV and the QuantiTect Probe® RT-PCR (Qiagen) kit for NoV GII and mengovirus was done as described previously (refer: Section 2.2.5). All samples were analysed in duplicate for the presence of HAV, mengovirus and NoV GII.

3.2.4.4 Quantification of viruses

Standard curves, constructed previously for each of the three viruses using the LightCycler v1.5 software (refer: Section 2.2.5), were available for each virus. After amplification the standard curves were used to calculate the RNA copies of each virus present in each *rt* RT-PCR reaction. This value was then used to

calculate the RNA viral copies per millilitre recovered from the fresh produce. Since a known concentration of mengovirus, HAV, and NoV GII was used to seed the fresh produce, extraction (elution) efficiencies could be calculated and expressed as a percentage. The effect of the different parameters was measured by comparing the percentages of recovery of mengovirus, HAV, and NoV GII.

3.3 RESULTS

The effect of: i) incubation or eluting time, ii) buffer type and iii) buffer pH on virus recovery from the surface of the seeded tomatoes (smooth surface) is presented in Tables 3.1 & 3.2.

Table 3.1: Comparison of the percentage of viruses recovered from seeded tomatoes using three different washing buffers (PBS, TGBE and GBEB) at two different pHs (7.2 and 9.5) with a 20 min elution time.

Elution buffer/ elution period	**Extraction efficiency of mengovirus**	**Extraction efficiency of HAV**	**Extraction efficiency of NoV GII**
PBS pH 7.2/ 20 min	0.53%	8.75%	3.03%
PBS pH 9.5/ 20 min	1.70%	12.60%	6.00%
TGBE pH 7.2/ 20 min	1.25%	47.48%	17.87%
TGBE pH 9.5/ 20 min	2.14%	21.69%	17.08%
GBEB pH 7.2/ 20 min	0.29%	31.54%	8.68%
GBEB pH 9.5/ 20 min	0.10%	11.40%	2.57%

Table 3.2: Comparison of the percentage of viruses recovered from seeded tomatoes using three different washing buffers (PBS, TGBE and GBEB) at two different pHs (7.2 and 9.5) with an overnight (12-14 hour) elution time.

Elution buffer/ elution period	**Extraction efficiency of mengovirus**	**Extraction efficiency of HAV**	**Extraction efficiency of NoV GII**
PBS pH 7.2/ Overnight	0.04%	29.88%	0%
PBS pH 9.5/ Overnight	0.01%	10.10%	0%
TGBE pH 7.2/ Overnight	0.006%	17.4%	14.82%
TGBE pH 9.5 Overnight	0.63%	23.66%	18.09%
GBEB pH 7.2/ Overnight	0.01%	23.90%	3.51%
GBEB pH 9.5/ Overnight	0.12%	12.00%	0.61%

From Table 3.1 and Table 3.2 it is evident that there was a marked difference in the recovery rates between the three different viruses. Not one of the elution buffers, at either of the elution times, performed equally well for all three viruses. Of the three viruses tested mengovirus, the proposed process control, showed the lowest recoveries in all elution buffers tested. Hepatitis A virus was more readily eluted from the tomato samples than NoV GII. Except for the recovery of HAV using the PBS (pH 7.2) elution buffer, an overnight elution time did not markedly improve the recovery rates of the three viruses, on the contrary the recovery rates remained similar or appeared to decrease. Although TGBE buffer (pH 7.2) elution buffer, with a 20 min elution time gave the highest percentage recovery for HAV, the TGBE buffer (pH 9.5) elution buffer, with a 20 min elution time, gave the best overall recovery for all three viruses.

The effect of: i) incubation or eluting time, ii) buffer type and iii) buffer pH on virus recovery from the surface of the green onions (intermediate/rough surface) is presented in Table 3.3 & 3.4. As was noted for the recovery efficiencies from tomatoes: i) there was a marked difference in the recovery rates of the three different viruses from the seeded green onions, and ii) not one of the elution buffers, at either of the elution times, performed equally well for all three viruses. Mengovirus again showed the lowest recoveries in all elution buffers tested. In contrast to what was noted for the tomatoes, the highest percentage recovery was recorded for HAV after an overnight elution period using elution buffers TGBE pH 9.5 and GBEB pH 7.2. The percentage recovery for mengovirus after overnight elution was however poor, ranging from 0.05% to 1.1%. Although GBEB buffer (pH 7.2) elution buffer, with an overnight elution time gave the highest percentage (116.4%) recovery for HAV, the TGBE buffer (pH 9.5) elution buffer, with a 20 min elution time, gave the best overall recovery for all three viruses.

Table 3.3: Comparison of the percentage of viruses recovered from seeded green onions using three different washing buffers (PBS, TGBE and GBEB) at two different pHs (7.2 and 9.5) with a 20 min elution time.

Elution buffer/ Elution period	**Extraction efficiency of mengovirus**	**Extraction efficiency of HAV**	**Extraction efficiency of NoV GII**
PBS pH 7.2/ 20 min	0.60%	20.39%	5.96%
PBS pH 9.5/ 20 min	0.72%	36.83%	6.23%
TGBE pH 7.2/ 20 min	6.44%	79.13%	19.05%
TGBE pH 9.5/ 20 min	14.07%	59.08%	36.90%
GBEB pH 7.2/ 20 min	10.30%	62.30%	29.50%
GBEB pH 9.5/ 20 min	4.21%	58.59%	13.10%

Table 3.4: Comparison of the percentage of viruses recovered from seeded green onions using three different washing buffers (PBS, TGBE and GBEB) at two different pHs (7.2 and 9.5) with an overnight (12-14 hour) elution time.

Elution buffer/ elution period	**Extraction efficiency of mengo virus**	**Extraction efficiency of HAV**	**Extraction efficiency of NoV GII**
PBS pH 7.2/ Overnight	0.06%	0%	7.78%
PBS pH 9.5/ Overnight	0.74%	0%	12.82%
TGBE pH 7.2/ Overnight	0.07%	35.06%	8.23%
TGBE pH 9.5/ Overnight	1.10%	113.06%	32.53%
GBEB pH 7.2/ Overnight	0.05%	116.43%	12.97%
GBEB pH 9.5/ Overnight	0.15%	0%	0%

3.4 DISCUSSION

In this study the percentage recovery of three viruses, namely HAV, NoV GII and mengovirus, from two food matrices (green onions and tomatoes) were investigated in relation to elution buffer, elution buffer pH and elution time. Two of the elution buffers, namely PBS and TGBE, used were commonly used in other laboratory studies investigating the recovery of viruses from food matrices (Bidawid et al., 2000; Leggitt and Jaykus, 2000; Croci et al., 2002; Dubois et al., 2002; Butot et al., 2007). The GBEB buffer was included in the study as it has successfully been applied in the elution of viruses from glass-wool used for recovery of enteric viruses from water by adsorption-

elution (Taylor et al., 2001; Venter, 2004; Vivier et al., 2004; Ehlers et al., 2005).

In general buffers with a high pH (pH 9.5) were found to be more effective for eluting viruses from the food matrices tested, i.e. tomatoes and green onions (Tables 3.1-3.4), which was consistent with findings of other studies (Butot et al., 2007; Baert et al., 2008; Cheong et al., 2009b). However, the recovery of HAV did not always follow the same trend as a high percentage (29.88%) recovery from tomatoes was noted with PBS at pH 7.2 with overnight incubation. The results from this study highlight the fact that no-one method is optimal for the recovery of all enteric viruses from all food matrices. A compromise must be reached in order to apply one method for the recovery of a number of viruses from one food matrix. Therefore, based on the results of this study the TGBE buffer at pH 9.5 with a 20 min elution time was selected for further application using field samples.

CHAPTER 4

DETECTION AND CHARACTERISATION OF HEPATITIS A VIRUS AND NOROVIRUSES IN IRRIGATION WATER AND ON FRESH PRODUCE

This Chapter of the dissertation was part of an ongoing solicited research project (K5/1773/4) funded by the Water Research Commission and co-funded by the Department of Agriculture, South Africa

4.1 INTRODUCTION

Fresh fruit and vegetables or fresh produce are essential requirements for living and consumers are aware of the health benefits derived from their consumption (Johnston et al., 2005; Newman 2005; Selma et al., 2007). It is widely accepted that a diet high in fruit and vegetables has been associated with a decrease in susceptibility to cancer, diabetes, heart diseases and also enhanced weight management (Yeh et al., 2008). Many countries have undertaken various initiatives to encourage consumers to eat more of these products (Yeh et al., 2008; Berger et al., 2010). Until recently, fruit and vegetable availability were considered to be seasonal, but advances in global trade and horticultural practice have enabled the fruit and vegetable industry to supply produce to consumers all year round (European Commission, 2002; WHO, 2008). The globalisation of the food industry, however, has contributed to the increase in national and international outbreaks of foodborne infections (Koopmans et al., 2002; Duizer and Koopmans, 2008; Verhoef et al., 2010).

Fresh produce is increasingly being recognised as a major source of foodborne infection (Steele and Odumeru, 2004; Baert et al., 2008; Fino and Kniel, 2008; Berger et al., 2010; Tuan Zainazor, et al., 2010) and it is estimated that 40% of NoV and 5% of HAV infections are due to the consumption of contaminated foods (Le Guyader and Atmar, 2008). Food and water contaminated with

viruses may conform to acceptable bacterial standards (Verhoef et al., 2009; Newell et al., 2010) and look, taste and smell normal (Koopmans and Duizer, 2004). Fresh produce is vulnerable to contamination by human microbial pathogens at any point along the food chain from pre-harvest in the field to post-harvest during processing and packaging (Berger et al., 2010). Pre-harvest contamination originates from human or animal faecal contamination which reach the crops via contaminated irrigation water, improperly composted manure or wastewater (Barrimah et al., 1999; Steele and Odumeru, 2004; Baert et al., 2008; Wei and Kniel, 2010). Post-harvest contamination can be due to contaminated washing water (Steele and Odumeru, 2004), or infected food-handlers during harvest and packaging (Steele and Odumeru, 2004; Barrabeig et al., 2010; Berger et al., 2010; Tuan Zainazor et al., 2010). Due to infrequency of testing for viruses, viral contamination of food and water is less likely to be recognised than bacterial contamination (Verhoef et al., 2009).

Very little information is available in SA with respect to virological quality of irrigation water and fresh produce which is often consumed raw or lightly cooked. There was therefore a need to assess the virological quality of water used for the irrigation of fresh produce as well as the virological quality of fresh produce in SA. The objective of this study was to: i) apply the techniques optimised in Chapters 2 & 3 to the recovery and detection of NoVs and HAV in field samples of irrigation water and fresh produce, ii) to try and establish a link between contamination in irrigation water and on irrigated fresh produce and iii) to determine the extent of virological contamination on commercially available fresh produce.

4.2 MATERIALS AND METHODS

4.2.1 Site and sample selection

As this study formed part of a larger Water Research Commission collaborative project (Project K5/1773/4) the sample selection criteria, which

took the irrigation water source and the type of vegetables or fruit grown into consideration, were governed by the terms of reference of the umbrella project. Sampling sites in four different provinces, namely Limpopo, Gauteng, Mpumalanga and Western Cape, South Africa were selected. Researchers and post-graduate students from collaborating institutions were trained with regard to the optimised methodology for the collection of samples and recovery of viruses from large volumes of water and from fresh produce. Viruses were recovered from selected irrigation water and fresh produce samples by collaborating laboratories and the recovered virus suspensions were referred to the Department of Medical Virology, University of Pretoria, Pretoria for viral analysis. The water and fresh produce samples were analysed by collaborating laboratories for microbial indicators of faecal contamination and foodborne bacterial pathogens.

4.2.2 Irrigation water and fresh produce samples

4.2.2.1 Irrigation water samples

Irrigation water samples (10 ℓ) included untreated river and irrigation canal water used by subsistence and/or small scale farmers, and surface river water used by commercial farmers for irrigating fresh produce. Water samples were collected in a sterile container and transported in cooler bags to the laboratory and stored at 4°C until further processing.

In Limpopo, the irrigation water sites included: i) Gobe river in the Vhembe region at Farmer 1's farm, ii) Mvudi river in the Vhembe region at Farmer 2's farm, and iii) irrigation canal water from the Phadzima river used by the Phadzima community farm. In Mpumalanga, the irrigation water sites included: (i) surface water from an irrigation canal fed from the Loskop Dam (Site A); (ii) surface water from the Olifants river (Site B), and (iii) surface water from the Wilge river (Site C). In the North West province irrigation water was collected at a single site on the Crocodile river (Site WIII). In the Western Cape, the irrigation water sites included: i) a concrete furrow, which

was a branch of the Mosselbank river, about 1 km downstream from a sewage works (Site 1); (ii) water originating from the Mosselbank river used for irrigating vegetables (Site 2); (iii) the Plankenburg river just downstream of an industrial area and the Kayamandi informal settlement (Site 4); (iv) the Plankenburg river approximately 200 m before the merger of the Plankenburg and Eerste Rivers (Site 5); (v) the Plankenburg river at the confluence of the Plankenburg and Eerste Rivers (Site 6); (vi) an irrigation canal fed from the Eerste River just after the confluence with the Plankenburg River (Site 10); (vii) tap water drawn from a dam filled from the Plankenburg River (Site 11 = Site 10a); (ix) Berg river downstream of the confluence of the Berg river and Franschhoek river (Site 16); (x) Berg river about 15 km downstream of Site 1 between Franschhoek and Paarl (R farm: Site 17) and (xi) Berg River downstream of Site 2 at the Lady Loch Bridge close to Wellington (Site 18).

4.2.2.2 Fresh produce samples

To ensure that each fresh produce sample was a representative sample, a sample comprised of three individual items of the fruit/vegetable collected from different parts or areas of the field, crate, box or punnet, and each item was analysed individually. Samples were collected in separate sterile plastic bags, transported to the laboratory in cooler bags and stored at 4ºC until further processing and analysis. Samples included fruit/vegetables with a rough or convoluted surface, namely lettuce, cabbage, rocket and beans and fruit/vegetables with a smooth surface, namely tomatoes and green onions. Samples were collected either pre-harvest or at harvest (directly in the field) or post-harvest (from commercial outlets or street vendors). A subset of the abovementioned samples included fruit/vegetable samples paired or linked with irrigation water samples, i.e. these were samples of vegetable/fruit for which a sample of irrigation water used to irrigate the produce could be collected at the same time.

4.2.3 Viral recovery

4.2.3.1 Viral recovery from irrigation water

Prior to the viral recovery process each water sample was seeded with 10 µℓ (1 x 10^5 $TCID_{50}$) mengovirus as a process control. Viruses were recovered from the irrigation water by means of a glass wool adsorption-elution technique based on the method of Vilaginès et al. (1993) as described previously by Wolfaardt et al. (1995b), Grabow et al., (1996) and Vivier et al. (2004), and further modified and optimised by Venter (2004). Briefly, irrigation water samples (10ℓ) were filtered through the positively charged glass wool columns using 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 of GBEB (0.05 M glycine [Merck], 0.5% beef extract [BBL™] pH 9.5). The eluting solution was left in contact with the glass wool for 5 min before being passed through the filter under pressure, whereafter the pH was adjusted to pH 7 using 1 M HCl (Merck). The recovered viruses were subjected to secondary concentration using PEG/NaCl (Merck) precipitation (Minor, 1985; Vilaginès et al., 1997). The resulting pellet was re-suspended in 20 mℓ of PBS pH 7.2 (Sigma-Aldrich) and recovered virus samples were stored at -20 ºC until further analysis.

4.2.3.2 Viral recovery from fresh produce

Prior to the viral recovery process each item of non-leafy fruit/vegetable was weighed and then seeded with 10 µℓ (1 x 10^5 $TCID_{50}$) mengovirus as a process control. For leafy vegetables, the outer leaves of the cabbage or lettuce were selected and cut to fit the area of a 140 mm Sterilin Petri dish (Bibby Sterilin Ltd, Stone, UK) and then seeded with mengovirus. Viruses were recovered from the fruit/vegetable samples using 40 mℓ TGBE buffer, pH 9.5, with elution for 20 min at room temperature (refer: Sections 3.2.3.2). Thereafter, the viruses were further concentrated to final volume of 500 µℓ in PBS pH 7.2 (Sigma-Aldrich) using PEG/NaCl (Merck) precipitation (refer: Section

3.2.3.3). The recovered virus suspension was stored at -20 ºC until further analysis.

4.2.4 Viral detection

4.2.4.1 Nucleic acid extraction

Genomic viral nucleic acid was extracted directly from either 1 ml (water) or 500 µℓ (fresh produce) of the recovered virus concentrate using the MagNA Pure LC Total Nucleic Acid Isolation Kit (large volume)(Roche), in a MagNA Pure LC instrument (Roche), following the manufacturer's instructions. An aliquot of nuclease-free water (Promega Corp., Madison, WI) was included in each batch of extractions as a negative extraction control. The purified nucleic acid (100 µℓ) was aliquoted and stored at -70 °C.

4.2.4.2 Viral amplification and detection

Primers and Probes: The primers and probes used for the detection of HAV, Nov GI, NoV GII and mengovirus were the same as those described previously (refer: Table 2.1).

Detection: Molecular amplification and real-time RT-PCR detection of NoV GI and NoV GII was done using the one-step QuantiTect Probe® RT-PCR kit (Qiagen) on the LightCycler 1.5 (Roche). Five microlitres of extracted viral RNA is added to the Quantitect Probe Master Mix, containing 4 pmol each of the forward primer, reverse primer and labelled TaqMan probe for the detection of NoV GI and GII, respectively. The cycling conditions were used as described by the manufacturer. For the detection of HAV the one-step RNA Ultrasense™ One-step qRT-PCR system (Invitrogen) was applied as it was found to be more sensitive than the Quantitect Probe RT-PCR kit (Qiagen). For the reaction, 5 µl RNA, 10 pmol of the forward primer, 18 pmol of the reverse primer and 5 pmol of the labelled probe was added to the Master Mix and using cycling conditions which were customised for HAV (refer: Section 2.2.5). The *rt* RT-PCR amplification for mengovirus was done using the QuantiTect Probe® RT-PCR kit (Qiagen) using five microlitres of nucleic acid and 10 pmol each of the forward primer, 18 pmol of the reverse primer

and 5 pmol of the labelled TaqMan probe. The reaction parameters were the same as for HAV. Included in each set or batch of *rt* RT-PCR reactions were a negative RNA extraction control, a negative *rt* RT-PCR control (nuclease-free water: Promega Corp.), and a *rt* RT-PCR positive control which comprised of RNA from the target virus.

4.2.5 Viral characterisation

4.2.5.1 Norovirus characterisation

Norovirus strains from samples that were NoV positive by real-time RT-PCR were genetically characterised, i.e. genotyped, by DNA sequence analysis of the 5' end of the NoV capsid gene (Region C) as described by Mans et al. (2010). Briefly, cDNA was prepared using the Transcriptor First Strand cDNA Synthesis (Roche). After reverse transcription, conventional PCR was performed where viral cDNA was amplified using primers G2SKF and G2SKR (Kojima et al., 2002) and HotStartTaq (Qiagen) according to the manufacturer's instructions. M13 (-21) and M13REV primer sequences (Messing, 1983) were attached at the 5' end of the primers to facilitate sequencing using the ABI PRISM BigDye® Terminator v3.1 Cycle Sequencing Kit on an ABI automated analyser (Applied Biosystems, Foster City, CA). Nucleotide sequences were analysed using Sequencer™ 4.9 (Gene CODES Corporation, Ann Arbor, MI), BioEdit Sequence Alignment Editor (V.7.09.0)(Hall, 1999) and BLAST-N (Altschul et al., 1997). Sequences were aligned with reference sequences from NoV GI and GII (Table 4.1) using MAFFT Version 6 (http://mafft.cbrc.jp/alignment/server/). After manual adjustment of the alignment, phylogenetic analysis was performed with MEGA 4 using neighbour-joining methods (Tamura et al., 2007). For determining the reliability of tree topology, bootstrap analysis was carried out on 1,000 replicates.

Table 4.1 Summary of norovirus gene sequences used in phylogenetic analysis.

Genotype	GenBank Accession no.	Strain	Year	Country of Origin
GII.1	AF414419 GU138768 U07611	283 8738 Hawaii	1994 2008 1971	United States South Africa: sporadic United States
GII.2	HQ003270 X81879	1605 Melksham	2009 1994	Russia United Kingdom
GII.4	HM635154 HQ008055 HQ003284 X76716	0399 8483 1296 Bristol	2009 2008 2010 1993	Korea South Africa: sporadic Russia United Kingdom
GII.6	HQ201670 HQ201668 GU969057 AJ277620	08-06-02b 08-05-26 9088/Maizuru Seacroft	2008 2008 2008 1990	South Africa: river water South Africa: river water Japan United Kingdom
GII.9	HQ201673 AY038599	08-08-18b-RV2 VA97207	2008 1997	South Africa: river water USA
GI.1	M87661	Norwalk virus	2006	USA

4.2.5.2 Hepatitis A virus characterisation

To confirm the identity of the detected HAV amplicons the strains were characterised by sequencing using the VP1/P2A junction region (Roberston et al., 1992). Viral RNA was transcribed to cDNA using the Transcriptor First Strand cDNA Synthesis (Roche). The VP1 region was amplified, by nested PCR, using 10 pmol of one or both RNA sense primers, 25 mM $MgCl_2$, 5 U Taq-polymerase and 5 X reaction buffer (Promega Corp.). Sequencing reactions were performed using the ABI PRISM BigDye® Terminator v3.1 Cycle Sequencing Kit on an ABI automated analyser (Applied Biosystems). The nucleotide sequences were entered into a database and verified using the BioEdit Sequence Alignment Editor V6.0.5 (Isis Pharmaceuticals, Inc.). Nucleotide sequences were analysed using Sequencer™ 4.9 (Gene CODES Corporation), BioEdit Sequence Alignment Editor (V.7.09.0) and BLAST-N. Sequences were aligned with reference sequences of HAV using MAFFT Version 6 (https://align.bmr.kyushu-u.ac.jp/mafft/online/server/). Published HAV gene sequences used in the phylogenetic analysis are listed in Table 4.2.

After manual adjustment of the alignment, phylogenetic analysis was performed with MEGA 4 using neighbour-joining methods. For determining the reliability of tree topology, bootstrap analysis was carried out on 1,000 replicates.

Table 4.2 Summary of hepatitis A virus gene sequences used in phylogenetic analysis.

Genotype	GenBank Accession no.	Strain	Country of Origin
IA	EU131373	HAV5	Uruguay : sporadic
	U66489	VDM	South Africa: Military recruit
	AF357222	LU38/WT	China:
	EF406357	H2	China:
	AY974170	M2	Cuba:
	M22825	MS1	Willowbrook, N.Y.
	U68696	SA-923359	South Africa: Outbreak
	L07676	GA88	Atlanta, Georgia
	EU930199	HAV	Kenya (Germany): Tourist
	U68695	SA-406808	South Africa: sporadic
IB	EU416266	034	South Africa (Germany): Tourist
	EU416265	166	South Africa (Germany): Tourist
	EU416264	126	South Africa (Germany): Tourist
	M59809	HM 175	Australia
	AF268396	HAF 203	Brazil
	AF314208	L-A-1	China
	U68698	SA-2333	South Africa: Outbreak
	U68692	SA-JVR	South Africa: Military recruit
	M20273	MBB	North Africa: (Germany): Tourist
	L07728	Jor88	Jordan
	U68699	SA-314274	South Africa: Sporadic
	U66485	SA-504184	South Africa: Sporadic
IIA	AY644676	CF53/Berne	Germany
IIB	AY644670	SLF88	Sierra Leone
IIIA	FJ227135	SIM27	India
	L07668	GA76	USA
	M34084	PA21	Panama: Owl Monkey
IIIB	AB279735	HAJ85-1	Japan
	AB258387	HA-JNG06-90F	Japan
IV	M59286	Cy145	Philippines (USA): Macaque Monkey
V	D00924	AGM-27	Kenya (USSR): African Green Monkey
VI	L07731	JM55	Indonesia (USSR): Macaque Monkey

4.3 RESULTS

4.3.1 Irrigation water and fresh produce samples

From April 2008 to November 2009, 72 fresh produce samples (cabbage, tomato, spinach, onion, lettuce, rocket, beans) were collected from commercial and subsistence farms (43), street vendors (11) and commercial outlets (18) (Table 4.3). Because of sampling limitations, small numbers of other produce items (rocket, onions and beans) were collected. Irrigation water samples (86) were collected from surface water sources (Table 4.4).

Table 4.3: Summary of the number and type of fresh produce samples collected according to their source and province of origin.

	Cabbage *(n = 34)*	Spinach *(n = 4)*	Lettuce *(n = 5)*	Rocket *(n = 1)*	Onion *(n = 2)*	Tomato *(n = 24)*	Beans *(n = 2)*
Gauteng							
Commercial Farm	0	0	0	0	0	0	0
Subsistence Farm	0	0	0	0	0	0	0
Street Vendor	2	2	1	0	0	5	0
Commercial outlet	5	1	1	1	1	5	0
Limpopo							
Commercial Farm	1	1	0	0	0	0	0
Subsistence Farm	22	0	0	0	0	13	0
Street Vendor	0	0	0	0	0	1	0
Commercial outlet	0	0	1	0	1	0	0
Western Cape							
Commercial Farm	3	0	1	0	0	0	2
Subsistence Farm	0	0	0	0	0	0	0
Street Vendor	0	0	0	0	0	0	0
Commercial outlet	0	0	0	0	0	0	0
Farm stall	1	0	1	0	0	0	0

Table 4.4: Summary of the number, source and province of origin of the irrigation water samples.

Geographical region	River *(n = 59)*	Tap *(n = 2)*	Irrigation canal *(n = 25)*
Limpopo	2	0	13
Mpumalanga	12	0	6
North West	1	0	0
Western Cape	44	2	6

4.3.2 Viral detection

4.3.2.1 Irrigation water

Among 86 water samples analysed, 11/59 (18.6 %) of river water samples, 0/2 of tap water samples, and 3/25 (12%) of irrigation canal samples were positive for the presence of one or more enteric viruses, respectively.

Limpopo: From Table 4.5, it was evident that no viruses were detected in the river water sources. Hepatitis A virus (untypable) was, however, detected in the irrigation canal water sample collected in June 2009 (winter) and NoV GII (untypable) in the water sample collected in October 2008 (late spring). In total, HAV was found to be present in 7.7 % and NoV GII in 7.7 % of the irrigation canal water samples, while NoV GI was not detected at all.

Table 4.5: Summary of results of viral analysis of irrigation water samples from Limpopo.

Sample site, name and location	Sample date	Real-time RT-PCR analysis		
		NoV GI	NoV GII	HAV
Farmer 1: River Water	2008.09.23	Neg	Neg	Neg
Farmer 2: River Water	2008.09.23	Neg	Neg	Neg
Site 1: Irrigation canal water (Phadzima community farm)	2008.10.27	Neg	**Pos**	Neg
	2008.11.17	Neg	Neg	Neg
	2008.12.01	Neg	Neg	Neg
	2009.01.26	Neg	Neg	Neg
	2009.02.16	Neg	Neg	Neg
	2009.03.16	Neg	Neg	Neg
	2009.05.18	Neg	Neg	Neg
	2009.06.24	Neg	Neg	**Pos**
	2009.07.20	⇦ Neg	Neg	Neg
	2009.08.17	⇦ Neg	Neg	Neg
	2009.09.07	Neg	Neg	Neg
	2009.10.13	Neg	Neg	Neg
	2009.11.02	Neg	Neg	Neg

⇨ : Real-time RT-PCR analysis for mengovirus = Positive
⇦ : Real-time RT-PCR analysis for mengovirus = Negative

North West: No enteric viruses, NoV GI, NoV GII and HAV, were detected in the single irrigation water sample from the North West Province (Table 4.6).

Table 4.6: Summary of results of viral analysis of irrigation water samples from North West.

Sample site, name and location	Sample date	Real-time RT-PCR analysis		
		NoV GI	NoV GII	HAV
Site WIII: River water (Crocodile)	2008.05.30	⇨ Neg	Neg	Neg

⇨ : Real-time RT-PCR analysis for mengovirus = Positive
⇦ : Real-time RT-PCR analysis for mengovirus = Negative

Mpumalanga: From February 2008 to September 2008, a total of 18 irrigation water samples, six from each site, were analysed on a monthly basis. Norovirus GII was detected in one or more samples from each site (Table 4.7). Of interest was the fact that NoV GII was detected at all three sites in the September (early spring) sampling period, while NoV GII was also detected in June (early winter). All four NoV GII strains could not be typed. Neither NoV GI nor HAV were detected in any of the samples analysed.

Western Cape: From May 2008 to December 2008, a total of 59 irrigation water samples were analysed for NoV GI, NoV GII and HAV. Norovirus GII was detected in ~12% (7/59) and HAV in ~5% (3/59) of the samples, while NoV GI was not detected in any of the samples (Table 4.8). In the Mosselbank river sites (Sites 1 & 2) NoV GII.4 (Figure 4.1) was detected in the river water (Site 1) in June (winter) 2008 and NoV GII.2 and HAV in September 2008, while no viruses were detected in the irrigation water (Site 2: Table 4.8). The Plankenburg river (Site 4) showed a high level of viral contamination in the late spring and summer months with NoV GII and HAV being detected in one of the samples drawn in October 2008 and HAV being detected in the sample of December 2008. No viruses were detected in the surface water from Sites 5, 6, 10 and 11. Two of the three sampling sites (Site 16 and Site 18) from the Berg river showed virological contamination in June

Table 4.7: Summary of results of viral analysis of irrigation water samples from Mpumalanga.

Sample site, name and location	Sample date	Real-time RT-PCR analysis		
		NoV GI	NoV GII	HAV
Site A: Irrigation canal water (from Loskop dam)	2008.02.20	⇦ Neg	Neg	Neg
	2008.03.?	⇦ Neg	Neg	Neg
	2008.04.02	⇦ Neg	Neg	Neg
	2008.04.28	⇦ Neg	Neg	Neg
	2008.07.09	⇨ Neg	Neg	Neg
	2008.09.09	⇦ Neg	**Pos**	Neg
Site B: River water (Olifants river)	2008.02.20	⇦ Neg	Neg	Neg
	2008.03.?	⇦ Neg	Neg	Neg
	2008.04.02	⇦ Neg	Neg	Neg
	2008.05.11	⇦ Neg	**Pos**	Neg
	2008.07.09	⇨ Neg	Neg	Neg
	2008.09.09	⇦ Neg	**Pos**	Neg
Site C: River water (Wilge river)	2008.02.20	⇦ Neg	Neg	Neg
	2008.03.?	⇦ Neg	Neg	Neg
	2008.04.02	⇦ Neg	Neg	Neg
	2008.04.28	⇦ Neg	Neg	Neg
	2008.07.09	⇨ Neg	Neg	Neg
	2008.09.09	⇦ Neg	**Pos**	Neg

⇨ : Real-time RT-PCR analysis for mengovirus = Positive
⇦ : Real-time RT-PCR analysis for mengovirus = Negative

and September 2008 with NoV GII being detected at both sites in both months (Table 4.8). Two NoV GII genotypes could be identified, i.e. NoV GII.4 and NoV GII.6 (Figure 4.1), with NoV GII.6 being detected upstream at Site 16 and NoV GII.4 being detected downstream at Site 18.

Table 4.8: Summary of results of viral analysis of irrigation water samples from Western Cape.

Sample site, name and location	Sample date	Real-time RT-PCR analysis		
		NoV GI	NoV GII	HAV
Site 1: River Water (B-R Buhr) (Mosselbank River)	2008.05.05	Neg	Neg	Neg
	2008.06.17	⇦ Neg	**Pos:GII.4**	Neg
	2008.09.08	⇨ Neg	**Pos: GII.2**	**Pos**
Site 2: Irrigation water (B-B Buhr) (Mosselbank River)	2008.06.17	Neg	Neg	Neg
	2008.08.08	Neg	Neg	Neg
	2008.09.08	Neg	Neg	Neg
Site 4: River water (Plankenburg River)	2008.05.05	Neg	Neg	Neg
	2008.05.19	⇦ Neg	Neg	Neg
	2008.05.?	Neg	Neg	Neg
	2008.06.17	Neg	Neg	Neg
	2008.09.08	Neg	Neg	Neg
	2008.10.06	⇦ Neg	Neg	Neg
	2008.10.?	⇦ Neg	Neg	Neg
	2008.10.?	⇦ Neg	**Pos**	**Pos**
	2008.12.?	⇦ Neg	Neg	**Pos**
Site 5: River water (Eerste River)	2008.05.19	⇦ Neg	Neg	Neg
	2008.06.17	Neg	Neg	Neg
	2008.09.08	Neg	Neg	Neg
	2008.10.06	⇦ Neg	Neg	Neg
	2008.10.?	⇦ Neg	Neg	Neg
	2008.10.?	⇦ Neg	Neg	Neg
	2008.12.?	⇦ Neg	Neg	Neg
Site 6: River water (Boord)	2008.09.08	Neg	Neg	Neg
	2008.10.06	⇦ Neg	Neg	Neg
	2008.10.?	⇦ Neg	Neg	Neg
	2008.10.?	⇦ Neg	Neg	Neg
	2008.12.?	⇦ Neg	Neg	Neg

⇨ : Real-time RT-PCR analysis for mengovirus = Positive
⇦ : Real-time RT-PCR analysis for mengovirus = Negative

Table 4.8 *continued*: Summary of results of viral analysis of irrigation water samples from Western Cape.

Sample site, name and location	Sample date	Real-time RT-PCR analysis		
		NoV GI	NoV GII	HAV
Site 10: Irrigation canal water (Roux sloot)	2008.05.?	Neg	Neg	Neg
	2008.05.?	Neg	Neg	Neg
	2008.10.06	⇦ Neg	Neg	Neg
	2008.10.?	⇦ Neg	Neg	Neg
	2008.10.?	Neg	Neg	Neg
	2008.12.?	⇦ Neg	Neg	Neg
Site 11: Irrigation tap water (Roux tap)	2008.05.19	Neg	Neg	Neg
	2008.12.?	⇨ Neg	Neg	Neg
Site 16: River water (Berg 1) (Berg river: Franschoek)	2008.06.17	⇦ Neg	**Pos**	Neg
	2008.09.08	Neg	Neg	Neg
	2008.09.?	Neg	**Pos:GII:6**	Neg
	2008.09.22	⇦ Neg	Neg	Neg
	2008.11.?	Neg	Neg	Neg
	2008.12.?	Neg	Neg	Neg
	2008.12.?	⇦ Neg	Neg	Neg
	2008.12.?	⇦ Neg	Neg	Neg
Site 17: River water (Berg 2) (Berg river: R-farm)	2008.06.17	⇦ Neg	Neg	Neg
	2008.09.08	Neg	Neg	Neg
	2008.09.?	Neg	Neg	Neg
	2008.09.22	⇦ Neg	Neg	Neg
	2008.09.?	Neg	Neg	Neg
	2008.11.?	Neg	Neg	Neg
	2008.12.?	Neg	Neg	Neg
	2008.12.?	⇦ Neg	Neg	Neg

⇨ : Real-time RT-PCR analysis for mengovirus = Positive
⇦ : Real-time RT-PCR analysis for mengovirus = Negative

Table 4.8 *continued*: Summary of results of viral analysis of irrigation water samples from Western Cape.

Sample site, name and location	Sample date	Real-time RT-PCR analysis		
		NoV GI	NoV GII	HAV
Site 18: River water (Berg 3)	2008.05.19	⇦ Neg	Neg	Neg
(Berg river: Lady Loch)	2008.06.17	⇦ Neg	**Pos:GII.4**	Neg
	2008.09.08	Neg	Neg	Neg
	2008.09.?	Neg	**Pos**	Neg
	2008.09.22	Neg	Neg	Neg
	2008.10.22	⇦ Neg	Neg	Neg
	2008.11.?	Neg	Neg	Neg
	2008.12.?	Neg	Neg	Neg
	2008.12.?	Neg	Neg	Neg

⇨ : Real-time RT-PCR analysis for mengovirus = Positive
⇦ : Real-time RT-PCR analysis for mengovirus = Negative

4.3.2.2 Fresh produce pre-harvest/at harvest

Among fresh produce samples collected pre-harvest or at harvest, 0/13 samples with smooth surfaces and 2/29 (6.9%) samples with rough or convoluted surfaces were positive for the presence of one enteric virus, respectively.

Limpopo: No viruses were detected on fresh produce with smooth or rough convoluted surfaces collected in the field from either Farmer 2 or Site 1 (Phadzima community farm). Norovirus GII (untypable) was detected on spinach (fresh produce with rough convoluted surface) from Farmer 1's field (Table 4.9).

Table 4.9: Summary of results of viral analysis of fresh produce samples from Limpopo.

Sample site, name, location	Fresh Produce	Sample date	Real-time RT-PCR analysis		
			NoV GI	NoV GII	HAV
Farmer 1:	Spinach (a)	2008.09.23	Neg	Neg	Neg
	Spinach (b)		Neg	Neg	Neg
	Spinach (c)		Neg	**Pos**	Neg
Farmer 2:	Cabbage (a)	2008.09.23	Neg	Neg	Neg
	Cabbage (b)		Neg	Neg	Neg
	Cabbage (c)		Neg	Neg	Neg
Site 1:	Tomato	2008.10.27	Neg	Neg	Neg
	Tomato	2008.11.17	Neg	Neg	Neg
	Tomato	2008.12.10	Neg	Neg	Neg
	Tomato	2009.01.26	Neg	Neg	Neg
	Tomato	2009.02.16	Neg	Neg	Neg
	Tomato	2009.03.16	Neg	Neg	Neg
	Tomato	2009.04.27	Neg	Neg	Neg
	Tomato	2009.05.18	Neg	Neg	Neg
	Tomato	2009.06.24	⇨ Neg	Neg	Neg
	Tomato	2009.07.20	⇦ Neg	Neg	Neg
	Tomato	2009.08.17	⇦ Neg	Neg	Neg
	Tomato	2009.10.13	⇨ Neg	Neg	Neg
	Tomato	2009.11.02	Neg	Neg	Neg

⇨ : Real-time RT-PCR analysis for mengovirus = Positive
⇦ : Real-time RT-PCR analysis for mengovirus = Negative

Table 4.9 *continued*: Summary of results of viral analysis of fresh produce samples from Limpopo.

Sample site, name, location	Fresh Produce	Sample date	Real-time RT-PCR analysis		
			NoV GI	NoV GII	HAV
Site 1:	Cabbage C*	2008.10.27	Neg	Neg	Neg
	Cabbage C*	2008.11.17	Neg	Neg	Neg
	Cabbage C*	2008.12.10	Neg	Neg	Neg
	Cabbage C*	2009.01.26	Neg	Neg	Neg
	Cabbage C*	2009.02.16	Neg	Neg	Neg
	Cabbage C*	2009.03.16	Neg	Neg	Neg
	Cabbage C*	2009.04.27	Neg	Neg	Neg
	Cabbage C*	2009.05.18	Neg	Neg	Neg
	Cabbage C*	2009.06.24	⇨ Neg	Neg	Neg
	Cabbage C*	2009.07.20	⇨ Neg	Neg	Neg
	Cabbage C*	2009.08.17	⇨ Neg	Neg	Neg
	Cabbage C*	2009.09.07	⇨ Neg	Neg	Neg
	Cabbage C*	2009.10.13	⇨ Neg	Neg	Neg
	Cabbage C*	2009.11.02	Neg	Neg	Neg
	Cabbage T†	2008.10.27	Neg	Neg	Neg
	Cabbage T†	2008.11.17	Neg	Neg	Neg
	Cabbage T†	2008.12.10	Neg	Neg	Neg
	Cabbage T†	2009.01.26	Neg	Neg	Neg
	Cabbage T†	2009.02.16	Neg	Neg	Neg
	Cabbage T†	2009.03.16	Neg	Neg	Neg
	Cabbage T†	2009.04.27	Neg	Neg	Neg
	Cabbage T†	2009.11.02	Neg	Neg	Neg

C*: Variety Conquest
T†: Variety Tennesse
⇨ : Real-time RT-PCR analysis for mengovirus = Positive
⇦ : Real-time RT-PCR analysis for mengovirus = Negative

Western Cape: No viruses were detected on cabbage (rough convoluted surface) collected in the field and which had been irrigated with water from the Mosselbank river (Site 2), or from beans irrigated with tap water drawn

from a dam filled from the Plankenburg River (Table 4.10). Norovirus GII.1 was detected on a cabbage sample, at harvest, which had been irrigated with water from the Berg river (Site 17: R-farm).

Table 4.10: Summary of results of viral analysis of fresh produce samples from Western Cape.

Sample site, name, location	Fresh Produce	Sample date	Real-time RT-PCR analysis		
			NoV GI	NoV GII	HAV
Site 3b/B-K (Buhr)/ Mosselbank	Cabbage	2008.09.08	Neg	Neg	Neg
Site 3b/B-K (Buhr)/ Mosselbank	Cabbage	2008.09.08	Neg	Neg	Neg
Twkloof /Berg 2	Cabbage	2008-08	⇨ Neg	**Pos:GII.1**	Neg
Twkloof/Berg 2	Lettuce	2008-08	Neg	Neg	Neg
Site 11: Roux tap	Beans		Neg	Neg	Neg
Site 11: Roux tap	Beans		Neg	Neg	Neg

⇨ : Real-time RT-PCR analysis for mengovirus = Positive
⇦ : Real-time RT-PCR analysis for mengovirus = Negative

4.3.2.3 Fresh produce post-harvest

Among fresh produce samples collected post-harvest, 2/13 (15.4%) samples with smooth surfaces and 5/17 (29.4%) samples with rough or convoluted surfaces tested positive for one or more enteric viruses, respectively (Table 4.11; 4.12 and 4.13).

Limpopo: Viruses were detected on two vegetable samples, namely one lettuce sample from a supermarket and an onion sample from a street vendor, both from Thohoyandou (Table 4.11).

Table 4.11: Summary of results of viral analysis of fresh produce samples from Limpopo.

Sample site, name, location	Fresh produce	Sample date	Real-time RT-PCR analysis		
			NoV GI	NoV GII	HAV
Supermarket (Thohoyandou)	Lettuce (a)	2008.09.26	Neg	Neg	Neg
	Lettuce (b)		Neg	**Pos**	Neg
Street Vendor (Thohoyandou)	Tomato (a)	2008.09.26	Neg	Neg	Neg
	Tomato (b)		Neg	Neg	Neg
	Tomato (c)		Neg	Neg	Neg
	Onion (a)	2008.09.26	⇨ Neg	Neg	Neg
	Onion (b)		⇨ Neg	**Pos**	Neg
	Onion (c)		Neg	Neg	Neg

⇨ : Real-time RT-PCR analysis for mengovirus = Positive
⇦ : Real-time RT-PCR analysis for mengovirus = Negative

Gauteng: Enteric viruses were detected on fresh produce from both commercial outlets and the informal sector. However, from Table 4.12 it was evident that fresh produce available from street vendors was more likely to be contaminated with enteric viruses than fresh produce from the formal commercial sector. Multiple enteric viruses were detected on the fresh produce from street vendors (street vendors 7 and 8) from Marabastad, Pretoria, Tshwane Metropolitan Area. A lettuce sample from street vendor 7 yielded untypable NoVs and HAV on two of the three items which comprised a sample. No viruses were detected on the third item of the sample. The tomato sample from street vendor 8 yielded NoV GII.9 and HAV IB on one of the three items which comprised the single sample.

Table 4.12: Summary of results of viral analysis of fresh produce samples from Gauteng.

Sample site, name, location	Fresh produce	Sample date	Real-time RT-PCR analysis		
			NoV GI	NoV GII	HAV
Greengrocer (Housewive's market)	Tomato (a)	2009.07.22	Neg	Neg	Neg
	Tomato (b)		Neg	Neg	Neg
	Tomato (c)		Neg	Neg	Neg
	Cabbage (a)	2009.07.22	Neg	Neg	Neg
	Cabbage (b)		Neg	Neg	Neg
	Cabbage (c)		Neg	Neg	Neg
	Spinach (a)	2009.07.22	Neg	Neg	Neg
	Spinach (b)		Neg	Neg	Neg
	Spinach (c)		Neg	Neg	Neg
	Tomato (a)	2009.08.15	Neg	Neg	Neg
	Tomato (b)		Neg	Neg	Neg
	Tomato (c)		Neg	Neg	Neg
	Cabbage (a)	2009.08.15	Neg	Neg	**Pos**
	Cabbage (b)		Neg	Neg	Neg
	Cabbage (c)		Neg	Neg	Neg
	Spinach (a)	2009.08.15	Neg	Neg	Neg
	Spinach (b)		Neg	Neg	Neg
	Spinach (c)		Neg	Neg	Neg
Supermarket 1 (Upmarket food store)	Rocket (a)	2009.08.15	Neg	**Pos**	Neg
	Rocket (b)		Neg	Neg	Neg
	Rocket (c)		Neg	Neg	Neg
Supermarket 2	Tomato (a)	2009.09.19	⇨ Neg	Neg	Neg
	Tomato (b)		⇨ Neg	Neg	Neg
	Tomato (c)		⇨ Neg	Neg	Neg

⇨ : Real-time RT-PCR analysis for mengovirus = Positive
⇦ : Real-time RT-PCR analysis for mengovirus = Negative

Table 4.12 *continued:* Summary of results of viral analysis of fresh produce samples from Gauteng.

Sample site, name, location	Fresh produce	Sample date	Real-time RT-PCR analysis		
			NoV GI	NoV GII	HAV
Supermarket 3	Tomato (a)	2009.09.19	⇨ Neg	Neg	Neg
	Tomato (b)		⇨ Neg	Neg	Neg
	Tomato (c)		⇨ Neg	Neg	Neg
Farmer's market (Boeremark)	Tomato (a)	2009.07.	Neg	Neg	Neg
	Tomato (b)		Neg	Neg	Neg
	Tomato (c)		Neg	Neg	Neg
	Onion (a)	2009.07.	Neg	Neg	Neg
	Onion (b)		Neg	Neg	Neg
	Onion (c)		Neg	Neg	Neg
	Lettuce (a)	2009.07.	Neg	Neg	Neg
	Lettuce (b)		Neg	Neg	Neg
	Lettuce (c)		Neg	Neg	Neg
	Cabbage (a)	2009.10.10	⇨ Neg	Neg	Neg
	Cabbage (b)		⇨ Neg	Neg	Neg
	Cabbage (c)		⇨ Neg	Neg	Neg
	Cabbage (a)	2009.10.10	⇨ Neg	Neg	Neg
	Cabbage (b)		⇨ Neg	Neg	Neg
	Cabbage (c)		⇨ Neg	Neg	Neg
	Cabbage (a)	2009.10.10	⇨ Neg	Neg	Neg
	Cabbage (b)		⇨ Neg	Neg	Neg
	Cabbage (c)		⇨ Neg	Neg	Neg
Street Vendor 1	Tomato (a)	2009.09.19	⇨ Neg	Neg	Neg
	Tomato (b)		⇨ Neg	Neg	Neg
	Tomato (c)		⇨ Neg	Neg	Neg
Street Vendor 2 (Arcadia)	Tomato (a)	2009.09.26	⇨ Neg	Neg	Neg
	Tomato (b)		⇨ Neg	Neg	Neg
	Tomato (c)		⇨ Neg	Neg	Neg

⇨ : Real-time RT-PCR analysis for mengovirus = Positive
⇦ : Real-time RT-PCR analysis for mengovirus = Negative

Table 4.12 *continued:* Summary of results of viral analysis of fresh produce samples from Gauteng.

Sample site, name, location	Fresh produce	Sample date	Real-time RT-PCR analysis		
			NoV GI	NoV GII	HAV
Street Vendor 3 (Sunnyside)	Tomato (a)	2009.09.26	⇨ Neg	Neg	Neg
	Tomato (b)		⇨ Neg	Neg	Neg
	Tomato (c)		⇨ Neg	Neg	Neg
Street Vendor 4 (Central)	Tomato (a)	2009.09.26	⇨ Neg	Neg	Neg
	Tomato (b)		⇨ Neg	Neg	Neg
	Tomato (c)		⇨ Neg	Neg	Neg
Street Vendor 5 (Marabastad)	Spinach (a)	2009.10.17	⇨ Neg	Neg	Neg
	Spinach (b)		⇨ Neg	Neg	Neg
	Spinach (c)		⇨ Neg	Neg	Neg
Street Vendor 6 (Marabastad)	Cabbage (a)	2009.10.17	⇨ Neg	Neg	Neg
	Cabbage (b)		⇨ Neg	Neg	Neg
	Cabbage (c)		⇨ Neg	Neg	Neg
Street Vendor 7 (Marabastad)	Lettuce (a)	2009.10.17	⇨ Neg	**Pos**	**Pos**
	Lettuce (b)		⇨ Neg	**Pos**	**Pos: 1B**
	Lettuce (c)		⇨ Neg	Neg	Neg
Street Vendor 8 (Marabastad)	Tomato (a)	2009.11.07	⇨ Neg	Neg	Neg
	Tomato (b)		⇨ Neg	Neg	Neg
	Tomato (c)		⇨ Neg	**Pos: GII.9**	**Pos: 1B**
Street Vendor 9 (Marabastad)	Spinach (a)	2009.11.07	⇨ Neg	Neg	Neg
	Spinach (b)		⇨ Neg	Neg	Neg
	Spinach (c)		⇨ Neg	Neg	Neg
Street Vendor 10 (Marabastad)	Cabbage (a)	2009.11.07	⇨ Neg	Neg	Neg
	Cabbage (b)		⇨ Neg	Neg	**Pos**
	Cabbage (c)		⇨ Neg	Neg	Neg

⇨ : Real-time RT-PCR analysis for mengovirus = Positive
⇦ : Real-time RT-PCR analysis for mengovirus = Negative

Western Cape: Although only a limited number of post-harvest fresh produce samples were tested, no viruses were detected in the fresh produce from a farm stall which had been irrigated with water from Plankenburg river.

Table 4.13: Summary of results of viral analysis of fresh produce samples from Western Cape.

Sample site, name, location	Fresh produce	Sample date	Real-time RT-PCR analysis		
			NoV GI	NoV GII	HAV
Friend farm	Lettuce	2008.08.08	Neg	Neg	Neg
	Cabbage		⇨ Neg	Neg	Neg

⇨ : Real-time RT-PCR analysis for mengovirus = Positive
⇦ : Real-time RT-PCR analysis for mengovirus = Negative

4.3.2.4 Linked (paired) irrigation water and vegetable samples

Limpopo: Irrespective of the farms from which they were collected, there was no correlation between the occurrence of viruses in the irrigation water samples and in the linked fresh produce samples collected in the field or at harvest (Table 4.14).

Table 4.14: Summary of results of viral analysis of linked irrigation water and fresh produce samples from Limpopo.

Sample site, name, location	Samples	Sample date	Real-time RT-PCR analysis		
			NoV GI	NoV GII	HAV
Set 1: Farmer 1	River Water	2008.09.23	Neg	Neg	Neg
	Spinach (a)		⇨ Neg	Neg	Neg
	Spinach (b)		⇨ Neg	Neg	Neg
	Spinach (c)		⇨ Neg	**Pos**	Neg

⇨ : Real-time RT-PCR analysis for mengovirus = Positive
⇦ : Real-time RT-PCR analysis for mengovirus = Negative

Table 4.14 *continued:* Summary of results of viral analysis of linked irrigation water and fresh produce samples from Limpopo.

Sample site, name, location	Samples	Sample date	Real-time RT-PCR analysis		
			NoV GI	NoV GII	HAV
Set 2: Farmer 2	River Water	2008.09.23	Neg	Neg	Neg
	Cabbage (a)		Neg	Neg	Neg
	Cabbage (b)		⇨ Neg	Neg	Neg
	Cabbage (c)		⇨ Neg	Neg	Neg
Set 3: Site 1	Canal water	2008.10.27	Neg	**Pos**	Neg
	Tomato		Neg	Neg	Neg
	Cabbage C*		Neg	Neg	Neg
	Cabbage T†		Neg	Neg	Neg
Set 4: Site 1	Canal water	2009.06.24	Neg	Neg	**Pos**
	Tomato		Neg	Neg	Neg
	Cabbage C*		Neg	Neg	Neg
Set 5: Site 1	Canal water	2009.07.20	Neg	Neg	Neg
	Tomato		Neg	Neg	Neg
	Cabbage C*		Neg	Neg	Neg
Set 6: Site 1	Canal water	2009.08.17	Neg	Neg	Neg
	Tomato		Neg	Neg	Neg
	Cabbage C*		Neg	Neg	Neg
Set 7: Site 1	Canal water	2009.09.07	Neg	Neg	Neg
	Cabbage C*		Neg	Neg	Neg
Set 8: Site 1	Canal water	2009.10.13	Neg	Neg	Neg
	Tomato		Neg	Neg	Neg
	Cabbage C*		Neg	Neg	Neg

Canal water: Irrigation canal water
C*: Variety Conquest
T†: Variety Tennesse
⇨ : Real-time RT-PCR analysis for mengovirus = Positive
⇦ : Real-time RT-PCR analysis for mengovirus = Negative

Table 4.14 *continued:* Summary of results of viral analysis of linked irrigation water and fresh produce samples from Limpopo.

Sample site, name, location	Samples	Sample date	Real-time RT-PCR analysis		
			NoV GI	NoV GII	HAV
Set 9: Site 1	Canal water	2009.11.02	Neg	Neg	Neg
	Tomato		Neg	Neg	Neg
	Cabbage C*		Neg	Neg	Neg
	Cabbage T†		Neg	Neg	Neg

Canal water: Irrigation canal water
C*: Variety Conquest
T†: Variety Tennesse
⇨ : Real-time RT-PCR analysis for mengovirus = Positive
⇦ : Real-time RT-PCR analysis for mengovirus = Negative

Western Cape: No correlation between the occurrence of viruses in the irrigation water samples and in the linked fresh produce samples collected in the field or at harvest noted (Table 4.15).

Table 4.15: Summary of results of viral analysis of linked irrigation water and fresh produce samples from Western Cape.

Sample site, name, location	Fresh produce	Sample date	Real-time RT-PCR analysis		
			NoV GI	NoV GII	HAV
Site 1: B-K (Buhr)/ Mosselbank	River water	2008.09.08	Neg	Neg	**Pos**
	Cabbage		Neg	Neg	Neg
	Cabbage		Neg	Neg	Neg
Site 10: Roux Tap	Tap water		Neg	Neg	Neg
	Beans		⇨ Neg	Neg	Neg
	Beans		⇨ Neg	Neg	Neg

⇨ : Real-time RT-PCR analysis for mengovirus = Positive
⇦ : Real-time RT-PCR analysis for mengovirus = Negative

4.3.3 Viral characterisation

4.3.3.1 Norovirus

BLAST analysis of a 250 bp region of the 5'end of the capsid region of the NoV GII strains from irrigation water and fresh produce revealed the presence of four genotypes, namely GII.1, GII.4, GII.6 and GII.9. Pairwise analysis of the nucleotide sequences indicated that the most of the strains from irrigation water and fresh produce showed the highest percentage genetic relatedness to other South African clinical and environmental strains (Figure 4.1). Strain GII.1 from a cabbage sample from the Western Cape showed a 99% nucleotide and 100% amino acid sequence identity to a strain, 8738, from a case of sporadic gastroenteritis in a hospitalised paediatric patient from Gauteng. The NoV GII.2 strain detected in the Mosselbank river in 2008 had the highest genetic relatedness (94% nucleotide and 92% amino acid sequence identity) to a strain, 1605, detected in Russia in 2009. Norovirus GII.4 strains from the Berg River (Lady Loch) and the Mosselbank rivers in the Western Cape (Table 4.8) showed 100% nucleotide and 100% amino acid sequence identity to each other and a 99% nucleotide and 100% amino acid sequence identity to a strain, 8483, from a case of sporadic gastroenteritis in a hospitalised paediatric patient from Gauteng and strains 0399 and 1296 detected in Korea and Russia in 2009 and 2010, respectively. The GII.6 strain from the Berg River (Franschhoek) showed a 99% nucleotide and 100% amino acid sequence identity to two strains, 08-06-02b and 08-05-26, detected in river water in the Vaal catchment area of SA and a 99% nucleotide and 100% amino acid sequence identity to a strain, 9088, detected in Japan in 2008. The GII.9 strain (SA-2009-Tom) detected on a tomato purchased from a street vendor in the Tshwane Metropolitan area, Gauteng showed a 98% nucleotide and 96% amino acid sequence identity to a strain, 08-08-18b-RV2, detected in the Riespruit river in the Vaal catchment area in 2008 (Mans et al., submitted).

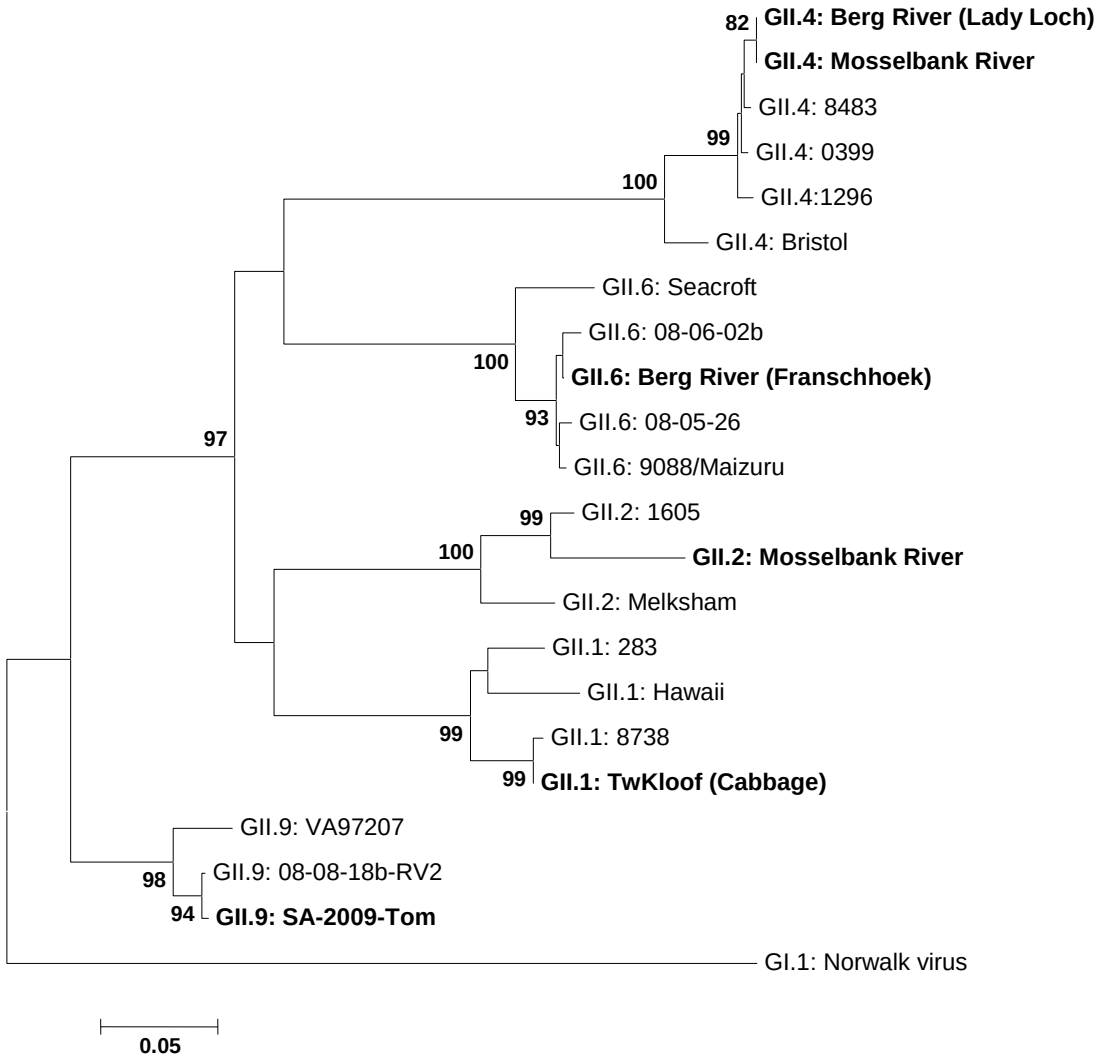

Figure 4.1: Neighbour-joining phylogenetic tree based on 250 base-pair fragment of the capsid region of the NoV strains from irrigation water and fresh produce and reference NoV GII strains using NoV G1.1 as an outgroup. The bar indicates nucleotide changes per site and bootstrap percentages are indicated.

4.3.3.2 Hepatitis A virus

Pairwise analysis of the nucleotide sequences of a 168 bp region of the putative VP1/P2A junction of the HAV strains detected on lettuce (SA-2009-Let) and tomatoes (SA-2009-Tom)(Table 4.12) with one another and to representative strains of all genotypes and subgenotypes revealed that the strains from South African fresh produce showed >92.5%, i.e. 95.96% sequence identity to the type strain, HM 175 and could therefore be grouped

within genotype IB. These strains showed a 98.3% nucleotide identity to each other and 98-99% nucleotide sequence identity to the most closely related HAV strains in GenBank, i.e. 034, 126 and 166, which were from German tourists who had acquired hepatitis A in SA. Phylogenetic analysis showed that these South African strains from the fresh produce and the German tourists, together with previously characterised South African strains SA-2333 and SA-JVR, formed a distinct cluster within subgenotype IB (Fig. 4.2). Pairwise analysis of the amino acid sequences for the same putative VP1/2A junction region indicated that the HAV strains from the fresh produce showed a 92% amino acid sequence identity to the type strain, HM175 and a 99-100% amino acid sequence identity to strains 034, 126 and 166 (results not shown).

4.4 DISCUSSION

Fresh produce may be contaminated with pathogenic microorganisms pre-harvest in the field through the use of polluted irrigation water or improperly treated manure (Steele and Odumera, 2004; Gerba and Choi, 2006), at harvest or post-harvest during handling, processing and distribution (Beuchat, 2002; Carter, 2005; Berger et al., 2010). In this investigation several factors may account for the viral contamination detected on the fresh produce analysed.

In this study 16.3 % (13/86) of irrigation water samples tested positive for one or more human pathogenic viruses, namely NoV GII and HAV. Similar findings were reported for previous studies in SA where human rotaviruses were detected in 16.7% of irrigation water samples analysed (Barnes and Taylor, 2004; van Zyl et al., 2006) and Tshivhandekano (2005) detected adenoviruses, enteroviruses and human rotaviruses in river water used by subsistence farmers for the irrigation of fresh produce. This level of viral contamination of irrigation water is not limited to SA as in South Korea, 17% of ground water used for irrigation of fresh produce was contaminated with enteric viruses (Cheong et al., 2009a).

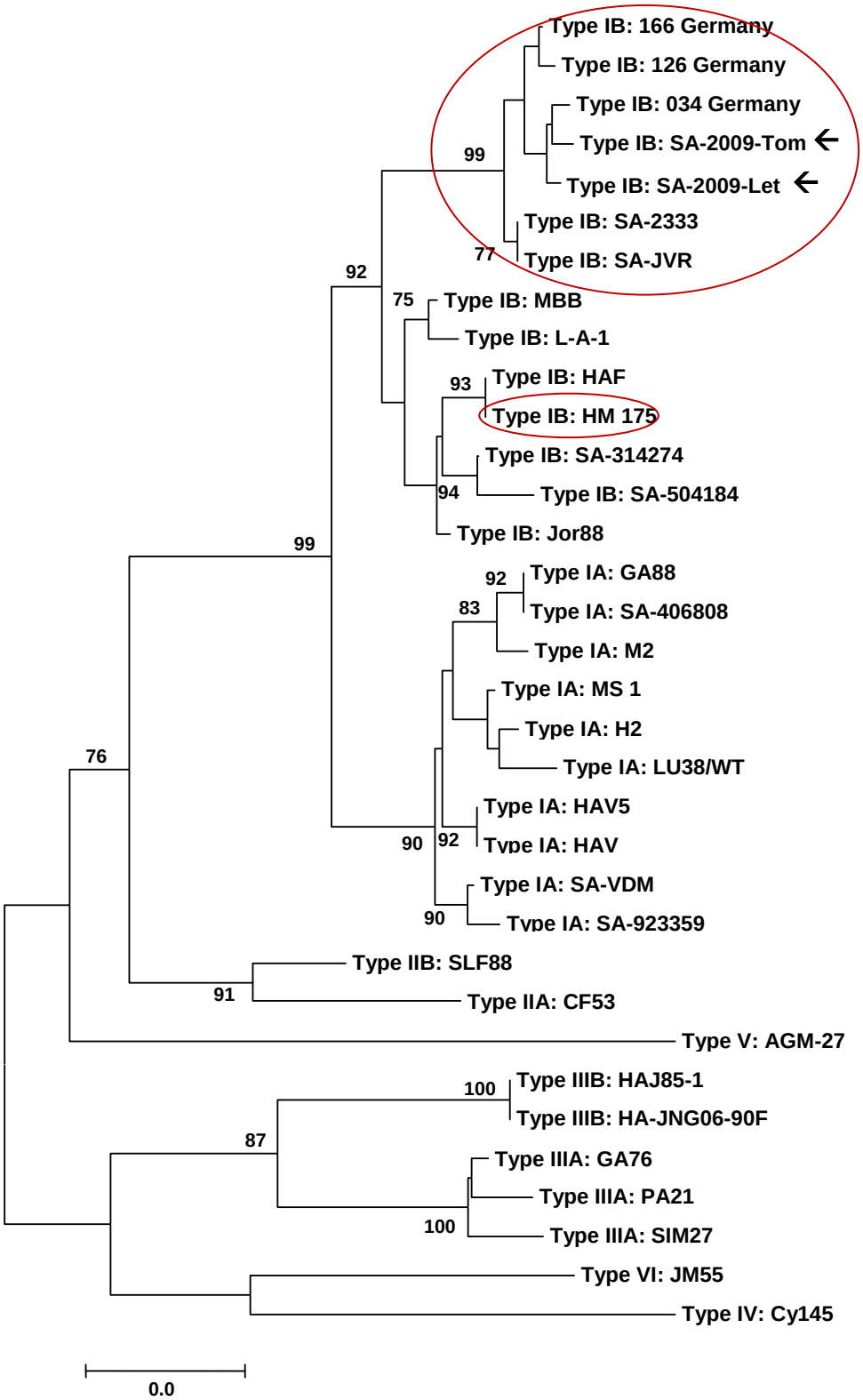

Figure 4.2: Neighbour-joining phylogenetic tree based on 168 base-pair fragment of the putative VP1/P2A region of the HAV strains from fresh produce and reference HAV strains. The bar indicates nucleotide changes per site and bootstrap percentages are indicated.

Norovirus GII was the most predominant virus detected in irrigation water samples in this study, being present in 11/86 (~13%) of samples with HAV being detected in 4/86 (~5%) of samples, respectively. Norovirus GI was not detected in any of the samples. When the occurrence of viruses in the irrigation water from the different geographical areas was compared, NoV GII was detected in 7.7% of samples from Limpopo, 0% from the North West, 10.2% of samples from the Western Cape and 22.2% of samples from Mpumalanga. Norovirus GII were detected at all three sites in Mpumalanga (Table 4.7) suggesting that the irrigation water in Mpumalanga had a higher level of contamination, but as only one sample from the North West was analysed there are insufficient data to make a definitive statement. These NoV GII strains, were, however untypable and therefore could not be compared to other environmental or clinical strains. Norovirus GII was detected in three different rivers in the Western Cape, namely Mosselbank river, Plankenburg river and the Berg river. Genotypes II.2 and II.4 was identified in samples from the Mosselbank river while genotypes II.4 and II.6 were detected at different sampling sites, Berg 1 and Berg 3, on the Berg river, respectively (Table 4.8). For the past 15 years NoV GII has been shown to be the most prevalent NoVs associated with infection worldwide with NoV GII.4 being the predominant genotype (Atmar, 2010). In a South African study NoV GII.4 was the most frequently detected NoV in diarrhoeal stool specimens in hospitialised paediatric patients (Mans et al., 2010). The detection of NoV GII.2 and GII.4 in water from the concrete furrow, which was a branch of the Mosselbank river, was not surprising as this sampling site was about 1 km downstream from the Kraaifontein sewage works and flows through a dilapidated area. The water is finally used for irrigation of a variety of vegetables mostly sold on the Cape fresh market. The detection of NoV GII.6 at the Berg 1 sampling site confirms bacteriological results (not reported) indicating gross human faecal contamination of the river which could have emanated from the Groenendal sewage works. Norovirus GII.4 was detected at the Berg 3 sampling site, approximately 30 km downstream of Berg 1. The Berg 3 sampling site is downstream from a wastewater treatment plant which

reportedly malfunctions at times (Barnes JM 2008, *confidential unpublished report*) and the bacteriological quality of the water exceeded safe levels for irrigation water (Britz TJ, Project leader WRC Project K5/1773/4, *personal communication*). There is only one other report of NoVs in surface water in SA (Mans et al., *submitted*), where high levels of NoV GII contamination of river water in the Vaal Catchment Area, Gauteng was described, with NoV GII.4 and GII.6 being the predominant genotype identified. This indicates that the contamination of river water with NoVs is not limited to the Western Cape and Mpumalanga but is a more widespread problem. The occurrence of NoVs in surface water has been reported in other regions of the world, namely The Netherlands (Lodder and de Roda Husman, 2005); South Korea (Lee and Kim, 2008), Brazil (Victoria et al., 2010) and Venezuela (Rodriguiz-Diaz et al., 2009) and have also been detected in groundwater used for irrigation purposes in South Korea (Cheong et al., 2009a). Hepatitis A virus was detected in 7.7% of irrigation water samples from Limpopo, 0% from North West, ~5% from Western Cape and 0% from Mpumalanga. In the Western Cape, HAV (untypable) was detected in the Mosselbank river (Site 1) and the Plankenburg river (Site 4) (Table 4.8). The Plankenburg river sampling site is downstream of the Plankenburg industries and Kayamandi informal settlement. The source of contamination in this area might therefore be the untreated sewage from the informal settlement as previously reported (Barnes and Taylor, 2004). In SA, large numbers of people live in areas where surface water is the only available water for irrigation and washing of fresh produce. Since water availability is often critical, little attention is given to the microbial or virological quality of irrigation water. Detection of the viral pathogens in large volumes of water will depend upon the sensitivity of the recovery and detection procedures, the concentration of viral pathogens in the point sources. The data from these samples demonstrated that some contamination of irrigation water by enteric viruses had taken place. The Western Cape falls within the winter rainfall region while Mpumalanga, Gauteng and Limpopo fall within the summer rainfall region of SA. During the rainy season, it is anticipated that runoff water could pollute surface water sources and disseminate enteric viruses from

contaminated point sources, e.g. from human defaecation close to the rivers. In only one province, i.e. Mpumalanga was the occurrence of enteric viruses in the irrigation water more noticeable in the rainy season.

The findings of the present study showed that 6.9% (2/29) of the irrigated pre-harvest produce had detectable enteric virus contamination, with NoV GII.1 being detected on a cabbage sample from the Western Cape. These results are higher than the 1.7 % for group A rotaviruses on fresh produce reported by van Zyl and colleagues (2006) and the 3% reported for fresh vegetables in South Korea (Cheong et al., 2009a). Although a direct link between contaminated irrigation water and contamination of fresh produce could not be shown, polluted irrigation water was identified as a possible source of contamination of fresh produce (Tables 4.14 & 4.15). Norovirus GII.1 was detected on a cabbage sample irrigated with water from the Berg River (Site 17 : Table 4.8), and although no NoV was detected in the irrigation water at this site, NoV GII.6 was detected upstream (Site 16) and NoV GII.4 downstream (Site 18) in the Berg River during the same time period (August – September 2008). The detection of identical or closely related NoV GII strains would have provided strong evidence of a direct link between viral contamination on fresh raw produce and irrigation water. The identification of distinct NoV GII strains, however, does not preclude the possibility of a link between the irrigation water and contamination of the produce as both the faecally contaminated irrigation water and the fresh produce may have been contaminated with multiple NoV strains (Le Guyader et al., 2004; CDC, 2011). The infectious and outbreak potential of NoV GII.1 detected on the cabbage is highlighted by the first report of possible foodborne NoV-associated gastroenteritis in SA which was ascribed to NoV GII.1 (Hawaii) (Taylor et al., 1993). In addition during the period 1990-2005 lettuce and salad greens contaminated with NoV were associated with ~25% of produce-associated foodborne outbreaks of NoV infection (Patel et al., 2009). In spite of the fact that very few samples were collected from Site 1 in the Western Cape, human pathogenic viruses were detected in the Mosselbank river water

in two consecutive months, i.e. NoV GII in August 2008 and NoV GII and HAV in September 2008 (Table 4.8). Similarly, NoV GII and HAV were detected concurrently in the Plankenburg river in October 2008, with HAV being detected in consecutive samples (October 2008 and December 2008). These results indicate that these water sources are subject to continual human faecal pollution, confirmed by concurrent bacteriological data (Britz TJ, Project leader WRC Project K5/1773/4, *personal communication*), and would therefore pose a risk if used for irrigation.

From this study it was evident that the fresh produce samples collected post-harvest were more likely to be contaminated with enteric viruses, with either NoV or HAV or both viruses being detected on 23% (7/30) samples tested. These results suggest that most of the viral contamination of the fresh produce occurred during harvesting, processing and packaging. Food handlers have typically been implicated in the contamination of foods implicated in foodborne outbreaks of NoV infection (Tuan Zainazor et al., 2010; CDC, 2011) and HAV infection (Fiore, 2004). It was noted that viral contamination was more prevalent on post-harvest fresh produce with rough convoluted surfaces, being present on ~29% of the samples, than on fresh produce with smooth surfaces where only ~9% of samples showed viral contamination. At pre-harvest enteric viruses were only detected on fresh produce with rough convoluted surfaces, i.e. cabbage and spinach (Tables 4.9 & 4.10). The higher viral contamination on produce such as lettuce, cabbage and spinach may be due to the large surface area of the leaves which also have many folds. Rotavirus and HAV have been detected on lettuce samples post-harvest in Costa Rica (Hernández et al., 1997), while NoV was detected on a pre-harvest spinach sample in Korea (Cheong et al., 2009a). The most common foods implicated in outbreaks of NoV and HAV infection are those ready-to-eat minimally processed foods with rough surfaces such as leafy vegetables and green onions (Rosenblum et al., 1990; Cuthbert, 2001; Fiore, 2004; Shan et al., 2005, Wheeler et al., 2005; Berger et al., 2010; CDC, 2011) and berry fruits (Fiore, 2004; CDC, 2011). Lettuce has been known to have a high

adsorption capacity for HAV and in a laboratory-based study, HAV was still detectable up to nine days after artificial contamination (Croci et al., 2002). The fresh produce from street vendors 7 & 8 from Marabastad, which included leafy green vegetables and tomatoes, was highly contaminated with NoV GII (33 %: 2/6) and HAV (50 %: 3/6). Outbreaks of NoV and HAV infection are thought to result predominantly from contamination of food via unwashed hands of infected food workers (Berger et al., 2010; CDC, 2010; Tuan Zainazor et al., 2010) suggesting that the lack of proper toilet and hand washing facilities and/or unhygienic practices of street vendors may be the cause of the post-harvest contamination of the fresh produce.

Due to the low titres of viruses in water and on food samples the analysis of food and water is a complex process (Mattison and Bidawid, 2009). As most food- and waterborne viruses have a low infectious dose, ranging from 1 to 100 particles, it is essential that sensitive and specific methods are applied to the viral analysis of food and water (Mattison and Bidawid, 2009). The sampling procedures required to obtain a representative sample for viruses on fresh produce are critical to obtain a high level of analytical sensitivity (Bosch et al., 2011). In this study the approach was to use a sample comprised of three randomly selected items of produce from the same batch, crate or field. The detection of viruses in one or two items in a sample comprised of three items (Tables 4.11 & 4.12), indicate the necessity of collecting a representative sample to get a true reflection of the virological quality of a particular crop. Effective quality assurance/quality control (QA/QC) procedures are also required to exclude false positive or false negative results (Bosch et al., 2011). False negative results usually result from poor virus recovery, inefficient nucleic acid extraction and inhibitory substances that affect the reverse transcription or amplification reaction, while false positive results could be due to cross-contamination (Bosch et al., 2011). In this study mengovirus was included as a process control to control for viral recovery and nucleic acid extraction efficiency. From Table 4.12 it is evident that items of fresh produce from street vendors 7 & 8 from Marabastad which tested

negative for the target viruses, namely NoV GI, NoV GII and HAV, tested positive for mengovirus highlighting the efficiency of the viral recovery and nucleic acid extraction methodology applied, and therefore the results are correctly reported for the target viruses. Mengovirus was, however, not detected in a number of irrigation water or on a number of fresh produce samples submitted from collaborating laboratories, e.g. Table 4.9. This could have been due to incorrect seeding, viral decay on the seeded vegetable and/or suboptimal and inefficient viral recovery in the referring laboratory or due to the presence of inhibitory substances in the extracted nucleic acid. Included in each of the individual *rt* RT-PCR runs were: i) a negative RNA extraction control to monitor for cross-contamination; ii) a negative RT-PCR control to control for contamination in the *rt* RT-PCR reagents, and iii) a positive *rt* RT-PCR control to control for the quality of the *rt* RT-PCR reagents. A limitation of this study, however, was the lack of internal controls to control for amplification in each individual reaction for each virus. This may have resulted in false negative results in selected samples where amplification of the target virus was inhibited, and therefore these results may represent an under-estimation of the true extent of viral contamination in the irrigation water and on the fresh produce.

In this study the detection of NoVs in the irrigation water samples from SA was not unexpected as a number of other enteric viruses, namely enteroviruses, HAdVs, HAstVs, RVs and HAV have been detected in surface water samples in SA (Taylor et al., 2001, Nadan et al., 2003; Barnes and Taylor, 2004; Vivier et al., 2004; van Heerden et al., 2003; 2005; Ehlers et al., 2005; Tshivhandekano, 2005; van Zyl et al., 2004, 2006). In addition, NoVs have been implicated in a number of waterborne outbreaks of gastroenteritis (Maunula et al., 2005; Hewitt et al., 2007, CDC, 2011) and have been detected in water sources worldwide, including The Netherlands (Lodder and de Roda Husman, 2005); Finland (Maunula et al., 2005); New Zealand (Hewitt et al., 2007), South Korea (Cheong et al., 2009a), Brazil (Victoria et al., 2010); China (He et al., 2010), and Ghana (Gibson et al., 2011). The presence of

NoVs on fresh produce in SA was previously suspected as salads were implicated in an outbreak of NoV-associated gastroenteritis in SA as early as 1991 (Taylor et al., 1993). The detection of genetically related NoVs in the Western Cape and Gauteng suggests that these strains have a widespread distribution in SA. From the results it is evident that the NoV GII.1, GII.4 and GII.6 strains detected in the irrigation water and on the fresh produce are of clinical importance as they showed a high genetic relatedness to strains associated with sporadic gastroenteritis in SA (Mans et al., 2010) and other regions of the world such as the US (Ando et al., 1997, 2000); Korea (Han et al., 2011); Russia (Epifanova et al., 2010 : *direct submission to GenBank*) and Japan (Chan-it 2010 : *direct submission to GenBank*). Worldwide NoVs are the most common cause of epidemic gastroenteritis in all age groups (Patel et al., 2009) and are becoming a prominent cause of travellers' diarrhoea (Apelt et al., 2010; Koo et al., 2010). The detection of common or closely related strains worldwide is of public health concern as they may be disseminated through a common vehicle such as the international food market (Glass et al., 2009). The presence of NoVs on fresh produce in SA has widespread implications for the domestic and international food market as well as the travel industry.

The identification of HAV IB on the lettuce and tomato sample confirms that the contamination on the fresh produce was of human origin as HAV 1B has only been described in association with human infection (Robertson et al., 1992; Nainan et al., 2006; Pintó et al., 2009) and subtype IB was the most prominent type detected in clinical specimens in SA (Taylor, 1997). In SA, HAV is endemic with epidemiological features of both lower socio-economic and higher socio-economic communities being evident (Venter et al., 2007). Hepatitis A virus has been detected in a variety of water sources (Taylor et al., 2001; Venter et al., 2007) and the potential risk of infection to the different South African communities has been quantified (Venter et al., 2007). Extrapolating from the latter study, fresh produce contaminated with HAV would pose a minimal risk to the predominantly immune lower socio-

economic community as the majority of this population group are immune to HAV infection. In this community only the very young, (children < 10 years), the immunocompromised or non-immune individuals would be at risk of infection. In the higher socio-economic income urbanised predominantly white communities the risk of clinical infection would be greater. The potential health impact of foodborne HAV from fresh produce would not be limited to the South African geographical region as non-immune unvaccinated tourists from non-endemic areas are also at risk of infection (Faber et al., 2009) which could be fatal (Oltmann et al., 2008). The economic impact of contaminated South African fresh produce therefore has important implications for the travel industry.

From the results of this study, it was evident that in selected areas of SA irrigation water and fresh produce, which is often eaten raw, was contaminated with potentially pathogenic human viruses, namely HAV and NoV GII. Irrigation water and food handling were identified as possible sources of contamination of the fresh produce. The detection of both NoV GII.9 and HAV on a single tomato sample (Table 4.12) is of concern as human rotaviruses were previously been detected on tomatoes in SA (van Zyl et al., 2006), indicating the vulnerability of tomatoes to viral contamination. As this study represented an exploratory study to ascertain the extent of viral contamination in irrigation water and on fresh produce, further in-depth studies are required to determine; i) the viral load on the fresh produce and the associated health risk; ii) where in the supply chain, from farm to fork, the contamination occurs, iii) what intervention strategies can be implemented to ensure the safety of the fresh produce for the consumer, and iv) the economic impact on health, agricultural and tourism industries.

CHAPTER 5

GENERAL DISCUSSION

Food- and waterborne viruses pose a significant health threat worldwide (Newell et al., 2010) and due to overlapping modes of transmission the burden of foodborne transmission is underestimated (Carter, 2005). The number of outbreaks associated with fresh produce consumption has steadily increased (Berger et al., 2010). In addition to the short and long-term health effects of such illnesses, there is also a substantial cost to the economy in terms of work days lost and the potential impact of a contamination incident on international trade. Globally food and-waterborne diseases are "grossly underreported owing to surveillance difficulties and also for fear of economic and social consequences" (Mead et al., 1999). Foodborne viruses can emerge at any point in the food chain, starting with the agricultural environment in which the food is grown, continuing through harvesting, processing and packaging procedures to storage, retail and food handling methods. Although infected food handlers have been identified as important sources of contamination (Barrabeig et al., 2010; Berger et al., 2010; Tuan Zainazor et al., 2010), the role of polluted irrigation water in the contamination of fresh produce is well recognised (Steele and Odumeru, 2004; Berger et al., 2010). To ensure food safety for the consumer, and considering the inadequacy of bacterial markers for indicating the presence of enteric viruses in food and water samples (Verhoef et al., 2009), there is a need for routine surveillance of food and water sources for the presence of enteric viruses (Bosch et al., 2011). The virological analysis of fresh produce and irrigation water is a complex multistep process (Mattison and Bidawid, 2009), with each stage adding to the variability of the result. Furthermore, no standard procedure for the detection of enteric viruses in environmental samples currently exists. Methodologies for the recovery and detection of enteric viruses from food and water sources vary from one study to another, with differences in the sensitivity, specificity

and detection limits of the individual assays. The aim of this investigation was therefore to develop and optimise a simple and efficient method for the recovery and detection of NoV GI, GII and HAV in irrigation water and fresh produce using mengovirus as a process control. These methods would then be applied to field samples of irrigation water and fresh produce to: i) assess their applicability for the routine detection of HAV and NoVs in irrigation water and food samples, and ii) to try and establish a link between viral contamination detected in irrigation water and that on associated irrigated fresh produce.

Real-time RT–PCR methods have been shown to be useful diagnostic assays for the qualitative and quantitative detection of viruses in environmental samples (Mattison and Bidawid, 2009, Bosch et al., 2011). In this study, the *rt* RT-PCR assays recommended by the CEN TC275/WG6/TAG4 Technical Committee for the detection of HAV, NoV GI, NoV GII and mengovirus, were optimised using commercial kits to achieve the best analytical sensitivity in the LightCycler® v1.5 (Roche). In addition, these optimised *rt* RT–PCR assays were applied to test naturally contaminated irrigation water and fresh produce. The specificity of the assays was confirmed and validated by nucleotide sequence analysis of strains present in the positive samples. The results of this study support the use of these optimised methods for the routine surveillance of food and water sources for HAV, NoV GI and NoV GII and for investigating enteric disease transmission through water and food.

The recovery and concentration of viruses from food and water sources is one of the most critical steps in the analysis of environmental samples for enteric viruses. In this study seeding experiments using HAV, NoV GII and mengovius, were conducted to optimise the recovery of viruses from various types of fresh produce. Three recovery buffers (GBEB, TGBE, and PBS) at two different pHs (pH 7.2 and pH 9.5), two elution/washing times (20 min and overnight) were assessed to ascertain which method would give the best percentage recovery for the viruses being tested. Not one of the elution

buffers, at either of the elution times, performed equally well for all three viruses. These results are consistent with those of other studies (Butot et al., 2007; Baert et al., 2008; Cheong et al., 2009b). The recovery of HAV had to be compromised in order to ensure the optimal recovery of mengovirus and NoV GII. The combination the TGBE buffer at pH 9.5 with a 20 min elution time was therefore used for the recovery of viruses from the field samples of fresh produce.

To address the frequency and pattern of viral contamination in irrigation water and fresh produce in SA, irrigation water and fresh produce samples from different geographical areas were analysed for the presence of NoVs and HAV. The results of this study showed virological contamination of fresh produce collected in the field and purchased from commercial outlets. Furthermore nucleotide sequence analysis of strains detected on food sources confirmed the presence of clinically relevant strains, HAV IB (Figure 4.2) and NoV GII (Figure 4.1), on commercially available fresh produce and in irrigation water sources. Polluted irrigation water was identified as a possible source of contamination of fresh produce (Tables 4.14 & 4.15) although a direct link between contaminated irrigation water and contamination of fresh produce could not be shown. The higher level of viral contamination on the fresh produce from commercial outlets, including street vendors, compared to that collected in the field suggests that food handlers contribute significantly to the viral contamination of the fresh produce, which is in keeping with findings from other regions of the world (Barrabeig et al., 2010; Berger et al., 2010). The frequent detection of viruses from different street vendors on separate occasions suggests that the viral contamination of fresh produce may be more prevalent than previously thought. In SA the question as to how much of the NoV-associated gastroenteritis and hepatitis A disease burden can be attributed to the consumption of irrigated fresh produce remains a challenging one as: i) food- and waterborne disease is underreported, ii) the HAV epidemiological data is outdated and there is underreporting of clinical cases (Venter et al., 2007) and there are few data on the current status of NoV

infection (Mans et al., 2010). However this study clearly shows that, in SA, fresh produce could be a potential source of enteric viruses for the consumer. More in-depth investigations are clearly needed to identify the source of the viral contamination, so that the appropriate authorities can implement the necessary action for disease control and prevention.

CHAPTER 6

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APPENDIX A

A:1: **Netshikweta R,** van Zyl WB, Mans J, Wolfaardt M, Taylor MB. The application of real-time RT-PCR for the detection of selected enteric viruses on fresh produce in South Africa [Poster] IUFoST 2010 15th World Congress of Food Science & Technology 22-26 August 2010: Cape Town International Convention Centre, South Africa

Introduction

Enteric viruses cause approximately 67% of foodborne outbreaks worldwide and the impact on human health and the economy is underestimated. Noroviruses (NoV) and hepatitis A virus (HAV) are most frequently associated with foodborne disease. In 1991 NoVs were implicated in an outbreak of gastroenteritis associated with the consumption of salads in South Africa (SA), but the source of contamination was not established. As there are no data regarding the occurrence of viruses on fresh produce in SA the aim of this study was to assess the virological quality of fresh produce in SA.

Methodology

Fresh produce samples were collected from farms, street vendors and commercial outlets in the Western Cape, the Vhembe region of Limpopo, and Pretoria. Viruses were recovered from the fresh produce samples by washing with tris-glycine-beef extract buffer and concentrated by polyethylene glycol/sodium chloride precipitation. Viral nucleic acid was extracted directly from the virus concentrate using a commercial nucleic acid extraction kit in the MagNA Pure LC robotic instrument. Noroviruses and HAV were detected by singleplex real-time RT-PCR assays and characterised by sequence analysis. Mengovirus was included as a process control.

Results

Noroviruses were detected from 9/100 (9%) and HAV from 5/100 (5%) of the fresh produce samples. Norovirus GII.1 was identified from a cabbage sample and HAV genotype IB from lettuce and tomato samples.

Discussion

The results reflect qualitative presence-absence data and give no indication as to the potential health risk to consumers. However the identification of NoV GII.1 and HAV IB indicate that the fresh produce had been exposed to faecal contamination of human origin.

Conclusion

The presence of potentially pathogenic viruses on fresh produce samples from farms, street vendors and commercial outlets emphasises the need for continued surveillance from farm-to-fork to establish the source of contamination to ensure the safety of consumers.

A.2: **Netshikweta R,** van Zyl WB, Mans J, Taylor MB. The application of real-time RT-PCR for the detection of selected enteric viruses in irrigation water in South Africa [Presentation] 1ST Regional Conference of the Southern African Young Water Professionals 19-20 January 2010: CSIR International Convention Centre, Pretoria, South Africa

There is an increasing awareness that fresh or minimally processed fruit and vegetables can be a source of pathogenic microorganisms including viruses. The fruit and vegetables can be contaminated by faecally polluted irrigation water, post-harvest washing water or by an infected food handler. Irrigation water sources include surface water, groundwater or wastewater. The microbiological quality of the irrigation or post-harvest washing water dictates the potential for pathogenic contamination and the water can either contaminate the fresh produce or spread micro-organisms from one product to the next. Outbreaks and sporadic occurrences of food- and waterborne viral infections have been documented worldwide but frequently go unrecognised, consequently the impact on human health and the economy is underestimated. From 1990-2006 fresh produce was responsible for the largest number of cases of foodborne illness in the United States although seafood was responsible for the highest number of outbreaks. Among all illness caused by foodborne pathogens approximately 67% were attributed to viruses. Of the enteric viruses hepatitis A virus (HAV) and noroviruses (NoVs) are the most frequently associated with food- and waterborne disease. In 1991 NoVs were implicated in an outbreak of gastroenteritis associated with the consumption of salads in South Africa (SA). However there was no information as to the source of contamination of the salads. One of the possible sources of contamination could have been the irrigation water. As there are little or no data with regard to the occurrence of viruses in irrigation water in SA the aim of this investigation was to assess the virological quality of water used for the irrigation of fresh produce in SA. Irrigation water samples (10L) were collected from surface and borehole water sources in the Western Cape, the Vhembe region of Limpopo and Mpumalanga. Enteric viruses were recovered from the water by means of a glass wool adsorption-elution technique. Recovered viruses were further concentrated by polyethylene glycol/sodium chloride precipitation. Viral nucleic acid was extracted directly from the virus concentrate using a commercial nucleic acid extraction kit in an automated platform. The purified nucleic acid was aliquoted and stored at -70°C. Five microlitres of the eluted nucleic acid was used for virus nucleic acid amplification. Optimised real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assays, using published primers and probes, were applied for the qualitative detection of HAV and NoV in the water samples. Mengovirus was used as a process control to evaluate the efficiency of virus recovery and nucleic acid extraction. Noroviruses were detected in 11/93(11.8%) and HAV in 3/93 (3.2%) of irrigation water samples tested. The study provides valuable new information as to the virological quality of irrigation water sources in SA and highlights the potential public health and economic impacts.

A.3: Netshikweta R, van Zyl WB, Mans J, Taylor MB. Optimisation and application of real-time RT-PCR techniques for the detection of selected food and waterborne viruses [Poster]. Faculty Day, Faculty of Health Sciences, University of Pretoria 18-19 August 2009: HW Snyman Building, Pretoria

Despite progress in recent times in both medical care and food technology, foodborne diseases are still of major concern for human health, both in developing and developed countries. Among all illness caused by foodborne pathogens approximately 67% have been attributed to viruses. Outbreaks and sporadic occurrences of food- and waterborne viral infections have been documented worldwide but frequently go unrecognized, consequently the impact on human health and economy is underestimated. Of the enteric viruses hepatitis A virus (HAV) and noroviruses (NoVs) are the most frequently associated with food- and waterborne disease. In 1991 NoVs were implicated in an outbreak of gastroenteritis associated with the consumption of salads in South Africa (SA).

In spite of the considerable progress in using molecular techniques for the detection of human enteric viruses few studies have applied these methods to the detection of viral contamination of food. As there are little or no data with regard to the occurrence of viruses on fresh produce in SA this study was focused on the assessment and application of methods for the recovery and detection of enteric viruses, namely NoV and HAV, from fresh produce and irrigation water. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assays were optimised for the qualitative and quantitative detection of HAV and NoV. Mengovirus was used as a process control to evaluate the efficiency critical steps for the quantification of viruses. A number of elution buffers were assessed for the recovery and extraction of viruses from fruit and vegetables. Viruses were recovered from the irrigation water by means of a glass wool adsorption-elution technique. Seeding experiments were conducted to determine the efficiency of recovery of each virus concentration procedure. In field studies NoV GII has been detected in 11/77 (14%) and HAV in 1/77 (1.3%) of irrigation water samples tested. NoV GII was detected in 3/14 (21%) samples of fresh produce. NoV GII.4, commonly associated with gastroenteritis outbreaks was identified among the NoV strains. The study provides valuable new information, with potential public health and economic impacts, regarding the virological quality of fresh produce and irrigation water sources in SA.

A.4: Netshikweta R, van Zyl WB, Wolfaardt M, Taylor MB. Comparison of conventional nested RT-PCR and real-time RT-PCR for the detection of enteroviruses in water samples. [Poster]. The 2007 MCBG symposium. Department of Medical Virology, University of Pretoria/Molecular and Cell Biology Group of South Africa. 17 October 2007. HW Snyman North, Faculty of Health Sciences, University of Pretoria, Pretoria

Enteroviruses (EVs) are members of the family Picornaviridae and are estimated to cause 30 million to 50 million infections per year in the United States. Enteroviruses are tolerant to residual chlorine from treatment processes and survive a wide range of temperatures and salinities thus facilitating their survival in environmental waters. Consequently they are one of the most commonly detected viruses in water. Given the load of EVs shed into waters and the role of EVs in waterborne disease, there is clearly a need for sensitive assays for the detection of EVs in water. The objective of this study was to develop a rapid, simple and efficient real-time RT-PCR procedure for the detection of to EVs in water samples. To this end real-time RT-PCR was compared to the conventional nested RT-PCR currently used for this purpose. Enteroviruses detected by either method were characterized, by sequence analysis, to determine whether any one type was preferentially detected by either method. In this study EVs were detected, by nested RT-PCR, in 27 of 271 (9.9%) water samples assayed and included 10 (7.1%) strains from treated drinking water and 17 (13.1%) from untreated and/or raw water sources. The results of a comparative study showed that the specificity of real-time RT-PCR and an in-house two-step nested RT-PCR assay were comparable, but that the sensitivity of the real-time RT-PCR was much less (3 $\log_{10}$) than the nested RT-PCR assay. This lack of sensitivity was further highlighted in this study where 27 of the 271 samples which tested positive for EV RNA by the in-house two-step nested PCR assay tested negative by real-time PCR. Sequencing of selected amplicons derived from the 5'-UTR region enabled differentiation between various serotypes of EVs. Enterovirus types CV-B1, CV-B3 and CV-A6 were identified but this was not unexpected as they have previously been detected in water samples. However the detection of rare and novel EV isolates, namely EV-68 and EV-89 was of great interest. Enterovirus 89 is one of four newly described EVs and the detection of EV89 in river water from the Western Cape suggests that this strain may be more widely spread than previously thought. This study indicates that the in-house nested RT-PCR assay is more suitable for the detection of EVs in water samples and valuable new data on the molecular epidemiology of novel EVs circulating in the community of South Africa was obtained.

A.5: **Netshikweta R,** van Zyl WB, Wolfaardt M, Taylor MB. Comparison of conventional nested RT-PCR and real-time RT-PCR for the detection of enteroviruses in water samples. [Poster/Presentation]. Faculty Day, Faculty of Health Sciences, University of Pretoria 14-15 August 2007, Pretoria

Enteroviruses (EVs) are members of the family Picornaviridae and are estimated to cause 30 million to 50 million infections per year in the United States. Enteroviruses are tolerant to residual chlorine from treatment processes and survive a wide range of temperatures and salinities thus facilitating their survival in environmental waters. Consequently they are one of the most commonly detected viruses in water. Given the load of EVs shed into waters and the role of EVs in waterborne disease, there is clearly a need for sensitive assays for the detection of EVs in water. The objective of this study was to develop a rapid, simple and efficient real-time RT-PCR procedure for the detection of to EVs in water samples. To this end real-time RT-PCR was compared to the conventional nested RT-PCR currently used for this purpose. Enteroviruses detected by either method were characterized, by sequence analysis, to determine whether any one type was preferentially detected by either method. In this study EVs were detected, by nested RT-PCR, in 27 of 271 (9.9%) water samples assayed and included 10 (7.1%) strains from treated drinking water and 17 (13.1%) from untreated and/or raw water sources. The results of a comparative study showed that the specificity of real-time RT-PCR and an in-house two-step nested RT-PCR assay were comparable, but that the sensitivity of the real-time RT-PCR was much less (3 $\log_{10}$) than the nested RT-PCR assay. This lack of sensitivity was further highlighted in this study where 27 of the 271 samples which tested positive for EV RNA by the in-house two-step nested PCR assay tested negative by real-time PCR. Sequencing of selected amplicons derived from the 5'-UTR region enabled differentiation between various serotypes of EVs. Enterovirus types CV-B1, CV-B3 and CV-A6 were identified but this was not unexpected as they have previously been detected in water samples. However the detection of rare and novel EV isolates, namely EV-68 and EV-89 was of great interest. Enterovirus 89 is one of four newly described EVs and the detection of EV89 in river water from the Western Cape suggests that this strain may be more widely spread than previously thought. This study indicates that the in-house nested RT-PCR assay is more suitable for the detection of EVs in water samples and valuable new data on the molecular epidemiology of novel EVs circulating in the community of South Africa was obtained.

APPENDIX B

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Analytical Methods for Virus Detection in Water and Food

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Abstract Potential ways to address the issues that relate to the techniques for analyzing food and environmental samples for the presence of enteric viruses are discussed. It is not the authors' remit to produce or recommend standard or reference methods but to address specific issues in the analytical procedures. Foods of primary importance are bivalve molluscs, particularly, oysters, clams, and mussels; salad crops such as lettuce, green onions and other greens; and soft fruits such as raspberries and strawberries. All types of water, not only drinking water but also recreational water (fresh, marine, and swimming pool), river water (irrigation water), raw and treated sewage are potential vehicles for virus transmission. Well over 100 different enteric viruses could be food or water contaminants; however, with few exceptions, most well-characterized foodborne or waterborne viral outbreaks are restricted to hepatitis A virus (HAV) and calicivirus, essentially norovirus (NoV). Target viruses for analytical methods include, in addition to NoV and HAV, hepatitis E virus (HEV), enteroviruses (e.g., poliovirus), adenovirus, rotavirus, astrovirus, and any other relevant virus likely to be transmitted by food or water. A survey of the currently available methods for detection of viruses in food and environmental matrices was conducted, gathering information on protocols for extraction of viruses from various matrices and on the various specific detection techniques for each virus type.

Keywords Enteric viruses · Gastroenteritis · Hepatitis · Detection · Concentration

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Introduction

The transmission of viruses through consumption of or contact with contaminated water and food is well recognized. Transmission of viruses associated with the consumption of contaminated bivalve shellfish, particularly, oysters which are eaten uncooked, is regularly reported (Koopmans and Duizer 2004; Widdowson et al. 2005). Other foods, including raspberries (Cotterelle et al. 2005; Le Guyader et al. 2004; Gaulin et al. 1999; Korsager et al. 2005; Hedlund et al. 2000; Falkenhorst et al. 2005; Hjertqvist et al. 2006; Fell et al. 2007; Ponka et al. 1999) and salads (Vivancos et al. 2009), have caused outbreaks after being contaminated by polluted water or virus-infected food handlers. Polluted water, both drinking water and recreational water, have been shown to have transmitted viruses (Cannon et al. 1991; Lawson et al. 1991; Maunula et al. 2005). Because many people may consume a batch of food or come into contact with the contaminated material, outbreaks involving large numbers infected people are common. The outbreaks of viral gastroenteritis are known to be mainly caused by norovirus (NoV) and outbreaks of viral hepatitis are caused by Hepatitis A virus (HAV) (Ramsay and Upton 1989; Reid and Robinson 1987; Calder et al. 2003; Niu et al. 1992; Hutin et al. 1999) and, in the case of water, more rarely, Hepatitis E virus (HEV) (Jothikumar et al. 1993).

In all the above cases, it is important to have effective tools with which to analyze the food or water matrix for its viral content. The following sections address the issues of what and how to take samples of food and water, how to release the virus from each matrix type, nonmolecular virus detection, nucleic acid extraction methods, and molecular detection techniques.

Sampling for Viruses Associated with Food

When sampling procedures for food/fresh produce are considered, the questions which arise are as follows: (i) is a specific weight of a particular fruit or vegetable representative, (ii) is one item, e.g. tomato, per crate, representative, (iii) is one leaf of a lettuce or cabbage representative, and if so, which leaf (iv) should the food item be analyzed whole or chopped, and (v) will a single sample suffice or should the test be repeated in triplicate?

In order to assess the real role of food in virus transmission, cost-effective standardized or comparable methods need to be developed for application in reference laboratories. The infectious dose for viruses, such as HAV and NoV, is estimated to be about 10–100 infectious particles; therefore, although the viral load on fresh produce, minimally processed, and ready-to-eat foods may be low, it may still be a source of infection and illness. Data on shellfish is still scarce, but some publications reported NoV concentrations ranging from 10^2 to 10^4 copies per gram of digestive tissues (Nishida et al. 2007; Le Guyader et al. 2003, 2006a, 2008, 2009). HAV has recently been quantified in naturally contaminated shellfish samples, showing titers ranging from 10^3 to 10^5 HAV genomes per gram of clam (Costafreda et al. 2006; Pintó et al. 2009), and it has also been reported in titers of 0.2–224 infectious particles per 100 g shellfish meat (Williams and Fout 1992).

Therefore, the methods for detection of viruses on food samples must have a high level of analytical sensitivity and specificity. With regard to sample size, it should represent the whole batch or crop, and that the US Environmental Protection Agency (US EPA), the International Organization for Standardization (ISO), and the national quality assurance (QA) regulations must be followed. There is, however, very little information in the literature, as well as in the US EPA and the European Committee for Standardization or Comité Européen de Normalisation (CEN)/ISO guidelines, regarding sampling procedures for the viral analysis of food. References to sampling for microbiology usually refer to the statistical representativeness of the samples, and economic and logistical considerations usually limit the number, type, and location of samples to be taken (Murray et al. 2002). Sampling for virological analyses of food will not necessarily follow the bacterial approach since the low level of contamination and the complexity and cost of assays are greater. The US EPA Manual of Methods for Virology (EPA, U.S. Environmental Protection Agency, Cincinnati 1984) only addresses the virological examination of water, with no mention of the virological examination of food. The Health Protection Agency (HPA) of UK has a standard operating procedure (SOP) for the preparation of samples for the microbiological examination of food samples (BS 5763). In this procedure, a 25 g sample of food, which is homogenized, is used, but this SOP only relates to subsequent bacteriological analyses where the results are reported as colony forming unit per gram (CFU/g) or milliliter (ml). Many of the published or methods under development for the detection of viruses on fruit and vegetables use a 10–100 g sample size (a detailed overview of these methods have been described by Croci and co-authors (2008); however, there is no mention of how many 10–100 g samples need to be taken in from a crate, field, or truckload of the particular food matrix to have a statistically representative sample. Obviously, sampling procedures vary according to food matrix type and must have to take the quantity of sample, seasonality, rainfall, and probable amount of contamination or pollution into account.

From the literature, it is also not clear whether the food sample should be analyzed as a whole or chopped. As most

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viral contamination would be from external sources during spraying or irrigation, a critical factor influencing the decision to analyze vegetables, whole or chopped, would be whether the claims that viruses can enter plants through root damage are substantiated or not. It appears that internal contamination of the leaves of tomato plants and green onions can occur (Oron et al. 1995; Chancellor et al. 2006) and that the internal contamination is of a much lower level than external contamination (Carter 2005; Urbanucci et al. 2009).

The detection of viruses inside a plant crop could potentially be an indicator of higher levels of external contamination and would be significant as these viruses would not be removed or inactivated by washing or ultraviolet irradiation (Carter 2005). Sampling of foods implicated in an outbreak of viral disease would be focused on the particular batches consumed.

Sampling for Viruses in Water

With regard to the virological analysis of water, a similar question arises as to those encountered with the virological analysis of food, namely, "What is a representative sample?" When sampling procedures for irrigation and washing water are considered the questions which come up are: (i) will a specific volume of water be representative, and (ii) will the water quality influence the sampling procedure?

Microbes pose the most significant waterborne health risk (Carter 2005) with waterborne diseases being misdiagnosed or underdiagnosed (Meinhardt 2006). Contamination of surface water with enteric viruses through disposal of human waste is a concern for public health, especially if these surface waters are used for recreational water, irrigation water, and the production of drinking water (Rutjes et al. 2005). The surveillance of irrigation water and water for washing the fresh produce is therefore essential to facilitate correct management procedures for the protection of fruit and vegetable growers and the health of farm workers and the consumers. In order to monitor the virological quality of water, an efficient combination of techniques has to be applied for the optimal recovery and detection of the low titres of viruses present in water (Gilgen et al. 1997; Soule et al. 2000).

Sampling and analytical procedures for the virological analysis of water are well documented (EPA, U.S. Environmental Protection Agency, Cincinnati 1984; Gerba 1987; Hurst and Reynolds 2002); American Public Health Association, American Water Works Association, Water Environment Federation 2005; Wyn-Jones 2007. A variety of techniques have been described for the recovery of viruses from water—each with their own advantages and disadvantages as the physicochemical quality of the water, including but not limited to the pH, conductivity, turbidity, presence of particulate matter, and organic acids, can all affect the efficiency of recovery of viruses (Richards et al. 2004). Viral recovery and concentration techniques include ultrafiltration (Soule et al. 2000; Divizia et al. 1989a; b; Garin et al. 1994), adsorption–elution using filters or membranes (Gilgen et al. 1997; Passagot et al. 1985; Senouci et al. 1996), glass wool (Vilaginès et al. 1993; Vilaginès et al. 1997) or glass powder (Gajardo et al. 1991; Menut et al. 1993), two-phase separation with polymers (Schwab et al. 1993), flocculation (Nasser et al. 1995; Backer 2002), and the use of monolithic chromatographic columns (Branovic et al. 2003; Kramberger et al. 2004; Kovac et al. 2009; Gutierrez-Aguirre et al. 2009). The use of the glass wool adsorption–elution procedure for the recovery of enteric viruses from large volumes of water has proven to be a cost-effective method and has successfully been applied for the routine recovery of human enteric viruses from large volumes of water in the South African setting (Taylor et al. 2001; Van Heerden et al. 2004, 2005; Vivier et al. 2001, 2002, 2004; Van Zyl et al. 2004, 2006; Venter 2004). This method can be adapted for the in-line recovery of viruses from water (Grabow et al. 1996) which circumvents transporting of large volumes of potentially highly polluted water great distances to a central laboratory which would be expensive and a potential health hazard.

It is important to acknowledge that no single method may universally be recognized as superior: efficiency, constancy of performance, robustness, cost, and complexity are all factors to be considered for each method and performance characteristics must be continually monitored.

Sampling Aerosols and Surfaces

There is concern over the potential transmission of viruses into the food chain through aerosols and from surface contamination. The crucial issues are sample collection and preparation for different virus detection techniques: different methods have been developed based on the attachment properties to surfaces of airborne particles (Verreault et al. 2008).

The most used air samplers are based on impact on solid surfaces, impingement, and filtration: all of them have been successfully used for virus detection, but have advantages and disadvantages.

Impact Samplers

An air flow with a fixed speed is directed to impact on a solid surface, generally an agar medium (Booth et al. 2005). Some equipment (Andersen sampler) has multiple stages and can sample separately particles with different size. After sampling, virus can be eluted from the solid medium, purified and prepared for subsequent virus analysis. This

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sampling method is easy, but dehydration or impact trauma can affect the virus survival; flow rate and sampling duration are critical.

Impinger Samplers

The air is forced to flow through a narrow orifice to make bubbles in a liquid medium (Pyankov et al. 2007). After sampling the medium can be concentrated or directly decontaminated, purified, and analyzed. The recovery efficiency of this method is high because it avoids dehydration but flow rate and the composition of the collection fluid are again critical for virus recovery (Hogan et al. 2005; Hermann et al. 2006).

Filter Samplers

The air passes through a filter and airborne particles are retained as a function of their aerodynamic size and surface properties, such as electrostatic charge. For aerosol sampling, membranes with 1–3 μm pore size can retain droplets with an aerodynamic size <500 nm more efficiently than other samplers (Verreault et al. 2008). Viruses in an aerosol are associated with particles and can be collected. The membrane material can be polytetrafluoroethylene (PTFE), cellulose, polycarbonate, or gelatine (Burton et al. 2007). The last one is easier for viral sampling, because it can be directly dissolved in an appropriate liquid medium. This sampling method is easy to use, but the flow rate, the sampling duration, and the membrane composition have to be strictly controlled to avoid dehydration.

Further methods for the analysis of viruses in aerosols include cyclone (Alexandersen et al. 2002) or electrostatic precipitators (Moore et al. 1979), and in the last years, the fear of bioterrorism stimulated the study of new methodologies (like mass spectrometry) (Johnston 2000) that are able to identify pathogens in air. However, their application to the routine environmental analysis is still far in the future and will require very large result data bases from many environmental samples.

To better understand the fate of virus dispersed through air, surface monitoring should be also performed, because of the settling of droplets with greater size. Surface sampling has its major indication in health care settings (Carducci et al. 2002) and in food production (Scherer et al. 2009) to assess not only viral contamination but also efficiency and correct application of disinfection procedures. To this aim, a definite surface area (i.e., 10 cm^2) is swabbed, then the swab is eluted and the eluate is processed as a liquid sample. For biomolecular tests, some swabs can be submitted directly for nucleic acid extraction. Alternative methods are contact plates that can be eluted.

Virus Release from Food Matrices

The food matrix and the route of contamination involved, determines the way of virus release prior to nucleic acid extraction. Viral particles need to be extracted from homogenized tissues in case of intrinsic contamination (i.e., oysters tissues, pig liver) or eluted from the surface of the food item (i.e., contamination by irrigation water or food handling).

Shellfish are filter feeders and concentrate enteric viruses from their environment while feeding. The majority of accumulated virus is found in the pancreatic tissue, also called the digestive diverticula. Mechanical entrapment and ionic bonding are among the mechanisms that have been suggested to explain observed differences in accumulation of different viruses and among different oyster species (Burkhardt and Calci 2000; Di Girolamo et al. 1977). Another potential mechanism for the uptake and concentration of viruses in shellfish has been proposed based upon the observation of specific binding of a NoV genogroup I to shellfish tissues (Le Guyader et al. 2006b).

Several efficient methods are now available for shellfish analysis; for example, Atmar et al. (1995), proposed the dissection of digestive tissues for virus extraction. Testing the stomach and digestive gland for virus presented several advantages in comparison with testing whole shellfish: less time-consuming procedure, increased test sensitivity, and decreased sample-associated interference with reverse transcription polymerase chain reaction (RT-PCR) (Atmar et al. 1995). Since the initial description of analyzing only digestive tissues, a number of variations have been published, and most have analyzed the same weight (1.5–2 g) of digestive tissues. Viruses are eluted using various buffers (e.g., chloroform-butanol or glycine) before concentration by polyethylene glycol (PEG) or ultracentrifugation (Nishida et al. 2007; Schwab et al. 2001; Fukuda et al. 2008; Milne et al. 2007). Direct lysis of virus particles is used more and more frequently. For example, proteinase K or Trizol and lysis of shellfish tissues using Zirconia beads and a denaturing buffer have all been used for virus elution (Jothikumar et al. 2005; Lodder-Verschoor et al. 2005).

Methods that have currently been developed and optimized for virus detection from fruit and salad vegetables focus on elution of the virus from the surface (Croci et al. 2008). A number of washing procedures and buffer systems have been described for the recovery of viruses from fruits and vegetables. The average recovery rates vary depending on the food matrix and virus (Croci et al. 2008). Dubois et al. (2007) described a protocol that included the rinsing of fruit and vegetable surfaces with a buffer of pH 9.5, supplemented with 100 mM Tris, 50 mM glycine, and 1% beef extract, a protein- and nucleic acid-rich substance. This buffer ruptures the electrostatic and hydrophobic

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interactions between fruit or vegetable surfaces and virus. In the case of soft fruits, pectinase has to be added to prevent the formation of a gelatinous substance (Rzezutka et al. 2005; Butot et al. 2007).

Some authors have reported the presence of viral particles trapped inside vegetables taken up intracellularly through the roots (Oron et al. 1995; Chancellor et al. 2006; Carter 2005; Urbanucci et al. 2009). This mechanism warrants further examination, and if confirmed, it will change future approaches for the detection of viruses from vegetables.

Virus concentrations on food are likely to be low, indicating that the virus which is present in the relatively large volumes of elution buffer, needs to be concentrated prior to detection. The choice of virus concentration method is dependent on the food matrix and eluant. Frequently used concentration methods include precipitation by PEG, ultrafiltration, and ultracentrifugation.

Immunological methods have also been applied to concentrate virus in food (Bidawid et al. 2000; Kobayashi et al. 2004; Shan et al. 2005; Tian and Mandrell 2006). However, NoV immunoconcentration is unlikely to be adapted widely for NoV detection due to the difficulties in obtaining antibodies and its variability at the capsid level.

Nucleic Acid Extraction and Purification

Following virus elution or concentration, a variety of subsequent nucleic acid extraction and purification protocols may be employed. Recently, a number of methods using kits have been published. A wide variety of commercial kits has been applied for nucleic acid purification, offering reliability, reproducibility, and they are quite easy to use. Most of these kits are based on guanidinium lysis and then the capture of nucleic acids on a column or bead of silica (commonly called Boom's method (1990)). Although most perform well, differences can be found depending on the virus and/or matrix analyzed (Rutjes et al. 2005; Hourfar et al. 2005; Knepp et al. 2003; Kok et al. 2000; Le Guyader et al. 2009; Burgener et al. 2003). In the last years, automated nucleic acid extraction platforms have been developed by commercial companies, which have been shown to be suitable for the analysis of virus in water samples (Rutjes et al. 2005; Perelle et al. 2009).

Virus Detection

Virus detection is mainly based on two principles: the detection of infectious viruses by propagation in cell culture or the detection of the viral genomes by molecular amplification techniques such as PCR or RT-PCR. Detection by cell culture is mainly based on the formation of cytopathic effects, with the quantification of the viruses by plaque assay, with the most probable number or tissue culture infectious dose 50 ($TCID_{50}$). Virus typing may be done by immunofluorescence or neutralization assays. However, efficient cell culture systems are not available for all viruses, and others grow slowly or do not produce a cytopathic effect.

In the last decade, real-time PCR assays have revolutionized nucleic acid detection by the high speed, sensitivity, reproducibility, and minimization of contamination. These methods are widely used in the field of food virology and are continuously evolving. For instance, Sanchez et al. (2007) summarized published real-time RT-PCR methods for HAV detection in food, and since then, several new methods have become available (Houde et al. 2007; Casas et al. 2007). It is essential that the specificity, the range of viruses detectable, and the sensitivity of real-time RT-PCR assays are demonstrated. All these points are interconnected and depend mostly on the target sequences for primers and probe. The selected targets must guarantee an absolute specificity and must reach equilibrium between high sensitivity, broad reactivity, and reliability of quantification.

Real-time RT-PCR procedures enable not only the qualitative but also the quantitative detection, which opens the possibility of quantitative hazard risk assessment analysis critical for several public health actions or food ban regulations. Quantification can also be performed as most probable number by conventional PCR (Rutjes et al. 2005, 2006a). Qualitative real-time PCR producing a positive or negative result is most appropriate when testing matrices that are unlikely to be contaminated with virus as it is least expensive and straightforward. Quantitative real-time PCR is required when a sample, such as shellfish, is likely to contain viruses and the degree of contamination needs to be ascertained.

Molecular assays by conventional PCR, i.e., gel-based, remain useful, as larger volumes of sample can be tested, larger PCR products can be obtained, and it is less expensive. The alternative molecular technique nucleic acid sequence-based amplification (NASBA) was shown to be less prone to environmental PCR inhibitors present in large volumes of surface water samples (Rutjes et al. 2006b).

Although the detection of enteric viruses in food is mainly done by molecular techniques, there are several limitations. The method is prone to inhibition, favoring false negative results and demonstrating the need for proper quality control (QC). Several ways have been described to overcome this inhibition, such as the analysis of samples dilutions, smaller sample sizes, adaptation of the PCR by, e.g., the addition of Tween, BSA, or commercial reagents (Rutjes et al. 2005; Butot et al. 2007; Al-Soud and Radstrom 2001).

One of the major limitations of PCR is its inability to differentiate between infectious and noninfectious viruses.

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Various approaches to overcome this limitation have been evaluated. Of them, integrated systems based on the molecular detection of viruses after cell culture infection are the most promising techniques (Pintó et al. 1994; 1995; Reynolds et al. 2001); a detailed overview of these approaches can be found elsewhere (Rodriguez et al. 2009). The integrate cell culture (ICC)-PCR assay is based on a selective enumeration of infectious viruses in combination with a rapid molecular detection, circumventing long incubation periods for cytopathic effect formation. Such ICC-PCR assays have been successfully utilized for the detection of several enteric viruses in environmental samples (Reynolds et al. 2001). Other alternatives, such as a protease and RNase pretreatment, have successfully been used to differentiate between infectious and noninfectious virus (Nuanualsuwan and Cliver 2002, 2003; Lamhoujeb et al. 2008; Topping et al. 2009), although Baert and collaborators (2008a) did not find correlation for murine NoV.

Quality Controls

One of the most critical challenges is the implementation of novel molecular-based methods for the detection of enteric viruses in the routine food analytical laboratories. However, obstacles that influence routine virus detection in foods include the low efficiency of concentration and nucleic acid extraction procedures and the presence of inhibitors to the molecular reactions. It seems obvious that harmonization of the molecular techniques, as well as addressing QA/QC (quality assurance/quality control) issues is required before adoption of the procedures by routine monitoring laboratories. QA/QC measures include the use of positive and negative controls, thus tracing any false negative or false positive result, respectively. Most false negatives are consequence of inefficient virus and/or nucleic acid extraction and of inhibition of the RT reaction. Most false positives result from cross-contamination.

The first dilemma is to choose between an actual internal control and an added external control for the extraction

Table 1 Terms and definitions in standardized molecular detection assays for virus detection in food

Process control virus	A virus added to the sample portion at the earliest opportunity prior to virus extraction to control for extraction efficiency
Process control virus RNA	RNA released from the process control virus in order to produce a standard curve data for the estimation of extraction efficiency
Negative RNA extraction control	Control free of target RNA carried through all steps of the RNA extraction and detection procedure to monitor any cross-contamination events
Negative process control	Control free of target RNA carried through all steps of the virus extraction, RNA extraction, and detection procedure to monitor any cross-contamination events
Hydrolysis probe	An oligonucleotide probe labeled with a fluorescent reporter and quencher at the 5′ and 3′ ends, respectively. Hydrolysis of the probe during real-time PCR due to the 5′–3′ exonuclease activity of Taq DNA polymerase results in an increase in measurable fluorescence from the reporter
Negative RT-PCR control	An aliquot of highly pure water used as template in a real-time RT-PCR reaction to control for contamination in the real-time RT-PCR reagents
External control RNA	Reference RNA that can serve as target for the real-time PCR assay of relevance, e.g., run-off transcripts from a plasmid carrying a copy of the target gene, which is added to an aliquot of sample RNA in a defined amount to serve as a control for amplification in a separate reaction
Cq value	Quantification cycle; the PCR cycle at which the target is quantified in a given real-time PCR reaction. This corresponds to the point at which reaction fluorescence rises above a threshold level
Theoretical limit of detection (tLOD)	A level that constitutes the smallest quantity of target that can, in theory, be detected. This corresponds to one genome copy per volume of RNA tested in the target assay, but will vary according to the test matrix and the quantity of starting material
Practical limit of detection (pLOD)	The lowest concentration of target in a test sample that can be reproducibly detected (95% confidence interval), as demonstrated by a collaborative trial or other validation (Annex L)
Limit of quantification (LOQ)	The lowest concentration of target in a test sample that can be quantitatively determined with acceptable level of precision and accuracy, as demonstrated by a collaborative trial or another

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procedure. For the diagnosis of an RNA virus, the use of an internal control based on the detection of the expression of a housekeeping gene, ideally containing introns, through the amplification of its messenger RNA (mRNA) in the target tissues is a clear first choice. However, this is an unrealistic approach for its application in food virology, which involves an increasingly heterogeneous selection of food matrices. For instance, in shellfish only, a pair of primers to amplify an mRNA for a specific hepatopancreas transcribed gene would be required for each species. It is impossible to apply this for the range of foodstuffs susceptible to be assayed for viruses, which leads to compromise in the use of an external control, applicable to all matrices under assay. Table 1 depicts the complete list of terms and definitions in standardized molecular detection assays for virus detection in food (Hoorfar et al. 2003, 2004; Rodríguez-Lázaro et al. 2007; Costafreda et al. 2006; Hoorfar and Cook 2003; Rodriguez-Lazaro et al. 2004, 2005).

One of the most important issues is the control of nucleic acid extraction efficiency. Recently, the use of a nonpathogenic viruses, mengovirus MC0 (Mattison et al. 2009) and feline calicivirus (Butot et al. 2008; Cannon et al. 2006; Hewitt and Greening 2004; Pintó et al. 2009), have been proposed as process control, although the latter has been reported to be an inappropriate surrogate for NoV in acid conditions (Pintó et al. 2009). Quantitative standardized procedures presently enable to perform quantitative microbial risk assessment (QMRA) in food samples (Gassilloud et al. 2003; Arnal et al. 1998).

Several authors have reported that the number of infectious viruses did not correlate with the number of genomes detected by real-time RT-PCR in water samples (Baert et al. 2008a, b; Butot et al. 2008, 2009; Hewitt and Greening 2004). This is more evident when water or food undergoes a removal/inactivation process.

It is a matter of debate whether the detection of enteric viruses in food or water by PCR or real-time PCR should be considered a safety issue and confirmation of a public health risk. However, one can argue that if viruses were found, even if not infectious, it would be an indication that the food or water is contaminated and that viruses were present in the food or water at some point.

Summary

The analysis of food and water matrices for the detection of viruses is now well established to the extent that European Standards are in draft:

- Microbiology of food and animal feeding stuffs—Horizontal method for detection of hepatitis A virus and norovirus in food using real-time RT-PCR—Part 1: Method for quantitative determination
- Microbiology of food and animal feeding stuffs—Horizontal method for detection of hepatitis A virus and norovirus in food using real-time RT-PCR—Part 2: Method for qualitative detection

Validation studies are expected to be undertaken for each of the process stages before the standard is confirmed. This QA will ensure that the highest level of QA is achieved. Developmental studies on matrices not covered by the standard will continue to be required to reach consensus on the optimum techniques necessary to ensure effective systems.

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ORIGINAL ARTICLE

The detection of enteric viruses in selected urban and rural river water and sewage in Kenya, with special reference to rotaviruses

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Abstract

Aim: To determine the occurrence of eight human enteric viruses in surface water and sewage samples from different geographical areas in Kenya.
Methods and Results: Enteric viruses were recovered from the water and sewage sources by glass-wool adsorption elution and/or polyethylene glycol/NaCl precipitation and detected by singleplex real-time and conventional PCR and reverse transcriptase-PCR assays. One or more enteric viruses were detected in nearly all sewage and river water samples except the urban Mbagathi River. The VP7 (G types) and the VP4 (P types) of the rotaviruses (RV) were characterized by multiplex nested PCR methods. The G and P types could be determined in 95·5% of the RV strains, respectively. Mixed G types were detected with G12 and G1 predominating, and unusual G types, G5 and G10, were present. P[4] predominated in the urban Karen sewage samples, while P[8] predominated in the urban and rural streams.
Conclusions: The high prevalence of RVs in surface water highlights the importance of assessing the water sources used for domestic purposes for viral contamination.
Significance and Impact of the Study: This study demonstrates the benefit of environmental surveillance as an additional tool to determine the epidemiology of RVs and other enteric viruses circulating in a given community.

Introduction

Worldwide, an estimated 1·1 billion people do not have access to safe drinking water, and 2·6 billion people lack adequate sanitation resulting in an estimated 4000–6000 children dying daily because of these adverse conditions (Moe and Rheingans 2006). In low-income countries, hygiene and sanitation impact heavily on population health and the burden of waterborne disease (Ford 2006). Viruses are a major cause of waterborne disease, but the health impact of waterborne viral infections is underestimated (Grabow 2007; Mena 2007). Human enteric viruses, which primarily infect and replicate in the gastrointestinal tract, have been associated with waterborne transmission (Carter 2005; Grabow 2007). The enteric viruses, which are shed in large numbers in the faeces of infected individuals, are stable in the environment and may survive wastewater treatment (Baggi and Peduzzi 2000; Carter 2005). These viruses therefore have the potential to pollute surface (Pintó and Saiz 2007), ground (Gerba 2007) and drinking water (Carter 2005). More than 100 types of enteric viruses may be present in faecally contaminated water (Fong and Lipp 2005), and their presence in water sources is of public health concern because of their low infectious dose (Wyn-Jones and Sellwood 2001; Fong and Lipp 2005; Teunis *et al.* 2008). Indicator

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micro-organisms have traditionally been used to assess the microbial quality of water (Ashbolt *et al.* 2001), but enteric viruses have been detected in water which conform to quality limits for indicator organisms (van Heerden *et al.* 2004; Pusch *et al.* 2005), consequently the enteric virus group is considered by the United States of America (USA) Environmental Protection Agency as the most meaningful, reliable and effective index for environmental monitoring (Vantarakis and Papapetropoulou 1998).

Worldwide, enteric viruses such as rotaviruses (RV), noroviruses (NoV), sapoviruses (SaV), human astroviruses (HAstV), human adenoviruses (HAdVs) and hepatitis A virus (HAV) are important agents of gastroenteritis and hepatitis, respectively (Bern and Glass 1994; Parashar and Glass 2003; Hollinger and Emerson 2007). RV is the most common cause of viral diarrhoeal disease in infants with virtually all children being infected by the age of 5 (Parashar *et al.* 2006). However, infection in older children and adults can occur (Bishop 1994; Anderson and Weber 2004; Estes and Kapikian 2007). Although water was previously not considered to be an important route of RV transmission (Grabow 2007), there is an increase in RV-associated waterborne outbreaks (Gratacap-Cavallier *et al.* 2000; Villena *et al.* 2003a). NoVs are an important cause of epidemic acute gastroenteritis (Green 2007), and waterborne outbreaks of NoV-associated gastroenteritis are well documented (Kukkula *et al.* 1999; Wyn-Jones and Sellwood 2001; Schwab 2007). HAdVs and HAstVs, which are associated with sporadic cases and occasional outbreaks of gastroenteritis (Bern and Glass 1994), have been detected in water sources, but the role of water in their transmission is as yet undetermined (Wyn-Jones and Sellwood 2001). The waterborne transmission of HAV, the most common cause of hepatitis in man globally, is well recognized (Bosch *et al.* 1991; Hollinger and Emerson 2007). Although there can be a lack of correlation between the presence of enteroviruses and pathogens such as HAV, enteroviruses are useful targets for the assessment of viral pollution (Formiga-Cruz *et al.* 2005; Shieh *et al.* 2008). Routine conventional diagnostic assays cannot be applied to the detection of enteric viruses in water as these viruses are usually present in low titres which are below the detection limits of the assays (Koopmans and Duizer 2004). Routine cell culture techniques, the gold standard for the isolation and detection of enteroviruses, cannot be applied for wild-type strains of HAV, HAstV and human RV as they are difficult to culture *in vitro* (Formiga-Cruz *et al.* 2005; Greening 2006), and NoVs cannot be propagated at all in cell culture (Duizer *et al.* 2004; Greening 2006). The development of molecular-based detection assays such as the polymerase chain reaction (PCR) and reverse transcriptase-PCR (RT-PCR) has improved the detection of viruses in water (Fong and Lipp 2005), and PCR and RT-PCR is now considered to be the gold standard for enteric virus detection in water (Bosch *et al.* 2008).

RVs have been identified as a significant cause of morbidity in Kenya, and the epidemiology of RV-associated diarrhoea is well described (Kiulia *et al.* 2006, 2008, 2009). The prevalence of HAstV and HAdV in diarrhoeal stool specimens has also been documented (Kiulia *et al.* 2007; Magwalivha *et al.* 2010), but there are no data on the occurrence of NoV-associated diarrhoea. However, as is the case for most of the low-income countries, the potential waterborne transmission of these enteric viruses in Kenya has not been investigated. There are no reports on the occurrence of enteric viruses in treated and untreated water sources or wastewater. Data regarding the presence of enteric viruses in rivers and wastewater treatment plants will be useful in assessing the potential risk of infection through waterborne transmission and the effectiveness of water treatment procedures. As wastewater treatment plants collect and treat community effluent, evaluation of viruses occurring in sewage samples may be used as an additional indicator of viruses circulating in a community (Katayama *et al.* 2008). This study represents a pilot investigation into the virological quality of selected urban and rural rivers and sewage water in Kenya. Selected river water and sewage samples were analysed for the possible presence of NoV genogroup I (GI), NoV genogroup II (GII), HAV, RV, SaV, HAstV, HAdV and enteroviruses using highly specific and sensitive qualitative PCR, RT-PCR and real-time RT-PCR assays. These data will provide new information as to the contamination of Kenyan water sources by human enteric viruses as well as provide valuable data regarding enteric viruses circulating in the surrounding communities.

Materials and methods

Water sample and sewage collection

From May 2007 to February 2008, water samples were collected in Kenya namely: surface water samples from the Mboone River in the rural Maua region ($n = 12$), surface water samples from the Mbagathi River in an urban setting in Nairobi ($n = 7$) and surface water samples from a Kibera wastewater stream which flows into the Nairobi dam ($n = 10$). The sampling site for the urban Kibera wastewater stream was located close to an area with a high human population density, while the sample collection site for the urban Mbagathi river was located within the Oloolua forest. The sample collection site from the Mboone river was close to a densely populated area close to the town of Maua. The river water (10 l) from Mbagathi and Mboone and Kibera wastewater stream

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(1 l) samples were collected in sterile containers and transported on ice to the laboratory where they were kept at 4°C until processing. During the same period, 13 grab samples of raw sewage (1 l) were collected at monthly intervals from the Institute of Primate Research (IPR) and the Karen sewage treatment plants, Nairobi, and kept at 4°C until processing. The Karen sewage plant serves an up-market low density housing estate with little or no industrial effluent. Details regarding the prevailing weather conditions, i.e. wet or dry season, were noted for each sampling date.

Virus recovery and concentration

Viruses were recovered from the river water samples using a glass-wool adsorption elution procedure, with an estimated efficiency of recovery of ≤40% (Venter *et al.* 2007), based on the method of Vilaginès *et al.* (1993) as described by Wolfaardt *et al.* (1995) and further modified and optimized by Venter (2004). Briefly, water samples (10 l) were filtered through the positively charged glass wool columns using negative pressure at a flow rate of 10 l h^{-1}. The negatively charged viruses, which adsorbed to the glass wool, were eluted twice with 50 ml glycine-beef extract buffer pH 9·5 [GBEB; 0·05 mol l^{-1} glycine; 0·5% beef extract (Merck, Darmstadt, Germany)]. Immediately after elution, the pH of the eluate was neutralized to pH 7 using 1 mol l^{-1} HCl (Merck). The 100 ml eluate was subjected to secondary concentration using a polyethylene glycol (PEG)/sodium chloride (NaCl) precipitation method (Minor 1985). The resulting pellet was resuspended in 20 ml phosphate-buffered saline pH 7·4 (PBS; Sigma-Aldrich Inc., St Louis, MO, USA), and the recovered virus samples were stored at −20°C until further analysis. Enteric viruses were recovered from the raw sewage (1 l) and wastewater (1 l) into a final volume of 20 ml PBS using the PEG/NaCl precipitation method described by Nadan *et al.* (2003), but without the final ultrafiltration step.

Nucleic acid extraction

The recovered virus suspensions were clarified by adding 200 μl of chloroform (Merck) to 1·5 ml of the suspension followed by rigorous vortexing for 30 s. The mixture was centrifuged at 3000 ***g*** (Eppendorf 5402 microcentrifuge; Eppendorf Gerätebau, Netheler & Hinz GmbH, Hamburg, Germany) for 30 s, and 1 ml of the supernatant was used for nucleic acid extraction. Genomic viral nucleic acid was extracted directly from 1 ml of the recovered virus concentrate using the MagNA Pure LC Total Nucleic Acid Isolation kit (large volume) (Roche Diagnostics GmbH, Mannheim, Germany) in a MagNA Pure LC instrument (Roche), following the manufacturer's instructions. As extracted nucleic acid is more prone to degradation than nucleic acid in intact virions, the eluted nucleic acid (100 μl) was aliquoted and stored at −70°C until use. To monitor any cross-contamination between specimens, nuclease-free water (Promega Corp., Madison, WI, USA) was introduced as a negative extraction control in every fourth well of the sample cartridge and as the water samples were highly contaminated, no positive extraction control was included.

Viral amplification and detection

The HAdVs and group A RVs were amplified, by conventional nested PCR and nested RT-PCR, respectively, using 8 and 10 μl of the undiluted purified nucleic acid template, respectively, for each reaction. Adenoviruses were detected using the method described by Avellón *et al.* (2001) and modified by van Heerden *et al.* (2004), and RVs were detected using the method described by van Zyl *et al.* (2004, 2006). The PCR products were analysed by 2% agarose gel (Seakem® LE agarose; Lonza, Rockland, ME, USA) electrophoresis and visualized by ethidium bromide staining and UV illumination using a 100-bp marker (O'GeneRuler; Fermentas Life Sciences, Burlington, ON, Canada) to determine amplicon size. The HAstVs, enteroviruses, NoV GI, NoV GII, HAV and SaV were detected using qualitative singleplex real-time RT-PCR assays based on TaqMan technology and published primers and probes, namely: HAstVs (Le Cann *et al.* 2004); enteroviruses (Fuhrman *et al.* 2005); HAV (Costafreda *et al.* 2006); NoV GI (Da Silva *et al.* 2007; Svraka *et al.* 2007); NoV GII (Jothikumar *et al.* 2005; Loisy *et al.* 2005) and SaV (Chan *et al.* 2006). Reverse transcription was carried out using 10 μl of the undiluted purified nucleic acid template in the Transcriptor First Strand cDNA synthesis kit (Roche), and amplification was performed using the Lightcycler® Taqman® Master kit (Roche) following manufacturer's instructions. The reaction, using 5 μl of cDNA, was carried out in a total volume of 20 μl in sealed glass capillaries in a Lightcycler® v1.5 instrument (Roche).

Quality control

Standard precautions were applied in all the manipulations to reduce the possibility of cross-contamination between samples, and sample and reaction contamination by DNA amplicons. Separate laboratories, each equipped with its own apparatus, pipettes, filter-tips and reagents tubes, were used for sample processing and preparation, reagent preparation, reaction preparation and manipulation of amplified fragments. The negative extraction controls, a negative PCR/RT-PCR control (nuclease-free water), and a positive

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control (nucleic acid from an appropriate characterized cell-cultured adapted or wild-type virus) were included in each virus detection reaction to monitor for false-positive and false-negative reactions.

Typing of rotaviruses

RVs are classified according to G and P genotypes and may be called a strain, genotype or type. Multiplex nested PCR methods and primers initially developed for the G typing (Gouvea *et al.* 1990, 1994a; Das *et al.* 1994; Gault *et al.* 1999; Iturriza-Gómara *et al.* 2004) and the P typing (Gentsch *et al.* 1992; Gouvea *et al.* 1994b; Mphahlele *et al.* 1999; Simmonds *et al.* 2008) of human and animal RVs from clinical samples were applied for the characterization of the VP7 (G types) and the VP4 (P types) genes of the strains from RV-positive samples.

Statistical analysis

Analysis of the viral prevalence rates in river samples and sewage samples was carried out using Fisher's exact test using StatView software (ver. 5.0; SAS Institute Inc, Cary, NC, USA). Differences with *P*-values >0·05 were considered not significant at 95% confidence interval.

Results

Prevalence of enteric viruses in the river water samples

The prevalence of the enteric viruses detected in the river water samples is summarized in Table 1 and reflect qualitative presence–absence data. In the rural Meru North district, one or more enteric viruses were detected in 8/12 (66·7%) of the water samples drawn from the Mboone river, Maua. HAstVs, which were present in 5/12 (41·7%) of the samples, were the most prevalent viruses detected. Norovirus GII, enteroviruses and RVs were detected in 3/12 (25%), HAdVs in 2/12 (16·7%) and NoV GI in 1/12 (8·3%) of the samples. HAV and SaV were not detected in any of the samples. No seasonal pattern was noted as enteric viruses were detected in both the wet and dry seasons. HAstVs were, however, detected in four consecutive samples drawn in November and December 2007 in the wet season.

From the urban Mbagathi River, only two samples (2/7; 28·6%) that were drawn at different times were shown to be positive for enteric viruses, namely SaV and HAstV (Table 1). Of the two urban rivers, the Kibera wastewater stream, a tributary of Nairobi dam, showed the highest level of contamination. Four or more enteric viruses were detected in all 10 (100%) of the samples analysed, but no sample tested positive for all viruses.

Table 1 Prevalence of enteric viruses in selected urban and rural river water in Kenya

	Number (%) enteric viruses detected in			
	Urban rivers and streams		Rural river	
Enteric virus	Kibera (*n* = 10)	Mbagathi (*n* = 7)	Mboone (*n* = 12)	*P* value*
Any enteric virus	10 (100%)	2 (28·6%)	8 (66·7%)	
Enteroviruses	10 (100%)	0	3 (25%)	<0·05
Hepatitis A virus	8 (80%)	0	0	<0·05
Human adenovirus	9 (90%)	0	2 (16·7%)	<0·05
Human astrovirus	6 (60%)	1 (14·3%)	5 (41·7%)	
Group A rotavirus	10 (100%)	0	3 (25%)	<0·05
Norovirus GI	9 (90%)	0	1 (8·3%)	<0·05
Norovirus GII	9 (90%)	0	3 (25%)	<0·05
Sapovirus	9 (90%)	1 (14·3%)	0	<0·05

GI, genogroup I; GII, genogroup II.
P value* denotes a statistically significant difference (Fishers' exact test) between the prevalence of enteric viruses in the Kibera stream and the prevalence of enteric viruses in both the Mbagathi river and the Mboone river.

Enteroviruses and RVs were present in all ten samples, while NoV GI, NoV GII, HAdV and SaV were detected in 9/10 (90·0%), HAV in 8/10 (80%) and HAstVs in 6/10 (60%) of the samples (Table 1). No seasonal pattern in the prevalence of the enteric viruses was noted as viruses were detected in both the wet and dry seasons (data not shown). The Kibera wastewater stream showed a statistically significant ($P < 0·05$) higher prevalence of enteric viruses, excluding HAstVs, than the other rivers.

Prevalence of enteric viruses in the sewage samples

A total of 13 raw sewage samples were analysed: five samples from the IPR sewage plant and eight from the Karen sewage plant (Table 2). One or more enteric viruses were detected in all the sewage samples analysed with RVs, HAdVs and enteroviruses being the most prevalent viruses (Table 2). The samples from the IPR sewage plant were all positive for enteroviruses and HAdVs, with SaV and NoV GI being detected in 3/5 (60%) samples, while NoV GII were detected in 2/5 (40%) and RVs in 1/5 (20%) of the sewage samples, respectively (Table 2). HAV and HAstVs were not detected in any of the IPR sewage samples. RVs were detected in eight (100%) of the samples from the Karen sewage plant, while HAdVs and HAstVs were each present in seven (87·5%) samples. Enteroviruses and NoV GII were detected in 4/8 (50%), and SaV and HAV were demonstrated in 1/8 (13%) samples, respectively. Of note was the absence of NoV GI

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Table 2 Prevalence of enteric viruses in urban sewage in Kenya

Enteric virus	Number (%) detected in Urban sewage		
	IPR* (n = 5)	Karen (n = 8)	P value†
Any enteric virus	5 (100%)	8 (100%)	
Enteroviruses	5 (100%)	4 (50%)	
Hepatitis A virus	0	1 (12·5%)	
Human adenovirus	5 (100%)	7 (87·5%)	
Human astrovirus	0	7 (87·5%)	<0·05
Group A rotavirus	1 (20%)	8 (100%)	<0·05
Norovirus GI	2 (40%)	0	
Norovirus GII	2 (40%)	4 (50%)	
Sapovirus	3 (60%)	1 (12·5%)	

GI, genogroup I; GII, genogroup II.
*IPR, Institute of Primate Research.
†P value: denotes a statistically significant difference (Fishers' exact test) between the prevalence of enteric viruses in the IPR sewage and the Karen sewage.

in the samples from the Karen sewage treatment works (Table 2). There was a statistically significant ($P < 0·05$) difference in prevalence of HAstVs and RVs in the two sewage plants (Table 2).

Distribution of RV genotypes

The G genotypes of 21/22 (95·5%) of the RV strains could be determined by multiplex RT-PCR of the VP7 gene. Mixed G types predominated (Table 3) with single strains only detected in the Mboone rural river in July 2007 and January 2008, genotypes G3P[8] and G2P[8], respectively. In the urban Kibera wastewater stream, types G1, G9, G10, G11 and G12 were present with G12 (100%) and G1 (90%) predominating. Genotype G9 was identified in four (40%) of the samples, all taken during the period November 2007 to January 2008, and G10 was identified in one (10%) sample. In comparison, types G1, G5, G8, G9 and G12 were identified amongst the strains detected in samples from the Karen sewage treatment plant in urban Nairobi. As was noted with the Kibera wastewater stream, types G1 (87·5%) and G12 (75%) predominated in the urban sewage. Genotype G9 was identified in four (50%) sewage samples, and G5 and G8 were presented in one sample each.

P types could be determined for all but one (95·5%) of the RV-containing samples (Table 3). Mixed P types were identified in both the urban (Kibera) and rural (Mboone) rivers as well as in the wastewater from the urban Karen sewage treatment plant. In the RV strains detected in the rural Mboone river, the predominant P type identified was P[8]. Genotype P[8] was demonstrated to be the predominant P type in 90% of the samples from the Kibera wastewater stream with P[4] and P[6] present in 40% and 20% of the samples, respectively. In the single RV-positive sample of sewage from the IPR, a P[8] P type was identified. However, in the samples from the Karen sewage treatment plant, P[4] predominated, being

Table 3 Rotavirus (RV) genotypes detected at the different sampling sites during the collection period

Sampling period	Season	RV genotypes			
		Urban river	Rural river	Urban sewage	
		Kibera	Mboone	IPR*	Karen
April 2007	Wet	–	–	G?, P[8]†	–
May 2007	Dry	–	–	–	G1, G9P[4], P[8]
June 2007	Wet	G1, G12P[8]	–	–	G1, G12P[4]
July 2007	Wet	G1, G12P[8]	G3, P[8]	–	G1, G12P[4], P[8]
August 2007	Dry	G10, G12P[?]	–	–	G4, G8P[4]
September 2007	Dry	G1, G12P[4], P[8]	–	–	G1, G9, G12P[4]
October 2007	Dry	–	–	–	G1, G9, G12P[4], P[8]
November 2007	Wet	G1, G9, G12P[4], P[8], P[6]‡	G1, P[6], P[8]	–	G1, G5, G9, G12P[4], P[8]
December 2007	Wet	G1, G9, G11, G12P[8] G1, G12P[8]	–	–	G1, G12P[4], P[8]
January 2008	Dry	G1, G9, G12P[4], P[8] G1, G12P[4], P[8]	G2P[8]	–	–

*IPR, Institute of Primate Research.
†'?' type could not be determined/ascertained.
‡The same RV genotype was detected in two consecutive samples taken in November 2007.

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identified in 8/8 (100%) of the RV strains with P[8] being present in 5/8 (62·5%) of the RV strains.

Discussion

Surface water can be contaminated with enteric viruses by a variety of sources, including raw and treated sewage, wastewater discharges, animal manure and unprotected connections (de Roda Husman and Bartram 2007). This article describes a pilot study to investigate the presence of enteric viruses in river and stream water and sewage in selected urban and rural areas in Kenya to assess whether surface water sources, used for domestic purposes, are a potential source or reservoir of enteric virus infection. Molecular-based assays such as PCR, RT-PCR and real-time RT-PCR were applied in this study for the detection of enteric viruses as these assays have been shown to be more advantageous than cell culture-based assays for the detection of enteric viruses in faecally contaminated water (Hot *et al.* 2003; Denis-Mize *et al.* 2004; Greening 2006; Miagostovich *et al.* 2008). While the molecular-based methods fail to distinguish between infectious and non-infectious virions, the detection of nucleic acid in polluted water suggests the presence of infective virus (Miagostovich *et al.* 2008) as survival of naked nucleic acid in aquatic environment is limited (Lambertini *et al.* 2008). This is further supported by the fact that the glass-wool adsorption elution method applied for the recovery of viruses from the water samples has been found to be more effective in retaining infectious intact virus rather than naked viral nucleic acid or partially degraded virions (Lambertini *et al.* 2008). The PEG precipitation procedure applied to the recovery of viruses from the wastewater and sewage samples has been shown to be effective for the concentration and detection of infectious RVs and enteroviruses from water samples by cell culture and molecular-based techniques (Vilaginés *et al.*, 1997). Therefore, the enteric viruses in the water and sewage samples in this study were probably intact and infectious.

From the results, it is evident that there is a wide disparity in the prevalence and profile of potentially pathogenic human enteric viruses between the urban and rural rivers and streams in Kenya. In the rural Mboone river, one or more of the eight potentially pathogenic human enteric viruses analysed were detected in 66·7% of the samples (Table 1). HAstV was the most prevalent virus, being detected in 41·7% of samples, and RVs were detected in 25% of samples, while HAV and SaV were not detected at all. The town of Maua does not have a sewage plant, and most of the households have pit latrines, consequently surrounding surface waters such as the Mboone river are vulnerable to human faecal pollution from various sources such as wastewater and storm water runoff. In the urban area the Mbagathi river, in a less densely populated forest area, showed a very low prevalence (28·6%) of enteric viruses. In contrast, the urban Kibera wastewater stream showed high levels of viral contamination with four or more of the eight potentially pathogenic human enteric viruses analysed being detected in all 10 (100%) samples (Table 1). RVs and enteroviruses were present in all ten samples, while HAstVs, HAdVs, NoVs and HAV were present in between 60 and 90% of the samples. The Kibera wastewater stream passes through the Kibera informal settlement, one of the largest slums in the south-east of sub-Saharan Africa, and is polluted with household waste and raw sewage from the surrounding communities which impacts negatively on the water quality of the stream. The results from the Kibera wastewater stream are comparable to recently reported results of an epidemiological survey of enteric viruses in surface water in Brazil (low income country) where one or more of four gastroenteritis viruses, namely HRV, HAstV, HAdV and NoV, were detected in 87·5% urban surface water samples collected close to an informal urban settlement in Manaus (Miagostovich *et al.* 2008). In high-income countries, enteric viruses have also been detected in surface water samples. For example, enteroviruses were detected in 88% of river water samples tested in France (Hot *et al.* 2003), NoVs were detected in surface water in the Netherlands (Lodder and de Roda Husman 2005), enteroviruses, NoVs, HAdVs and RVs were detected in surface water in Germany (Pusch *et al.* 2005), and RVs were detected in surface water in Italy (Grassi *et al.* 2009) and the Netherlands (Rutjes *et al.* 2009). Although there are no data on the concentration of faecal coliforms in the surface water samples from Kenya, other studies have shown a lack of positive correlation between the prevalence of enteric viruses and bacterial indicators of faecal pollution in water (Hot *et al.* 2003; Miagostovich *et al.* 2008; Grassi *et al.* 2009; Rutjes *et al.* 2009), and there is no reason to believe that the same would not be applicable to the Kenyan surface water samples in this study. Therefore, regardless of the socio-economic status of a country, the surveillance of surface water samples for enteric viruses is an important indicator of the level of human faecal pollution with the added benefit of providing information as to which enteric viruses are circulating in the community and have epidemic potential. This information is essential for further assessment of the public health risks associated with exposure to these water sources (Haas *et al.* 1993; van Heerden *et al.* 2005; Venter *et al.* 2007; Espinosa *et al.* 2008).

There was a marked difference in the profile of enteric viruses in the two sewage plants. In sewage from the Karen sewage plant, the predominant enteric viruses were RV, HAstV, HAdV and NoV GII, while in the sewage from the

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IPR sewage plant enteroviruses, HAdV and SaV predominated (Table 2). Although both sewage works are within the same locality, they each serve distinct communities with the IPR sewage plant serving the IPR primate animal housing, offices and staff residential houses, while the Karen sewage plant serves the adjacent Karen shopping centre and Karen estate. The diversity of the enteric viruses in the sewage from the different sewage treatment plants is a reflection of the enteric viruses circulating in the distinct communities and may be one of the most appropriate targets with which to assess the prevalence of viruses circulating among the distinct communities.

As children <14 years of age comprise 42·2% of the total Kenyan population (*c.* 37 953 840) (Central Intelligence Agency, 2009) and as RVs have been identified as an important cause of paediatric viral gastroenteritis in Kenya (Kiulia *et al.* 2006, 2008, 2009), the group A RVs detected in the surface water sources and sewage were genotyped to gain further insight into the RVs circulating in the water sources and surrounding communities. In this investigation, the group A RV G typing methods, as described by Gouvea *et al.* (1990, 1994a), Das *et al.* (1994), Gault *et al.* (1999) and Iturriza-Gómara *et al.* (2004), were successfully applied to type 95·5% of the RVs detected in the Kenyan surface water and sewage samples. This typing rate is comparable to that reported by van Zyl *et al.* (2006) who identified the G types in 94·7% of the RV-positive environmental water samples in South Africa compared to the typing rate of 70 and 43·6% reported for Egypt and Spain, respectively (Villena *et al.* 2003b), and 20% reported for Brazil (Miagostovich *et al.* 2008). The well-defined single bands of amplicons evident on the agarose gels (results not shown) and the use of multiple sets of primers (Gentsch *et al.* 1992; Gouvea *et al.* 1994b; Mphahlele *et al.* 1999; Simmonds *et al.* 2008) resulted in the successful P typing of 95·5% of the RV-containing samples. This P-typing rate is much higher than the rate of 31·6% described by van Zyl *et al.* (2006) and 73·3% described by Villena *et al.* (2003b), both of whom only used the P-typing primers described by Gentsch *et al.* (1992) and is similar to the P-typing rate of 86·6% described by Miagostovich *et al.* (2008).

Among the group A RVs detected in the water samples from the Mboone rural river was a single G2 strain in association with P[8]. G2 is one of the common G serotypes, while P[4] and P[8] are the most common P types associated with human infection globally (Iturriza Gómara *et al.* 2003). The identification of a RV strain with G2[P8] specificity is of interest as worldwide G2 is commonly associated with [P4], and the G2[P8] strain may be a naturally occurring re-assortant (Santos and Hoshino 2005). The occurrence of the G2 RV strain in the rural water source was unexpected as no G2 strains were detected in the faecal specimens from infants and children with acute diarrhoea in the same region (Kiulia *et al.* 2006). The stool specimens were, however, collected 2–3 years prior to the collection of the water samples, and as there is a natural fluctuation and seasonality in circulating RV genotypes, the occurrence of G2 in the water samples could reflect the emergence of G2 strains within the rural community in 2007. Notably in 2007, type G2 was found to be the most prevalent RV in paediatric diarrhoea in Portugal (Antunes *et al.* 2009) and in surface water samples in Italy (Grassi *et al.* 2009), suggesting its re-emergence during this time period. In contrast, mixed RV strains were identified in the Kibera urban wastewater stream samples with RV G1 and G12 predominating (Table 3). Type G9 was detected in 40% of the RV-positive samples, and single strains of G10 and G11 were amongst the mixed RV strains. P[4] and P[8] were the dominant P types detected throughout the sampling period, while P[6] was identified in the sample set taken in November 2007. Globally, the most common G and P types associated with RV infection are G1 to G4 and P[4] and P[8], respectively (Iturriza Gómara *et al.* 2003), and during the period 1980–2005, type G1 was the predominant type circulating in Nairobi over a number of seasons (Kiulia *et al.* 2008). The presence of G9 in the wastewater was not unexpected as recently G9 has emerged as an important cause of RV-associated gastroenteritis in Kenya (Kiulia *et al.* 2006, 2008) and is considered to be the fifth most important G type globally (O'Ryan 2009). The high prevalence of G12 strains in the Kibera wastewater was noteworthy as RV G12 strains have not previously been described in stool specimens from paediatric patients in the Nairobi environs. Prior to 1998, G12 was considered to be a rare type associated with human infection (Page *et al.* 2009). However, G12, in association with P[8] and P[6], is emerging worldwide (O'Ryan 2009; Page *et al.* 2009), and the presence of G12 in the wastewater from the Kibera informal settlement suggests that this type is emerging and circulating in the Kenyan population. The presence of G10 and G11 types in the mixed RV strains in the wastewater could be attributed to faecal contamination from animals, as these types are generally regarded as animal types, or attributed to animal to human transmission (Santos and Hoshino 2005). However, neither G10 nor G12 has been reported in association with human infection in Kenya (Kiulia *et al.* 2006, 2008, 2009). This could be ascribed to the absence of human infection with these types, inherent limitations in the surveillance sample collection or the fact that the primers applied for the G typing of the clinical specimens did not detect these types. The profile of the RV types in the sewage from the urban Karen sewage plant was similar to that described for the urban wastewater stream with the exception that types G5 and G8, and not G10 and

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G11, were amongst the mixed RV strains. G8 strains are increasingly being reported in Africa (O'Ryan 2009), and G5 RVs, commonly associated with pigs, are an uncommon RV type detected in humans worldwide (Santos and Hoshino 2005). The detection of these strains in urban sewage could indicate the presence of animal waste or represent re-assortants between human and animal strains.

Group A RVs are a leading cause of paediatric gastroenteritis and death worldwide (Vesikari *et al.* 2006). RVs exhibit a seasonal pattern of infection with epidemics in the cooler months of the year (Rutjes *et al.* 2009). In this study, no seasonal variation was noted in the occurrence or the types of RVs in the surface water and sewage samples. A similar lack of seasonality was noted in a study to determine the prevalence of RVs in sewage in Cairo and Barcelona (Villena *et al.* 2003b), whereas in Italy, RVs were more prevalent in sewage samples in the spring and summer months (Grassi *et al.* 2009). The significance of this lack of seasonal pattern of the RVs in the water and sewage samples is difficult to assess as the seasonality of virus infections is reportedly difficult to determine in surface water samples (Rutjes *et al.* 2009). In many countries, the licensing of the oral RV vaccines, namely the human monovalent strain G1P[8] (Rotarix™; GlaxoSmithKline Biologicals S.A./NV, Rixensart, Belgium) and the human-bovine pentavalent strains G1-G4P[8] (RotaTeq™; Merck & Co., Inc., Whitehouse Station, NJ) has refocused attention on the epidemiology of RVs as the vaccine efficacy may be affected by circulating RV strains (Santos and Hoshino 2005; Grimwood and Buttery 2007). Therefore, the typing of RVs in a particular geographical setting is important as this will give an indication as to the potential efficacy of the RV vaccine in the region. In Kenya, RV surveillance in clinical specimens has been ongoing since 1975, and types G8 and G9 have recently been identified in diarrhoeal stool specimens (Kiulia *et al.* 2006, 2008). The oral RV vaccines have been licensed in Kenya, and the identification of RVs occurring in the environment provides an additional source of information regarding the genotypes circulating in the community which provides an evidence base for policy makers with regard to decisions to introduce the RV vaccine.

Microbes pose the most significant waterborne health risk (Carter 2005) with waterborne diseases being misdiagnosed or underdiagnosed (Meinhardt 2006). The routine surveillance of water sources is therefore essential to facilitate correct management procedures for the protection of public health (Sobsey 2006). In low-income countries, poor water quality has been implicated in waterborne diarrhoeal disease (Miagostovich *et al.* 2008), and in Kenya, the majority of the population lack access to potable water and rely on untreated surface water for their domestic needs. Further characterization of these viruses is therefore warranted to establish the genetic relatedness of viruses from different water and sewage sources and to ascertain whether the contamination is of human or animal origin or not. Although quantitative data are required to determine the infectious risk to persons ingesting water from these sources, the data presented in this pilot study provide valuable new information on enteric viruses in Kenyan surface water sources and highlight the potential public health risk to communities, especially children, using these water sources for domestic purposes. Further nationwide surveillance of water sources used for domestic and irrigation purposes for enteric viruses is therefore warranted to promote and facilitate the protection of water sources from viral contamination and to ensure adequate water treatment and disinfection procedures.

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