The development and application of multiplex real-time reverse transcriptase polymerase chain reaction assays for the detection of enteric viruses on berry fruits and in water samples

by

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DECLARATION

I, Gabriël Adriaan de Ridder, 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 Sciences Research Ethics Committee, University of Pretoria

Signature…GdeR……

Date…8 Feb 2014……

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DEPARTMENT: MEDICAL VIROLOGY

DEGREE: MAGISTER SCIENTIAE (MEDICAL VIROLOGY)

SUMMARY

The transmission of enteric viruses by food, food products and water remains a well-recognised, largely underestimated widespread public health problem. Outbreaks of gastroenteritis and hepatitis A due to the consumption of contaminated berry fruits have become a growing phenomenon worldwide. Contamination of fresh produce and other minimally processed foods can be attributed to pre- and post-harvest irrigation and washing water and food handlers. The prevention of such outbreaks relies on the optimisation of adequate methods for the recovery and detection of enteric viruses from food matrices and irrigation water.

The aim of this study was to develop and apply optimised multiplex real-time reverse transcriptase-polymerase chain reaction (*rt* RT-PCR) assays for the detection of selected enteric viruses on berry fruits and in paired associated irrigation waters. In this study quality control measures were implemented by the development and optimisation of an

internal amplification control (IAC) for norovirus (NoV) GII to monitor for the success of the amplification process. Mengovirus was used as a process control to validate the recovery and nucleic acid extraction of selected enteric viruses from strawberries and in associated irrigation waters. Three multiplex *rt* RT-PCR assays for the detection of NoV GI, NoV GII, sapovirus , hepatitis A virus, human astrovirus, human rotavirus and mengovirus were optimised with the IAC. Blackberries and strawberries were used to assess the efficiency of three nucleic acid extraction kits with the most efficient used in further investigations. Three elution buffers based on protein concentration, pH, Tris and elution period were assessed for the recovery of the viruses from the berry fruits. The pH more so than the protein concentration proved to be more effective in the recovery of the selected enteric viruses from the strawberries with no analytical significant differences noted for the two 3% glycine-beef extract (GBE) buffers assessed, irrespective of the parameters considered. During the period September 2010 to August 2011, strawberries and associated irrigation water were collected from which NoV GII, NoV GI and HAV could be recovered using a 3% tris-GBE pH 9.5 elution buffer and a glass-wool absorption elution method, respectively, and detected using optimised singleplex *rt* RT-PCR assays. The irrigation water samples together with eight surface and three groundwater samples collected from the Limpopo area was retested using the optimised multiplex *rt* RT-PCR assays. The multiplex *rt* RT-PCR assays proved to be more efficient in the detection of NoVs than the commercial environmental *rt* RT-PCR assays with lower detection efficiencies noted for HAV. Commercially obtained strawberries were dipped in polluted surface water, the viruses recovered from both and detected using the optimised multiplex *rt* RT-PCR assays resulted in the detection of similar viruses on both the strawberries and polluted irrigation water. Norovirus GII.7 and swine NoV GII.18 were identified on the strawberries and in the associated irrigation water, respectively. This is the first report of swine NoVs in South Africa, and begs the question as to the possibility of zoonotic NoV infection. This link between the viruses detected on the surface of the strawberries and in the irrigation water could not be confirmed by typing data. From this study, a functional AC was developed and used in the development and optimisation of three multiplex *rt* RT-PCR assays which made the gathering of new data of the role of irrigation water as a source of contamination of irrigated berry fruits in SA possible.

PUBLICATIONS AND PRESENTATIONS

Publications

Cook N, **de Ridder GA**, d'Agostino M, Taylor MB. Internal amplification controls in real-time polymerase chain reaction-based methods for pathogen detection. In: Rodríguez-Lázaro D, editor. Real-time PCR in Food Science. Current Technology and Applications. Norfolk, UK: Caister Academic Press; 2013. p. 35-42.

Presentations

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TABLE OF CONTENTS

ABBREVIATIONS

AC	Amplification control
AdV(s)	Adenovirus(es)
AstV(s)	Astrovirus(es)
BGM	Buffalo green monkey kidney cells
bil	Billion
BIW	Bon Accord irrigation water
bp	Base Pair
BSA	Bovine serum albimum
BST	Bon Accord irrigation water
°C	Degree Celsius
CA	California
CDC	Centre of Disease Control and Prevention
cDNA	Complementary DNA
CEN	European Committee of Standardisation
Co.	Company
Corp.	Corporation
CPE	Cytopathic effect
Ct	Crossing threshold
DNA	Deoxyribonucleic acid
dNTP(s)	Deoxynucleotide triphosphate
ds	Double-stranded
“E” or “e”	Efficiency of the PCR reaction
EAC	External amplification control
EIA	Enzyme immunoassay
EM	Electron microscope
EtBr	Ethidium bromide
EtOH	Ethanol
EV(s)	Enterovirus(es)
FCV(s)	Feline calicivirus(es)
g	Gram
G	Genotype
GBE	Glycine beef extract
GBE-7.2	Glycine beef extract pH 7.2
GBE-9.5	Glycine beef extract pH 9.5
GP	Gauteng Province

HAV	Hepatitis A virus
HAdV(s)	Human adenovirus(es)
HAstV(s)	Human astrovirus(es)
HCl	Hydrochloric acid
HEV	Hepatitis E virus
h	Hour
HRV(s)	Human rotavirus(es)
IAC	Internal amplification control
kb	Kilobase pair
ℓ	Litre
LB	Luria-Bertani
LNA	Locked nucleic acid
M	Mole
MA	Massachusetts
mg	Milligram
mil	Million
mℓ	Millilitre
mM	Millimole
min	Minutes
MO	Missouri
mo	Month
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NC	North Carolina
NHLS	National Health Laboratory Service
nm	Nanometre
NoV(s)	Norovirus(es)
No.	Number
NY	New York
ORF(s)	Open reading frame(s)
p	Probability
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PCR	Polymerase chain reaction
PFU	Plaque forming units
PRF/PLC/5	Polio Research Foundation/Primary liver carcinoma

PV	Poliovirus
R^2	Pearson coefficient of determination
RNA	Ribonucleic acid
rt	Real-time
RT-PCR	Reverse transcriptase polymerase chain reaction
RRV	Rhesus rotavirus
RV(s)	Rotavirus(es)
RV-SA11	Simian rotavirus SA11
SA	South Africa
SaV(s)	Sapovirus(es)
ss	Single-stranded
Std. Dev.	Standard deviation
TAD	Tshwane Academic Division
TGBE	Tris (hydroxymethyl) amino methane glycine beef extract
TGBE-7.2	Tris (hydroxymethyl) amino methane glycine beef extract pH 7.2
TGBE-9.5	Tris (hydroxymethyl) amino methane glycine beef extract pH 9.5
TRIS	Tris (hydroxymethyl) amino methane
U	Units
UK	United Kingdom
µℓ	Microliter
µM	Micromole
US	United States of America
UV	Ultraviolet
Vero cell	African green monkey kidney cells
VDPV	Vaccine-derived poliovirus
VP	Viral protein
WHO	World Health Organisation
WI	Wisconsin
ZAR	Zuid African Rand

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Food- and waterborne transmission of enteric viruses remains largely underestimated and a widespread public health problem (Koopmans *et al*., 2002; Papafragkou *et al*., 2006; Cliver, 2008; O'Brein, 2008). Human enteric viruses are commonly associated with outbreaks of gastroenteritis, hepatitis and other diseases due to contaminated food and water (Greening, 2006; White *et al*., 2009). Mead *et al*. (1999) estimated that 67%, 30% and 3% of the foodborne associated illnesses in the United States (US) were due to viruses, bacteria and parasites, respectively. During the period of 1990-2006, viruses accounted for 24% of foodborne infections of which 90% were caused by noroviruses (NoVs), 4% due to hepatitis A virus (HAV) and a further 6% caused by other viruses (Smith De Waal *et al.*, 2008). More recent estimates suggest that 59% of the foodborne related illnesses in the US were due to viruses, with 39% and 2% due to bacteria and parasites, respectively (Scallan *et al*., 2011a).

Gastroenteritis outbreaks place an extensive economic burden on a country, with cost estimated to be hundred million (mil) pounds annually (UK) (Safefood, 2007). The National Health Service annually spent an estimated £1 bil as a result of hospitalised cases of gastroenteritis (Lee *et al*., 2011). An outbreak of hepatitis A in Denver involving 43 cases was estimated to cost over South African rand (ZAR)5.6 mil (Fiore, 2004). During the period 1980-2007 the US was burdened with 578, 171 cases of acute hepatitis with cost estimated in the billions of US dollars (Centre for Disease Control and Prevention [CDC], 2009).

Preventative measures such as the detection of enteric viruses in food and water remains problematical. Electron microscopy (EM) and enzyme immunoassays (EIA) can be used in the detection of enteric viruses in clinical samples. However, these techniques are not considered useful in detection of viruses in food and water due to their low concentrations in these matrices (Koopmans and Duizer, 2004). Cell culture assays

used for viral isolation, able to detect ≤10 particles of viruses per gram (g), has the drawback that they are unable to support the replication of all enteric viruses. Molecular-based techniques such as reverse transcriptase-polymerase chain reaction (RT-PCR) or real-time RT-PCR (*rt* RT-PCR) enables the detection of low concentrations of pathogens. Multiplex *rt* RT-PCR assays reduces the financial and labour burden by allowing the detection of multiple pathogens in a single reaction.

Fresh produce and other minimally processed foods have been implicated as primary sources of infection in gastroenteritis and hepatitis A outbreaks (Yano, 2007; Butot *et al*., 2008; Doyle and Erickson, 2008; Baert *et al*., 2009a). Contamination of food sources can possibly be attributed to the irrigation water or fertiliser used or as a result of infected food handlers (Koopmans and Duizer, 2004). Outbreaks of gastroenteritis and hepatitis, attributed to the consumption of berry fruits, have been reported worldwide and are becoming a growing phenomenon (Niu *et al*., 1992; Hutin *et al*., 1999; Calder *et al*., 2003; Fiore, 2004; Koopmans and Duizer, 2004; Le Guyader *et al*., 2004; Carter, 2005; Cotterelle *et al*., 2005; Falkenhorst *et al*., 2005; Korsager *et al*., 2005; Butot *et al*., 2008).

Current available data on the virological quality of crops cultivated in SA is limited to a few selected food crops with no data for berry fruits. This can be attributed to the lack of infrastructure for the detection and underreporting of such infections (Grabow, 1996; Netshikweta, 2012). Improved strategies to prevent viral contamination of foods are needed in SA. In addition, further research is needed to detect and identify food and waterborne viral pathogens with the aim of facilitating proper management procedures to reduce the burden of disease. Standardised methods for the detection and recovery of human enteric viruses have not been established and therefore routine surveillance for these viruses on berry fruits and other fresh produce is not practiced.

1.2 FOOD AND WATERBORNE VIRUSES

Human enteric viruses associated with food- and waterborne diseases can be divided into three different disease categories:

i) Gastroenteritis: NoVs, sapovirus (SaV), human rotavirus (HRV), human astrovirus (HAstV), human adenovirus (HAdV);

ii) Hepatitis: HAV and hepatitis E virus (HEV);
iii) Other diseases such as poliomyelitis, aseptic meningitis, etc.: human enteroviruses (EVs) (Koopmans and Duizer, 2004).

1.2.1 Enteric viruses associated with foodborne gastroenteritis

Noroviruses are the main cause of foodborne viral gastroenteritis worldwide (Greening, 2006; Rutjes *et al*., 2006; Nenonen *et al*., 2009). Other enteric viruses namely: HAstV, HRV and SaV are occasionally associated with foodborne gastroenteritis with minimal evidence to support the role of HAdV and human EVs in foodborne gastroenteritis (Fleet *et al*., 2000; Koopmans and Duizer, 2004; Carter, 2005; Greening, 2006; Le Guyader *et al*., 2008; Ethelberg *et al*., 2010; Mattison *et al*., 2010; Petrignani *et al* 2010). Enteric viruses associated with foodborne gastroenteritis are excreted in high concentrations in the faeces of infected individuals (Bosch, 1998). They are able to persist outside the human host for a prolonged period of time and remain infectious after temperature, ultraviolet (UV) irradiation and common disinfectant treatments (Duizer *et al.*, 2004a; Koopmans and Duizer, 2004; Carter, 2005; Cliver, 2008). In the majority of cases person-to-person transmission of enteric viruses predominates due to their high secondary attack rate, masking the role of food and waterborne transmission (Dalling, 2004; Carter, 2005; Baert *et al*., 2009b; Maunula *et al*., 2009; Ter Waarbeek *et al*., 2010)

1.2.1.1 Human caliciviruses

Caliciviruses, whose name is derived from the cup-like surface depressions which gives the virus its unique “Star of David” appearance, are non-enveloped icosahedral virions, 27-40 nm in diameter, with a single stranded (ss) positive-sense RNA genome of 7.4-8.3 kilobase (kb) (Moreno-Espinosa *et al*., 2004; Hansman *et al*., 2007; Ootsuka *et al*., 2009; Bank-Wolf *et al*., 2010). In some viruses the cup-shaped depressions are less prominent with some having an amorphous structure with a fuzzy ragged edge, hence the old name “small round structured viruses” (Figure 1.1) (Koopmans *et al*., 2005).

Viruses of the family *Caliciviridae* are divided into five major genera: *Norovirus*, *Vesivirus*, *Sapovirus*, *Nebovirus* and *Lagovirus* (Clarke *et al*., 2012). Norovirus include strains that can infect mice, cattle, pigs and humans (Zheng *et al*., 2006). Sapovirus

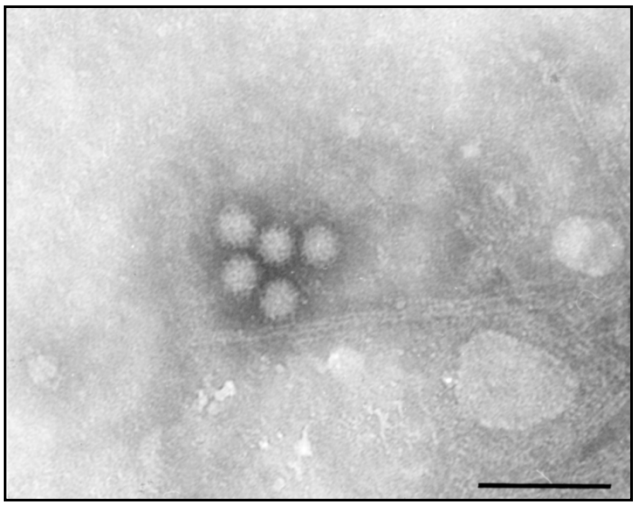

Figure 1.1: Electron micrograph of caliciviruses depicting virions with the amorphous structure with a fuzzy ragged edge (*bar = 100 nm*) (courtesy Prof MB Taylor).

include strains that can infect humans, mink and swine species (Green, 2007), with the swine strains sharing a genetic relatedness to human SaV strains (Farkas *et al*., 2004; Hansman *et al*., 2007). Nebovirus infect bovine calves, lagovirus strains infect rabbits and vesivirus include strains that infect feline, swine and possibly canine species (Clarke *et al*., 2012). In addition the new genus *Recovirus* was proposed for the Tulane virus from rhesus macaques (Farkas *et al*., 2008) and *Valovirus*, for the St-Valérien virus from swine (L'Homme *et al*., 2009). Chicken calicivirus may also be members of the *Caliciviridae* family (Clarke *et al.*, 2012).

1.2.1.1.1 Noroviruses

Noroviruses are considered the leading cause of both sporadic and epidemic acute gastroenteritis in both industrialised and developing countries, being responsible for 80-95% of all recorded non-bacterial acute gastroenteritis cases worldwide (Atmar, 2010; Bull and White, 2011). Over the past 15 years NoVs have been responsible for four global epidemics of gastroenteritis on three different continents (Bull and White, 2011).

(i) Virology

The NoV genome is approximately 7.3-8.5 kb in length with a poly A tail and viral protein (VP) attached to the genome (Green, 2007). The genome is divided into three open reading frames (ORFs): ORF1 encodes a large non-structural polyprotein needed

for viral replication, ORF2 encodes the major viral protein (VP1) which determines the antigenicity of the virus (Atmar and Estes, 2006; Bull and White, 2011), and ORF3 encodes the minor capsid protein (VP2). On the basis of nucleotide sequence analysis of the complete capsid (VP1) gene, NoVs can be divided into five distinct genogroups (GI-GV) with genogroups GI and GII further subdivided into 8 and 19 genotypes, respectively (Figure 1.2) (Green, 2007; Bull and White, 2011).

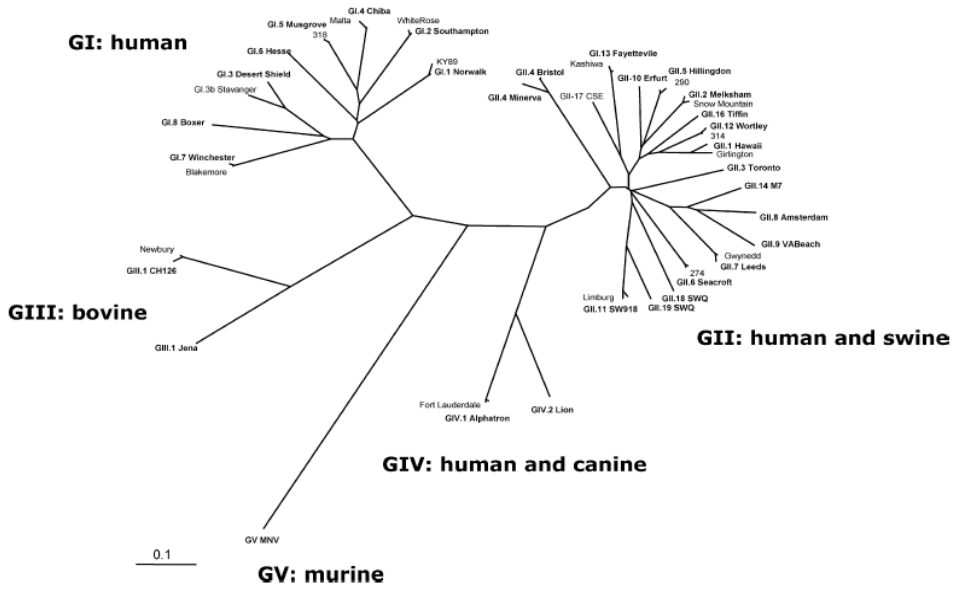

Figure 1.2: Phylogenetic tree depicting the genetic relatedness of NoV genogroups and genotypes (Patel *et al*., 2009)

New variants of NoVs arise almost every two to three years (Siebenga *et al*., 2010; Bull and White, 2011). Strains of genogroups GI, GII and GIV are associated with human infections with GII.4 strains predominating in outbreaks worldwide (Kearny *et al*., 2007; Nayak *et al*., 2009; Siebenga *et al*., 2010). Genogroup II can infect porcine species and genogroup IV dogs and lions. Genogroups III and V cause infections in bovine and murine species, respectively (Zheng *et al*., 2006; Bull and White, 2011).

Noroviruses can retain infectivity after being exposed to a pH 2.7 for 3 hours (h) at room temperature, 60ºC for 30 minutes (min), 20% ether at 4ºC for 18 h and 0.5-1 milligram/litre (mg/ℓ) free chlorine or 10 parts per mil chlorine. Noroviruses are more resistant to chlorine inactivation than poliovirus (PV) and simian RV (Green, 2007). However, a free residual chlorine concentration of 10 mg/ℓ has been shown to inactivate NoVs (Green, 2007). Studies done on feline caliciviruses (FCV), used as surrogate for

NoVs, showed that inactivation can be achieved by pasteurisation at 63ºC for 30 min or 72ºC for 2 min, and by UV radiation (253.7 nm and 280-320 nm). The infectivity of FCVs was greatly reduced when exposed to pH 2 at 37ºC for 30 min while the RNA of NoVs could still be detected after exposure to a pH of 2.7 at room temperature for 3 h (Duizer *et al*., 2004a; Dolin, 2007; Baert *et al*., 2009a).

(ii) Clinical

After an incubation period of 10-51 h, NoV infection is characterised by the sudden onset of projectile vomiting and/or profuse watery diarrhoea which usually lasts for 24-48 h but can range from 2 h to several days. (Atmar and Estes, 2006; Green, 2007; Nayak *et al*., 2009; Atmar, 2010). Infection can, however, be subclinical or mild with non-specific symptoms such as nausea, abdominal cramps, myalgia and fever (Patel *et al*., 2009; Atmar, 2010). Virus shedding can persist for up to three weeks with prolonged shedding, with or without symptoms, seen in immunocompromised and organ transplant patients (Ludwig *et al*., 2008; Patel *et al*., 2009; Bank-Wolf *et al*., 2010). Treatment of NoV-associated illness is usually supportive (Green, 2007). To date, there is no commercial available vaccine for NoVs (Bank-Wolf *et al*., 2010). Immunity is strain specific although there are conflicting results regarding the lasting effect of immunity. Data from recent studies support the possibility that immunity persists for a period of 6-12 months (mo), a period sufficient to drive the emergence of new variants (Atmar and Estes, 2006; Bull and White, 2011).

Efforts to isolate NoVs in cell culture has been unsuccessful (Duizer *et al*., 2004b; Atmar and Estes, 2006; CDC, 2011). The laboratory diagnosis of infection is dependent on the detection of the virus in stool or vomitus specimens by EM, EIAs or molecular-based assays (Glass *et al*., 2009; Patel al., 2009; Atmar, 2010). Reverse transcriptase-PCR has now become the ‘gold standard’ of diagnostic assessment for NoVs (Desselberger and Gray, 2003).

(iii) Epidemiology

Noroviruses are transmitted predominantly by the faecal-oral route (Patel *et al*., 2009; Atmar 2010), directly from person-to-person or indirectly via contaminated food and water (Atmar and Estes, 2006; Koopmans, 2008), and aerolised vomitus (Patel *et al*., 2009). Person-to-person transmission is seen as the primary route of infection with

outbreaks frequently occurring in closed settings such as cruise ships, healthcare institutions and schools (Kroneman *et al*., 2008; Patel *et al*., 2009; Atmar, 2010), Persons at greatest risk for infection include, but not limited to the young, the elderly, travellers and immunocompromised persons (Glass *et al*., 2009). After HRVs, NoVs are the second most common cause of severe childhood gastroenteritis (Matson and Szücs, 2003; Studdert and Symes, 2008; Patel *et al*., 2009; Koopmans, 2008; Mans *et al*., 2010). The infectious dose of NoVs is reported to be 10-100 infectious particles (Koopmans *et al*., 2002; Teunis *et al*., 2008; Bank-Wolf *et al*., 2010). The estimated risk of infection for a single infectious particle is ~49% (Teunis *et al*., 2008). Infections occur throughout the year with a peak in the cooler winter months, hence the name "winter vomiting disease" (Atmar and Estes, 2006; Patel *et al*., 2008; Harris *et al*., 2010; Bull and White, 2011). Zoonotic transmission of NoVs cannot be excluded although this is not well understood and thought to be a rare event (Bank-Wolf *et al*., 2010).

1.2.1.1.2 Sapovirus

Sapporo virus was first described in infants and young children in Sapporo, Japan in 1982. This strain was considered as the prototype strain and remains the only member of the genus *Sapovirus* (Green, 2007; Dos Anjos *et al*., 2011). Sapoviruses were initially thought to be a disease affecting only young children but studies have shown SaVs to cause infection in all age groups (Mikula *et al*., 2010). Sapovirus-associated diarrhoea is less severe than that caused by NoVs (Bank-Wolf *et al*., 2010; Dos Anjos *et al*., 2011).

(i) Virology

Sapoviruses have a ~7.5 kb genome with a poly A tail encapsidated in a 41-46 nm diameter capsid with a cup-shaped depression. Sapoviruses are divided into seven genogroups (GI-VII) and further subdivision into genotypes (Dos Anjos *et al*., 2011). Genogroups I, II, IV and V infect humans, with GI.1 being detected most frequently, while genogroups III and VI infects porcine species and genogroup VII infect mink species (Moreno-Espinosa *et al*., 2004; Phan *et al*., 2007; Johnsen *et al*., 2009; Cunha *et al*., 2010). Genogroup II is organised into two ORFs, whereas genogroups I, IV, and V genomes are organised into three ORFs. The third ORF encodes a protein of unknown function (Koopmans *et al*., 2005; Hansman *et al*., 2007). Both inter-and intragenogroup

recombination for SaVs has been identified with recombination mostly occurring at the RNA-dependent RNA polymerase-Vp1 junction (Hansman *et al*., 2007).

(ii) Clinical

The clinical manifestation of SaV-associated diarrhoea may require hospitalisation in sporadic cases of acute gastroenteritis. The clinical symptoms in infected children predominantly include diarrhoea (95%), vomiting (44%) and fever (18%). Other symptoms that may be experienced include nausea, headache, malaise and aching limbs (Desselberger and Gray, 2003). Sapovirus-associated diarrhoea has an incubation period of 12-72 h and lasts for 3-4 days although illness can persist for up to 11 days. These illnesses are considered to be self-limiting with the absence of chronic infections in immunocompromised individuals. Viral shedding can last for two weeks with prolonged shedding occurring in younger children. (Desselberger and Gray, 2003; Moreno-Espinosa *et al*., 2004). The treatment of SaVs is considered supportive with the administration of electrolytes to compensate for the fluid loss and no vaccine is as of yet available (Bank-Wolf *et al*., 2010). The laboratory diagnosis of infection is dependent on the detection of the virus in stool or vomitus specimens by EM or molecular-based assays (Hansman *et al*., 2007).

(iii) Epidemiology

Sapoviruses are transmitted predominantly through the faecal-oral route (Bon *et al*., 2005; Atmar and Estes, 2006; Hansman *et al*., 2007) with person-to person transmission perpetuating the spread. Sapoviruses are detected in all age groups with the majority of infections detected in infants and young children and rarely in adults and the elderly (Desselberger and Gray, 2003; Bank-Wolf *et al*., 2010). During the period 1991-1995 the prevalence of SaV-associated gastroenteritis in SA was estimated to be 0.95% in infants ≤11 mo. However, during the period of 2008 the prevalence of SaV-associated gastroenteritis increased to 6.7% in children ≤13 years (Wolfaardt *et al*., 1997; Mans *et al*., 2010). Infections are asymptomatic in the majority of cases with a suggested winter seasonal peak (Moreno-Espinosa *et al*., 2004; Bank-Wolf *et al*., 2010). Outbreaks have been reported from day-care centres, elementary schools, cruise ships and hospitals, but are considered to be less frequent than NoV-associated gastroenteritis outbreaks (Chan *et al*., 2006; Hansman *et al*., 2007; Bank-Wolf *et al*., 2010).

1.2.1.2 Human rotavirus

Human RV belongs to the genus *Rotavirus* in the family *Reoviridae* which includes the genera *Orthoreoviruses* and *Orbiviruses* (Estes and Kapikian, 2007). Rotaviruses (RVs) are divided into eight groups (A-G) and further into multiple serotypes. Groups A-C infects both humans and animals, with group A the most common in humans, and groups D-G predominantly infecting animals (Parashar *et al*., 2009; Linhares *et al*., 2011). Human RV is the leading cause of severe diarrhoea in young children and infants, both in industrialised and low to medium income class countries worldwide (Parashar *et al*., 2006; Estes and Kapikian, 2007). During the period 1986-1999, HRV was estimated to cause ~22% of all hospitalised childhood diarrhoea worldwide, a figure that increased to ~39% during 2000-2004 (Parashar *et al*., 2006).

(i) Virology

Rotaviruses contain 11 segments of double-stranded (ds) ribonucleic acid (RNA) genome of ~18.5kb that is enclosed into a triple-layered non-enveloped capsid (Estes and Kapikian, 2007). The mature virus particle is ~100 nm in diameter and composed of an outer layer, an intermediate layer and an inner core layer. Sixty protein spikes protrude from the outer layer, giving the virus its wheel-like resemblance (Estes and Kapikian, 2007; Li *et al*., 2009). Reassortment of the viral genome is group specific and occurs between serotypes of the same group (Estes and Kapikian, 2007). Rotavirus is shed in high concentration by an infected person (10^{11} particles/g) and shows a high resistance to degradation at ambient temperature (Franco and Greenberg, 2009). Studies on rhesus RV showed that; i) RVs have a longer survival time in non-polluted water or groundwater (64 days at 15 degree Celsius [°C]) as opposed to polluted water (10 days at 24°C) and ii) RV is inactivated in drinking water containing 1 mg/ℓ free chlorine for 120 min (Espinosa *et al*., 2008). Rotaviruses are inactivated by a UV dose between 120-360 mill joules/cubic centimetre (Li *et al*., 2009) and a temperature of 60°C for 10 min (O'Mahony, 2000).

(ii) Clinical

The clinical manifestation of HRV in children can range from subclinical infections to severe gastroenteritis with the onset of diarrhoea, vomiting, fever, and/or fatal dehydration (Estes and Kapikian, 2007; Kawai *et al*., 2012). Malnutrition, low birth weight and premature infants are at greater risk of contracting severe RV-induced

gastroenteritis, requiring hospitalisation (Franco and Greenberg, 2009). Minimal to no clinical symptoms have been reported for older adults (Anderson *et al*., 2012). Rotavirus-induced diarrhoea last for approximately 5 days with the onset of symptoms ranging from 1-4 days after an incubation period of less than 48 hr (Estes and Kapikian, 2007; Franco and Greenberg, 2009). Chronic cases of RV infections have been documented in immunosuppressed and severe combined immunodeficiency patients (Estes and Kapikian, 2007; Franco and Greenberg, 2009; Kiulia *et al*., 2009). Laboratory diagnosis of HRV relies on the use of EIA or EM for the screening of stool specimens (Khan and Bass, 2010). Viral isolation has been proven useful for the isolation and propagation of group A RVs, however this method is not used for routine diagnosis.

(iii) Epidemiology

Transmission of HRVs is by the faecal-oral route, direct or indirect contact with infected individuals and seldom by the respiratory, food (<1%) and water routes (O'Mahony, 2000; Franco and Greenberg, 2009; Rutjes *et al*., 2009; Khan and Bass, 2010; Scallan *et al*., 2011a; Rodríguez-Lázaro *et al*., 2012). Annually, 323 children <5 years of age die from HRV-associated gastroenteritis in SA, far less than that suffered by other sub-Saharan African countries such as Nigeria (71144), Kenya (8898), Malawi (7368) and Zambia (4111) (Sanchez-Padilla *et al*., 2009). The prevalence of HRV in children hospitalised with diarrhoea in SA was 32.8% (Steele and Glass, 2011), a number which has since decreased to ~24.2% in children ≤13 years presenting with gastroenteritis (Mans *et al*., 2010). Two vaccines; a monovalent attenuated (Rotarix™: GlaxoSmithKline Biologicals, Rixensart, Belgium (BE)) and a pentavalent human-bovine reassortant vaccine (RotaTeq™: Merck, West Point; PA) have significantly decreased severe HRV episodes worldwide (Khan and Bass, 2010; Walker and Black, 2011). Rotavirus infections may re-emerge placing adults, children and immunosuppressed patients at potential risk (Franco and Greenberg, 2009; Anderson *et al*., 2012). In temperate climates HRV infections peak during the cooler months of the year whereas in other countries a year-round endemic pattern of infection is noticed (Estes and Kapikian, 2007; Franco and Greenberg, 2009). Zoonotic transmission and genome reassortment of RVs leads to novel strains that could potentially increase disease severity in humans and animals (Abe *et al*., 2011; Midgley *et al*., 2011).

1.2.1.3 Human astrovirus

The name astrovirus (AstV) was derived from the Greek word “astron” (star) which describes the characteristic star-like surface structure when viewed by negative stain EM (Méndez and Arias, 2007; De Benedictis *et al*., 2011). Human AstVs are grouped in the genus *Mamastroviruses* which infects mammalian species in the family *Astroviridae* (Méndez and Arias, 2007). Astroviruses belonging to the genus *Avastroviruses* which have been detected in various bat, birds and mammal species (Chu *et al*., 2009; Zhu *et al*., 2009). In humans, eight so called “classic” serotypes of AstVs (HAstV 1-8) are known (Wang *et al*., 2001) with two newly proposed species, MLB1, which are genetically related to rat AstV, and VA1, recently described in patients with diarrhoea (Finkbeiner *et al*., 2008, 2009; De Benedictis *et al*., 2011). Phylogenetic analysis now distinguishes and recognises three new avian species and 19 new mammalian species (De Benedictis *et al*., 2011).

(i) Virology

Astroviruses are positive-sense, ss RNA viruses with a genome size of ~6.1-7.3 kb in length and a diameter of 28-30 nm with a characteristic five- to six-pointed star-like formation on the surface that is visible in approximately 10% of particles (Méndez and Arias, 2007). Infection of susceptible cells results in the formation of two RNA species, full length genomic RNA and a ~2.4 kb subgenomic RNA (Figure 1.3).

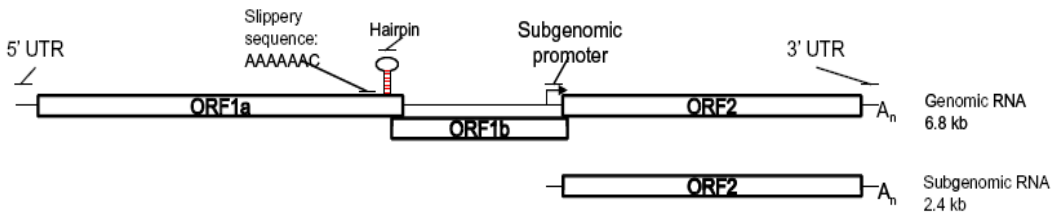

Figure 1.3: Genome structure of astroviruses (De Benedictis *et al*., 2011)

The genome is divided into three ORFs (1a, 1b, 2) with a non-translated region on both the a 3’ and 5’ ends (Méndez and Arias, 2007; Finkbeiner *et al*., 2008). Trypsin-treated HAstV can be propagated in human epithelial colorectal adenocarcinoma cells (CaCo-2) (Méndez and Arias, 2007). Co-infection by two different genotypes of HAstV have

resulted in recombination between the two strains (Wolfaardt *et al*., 2011). Human AstVs can persist for more than 90 days in the environment with a 3.3-5 $\log_{10}$ unit reduction in water at 4-20°C. Residual infectivity was found after 2 h in the presence of 1 mg/ℓ of free chlorine and a 2.4 $\log_{10}$ titre reduction in the presence of 0.5 mg/ℓ of free chlorine (Abad *et al*., 1997). Human AstVs are stable at a wide range of pH (3-10). A drastic reduction in infectivity is seen when incubated at 60°C for 5 min (Méndez and Arias, 2009).

(ii) Clinical

The incubation period prior to the onset of symptoms is estimated to be 3-4 days in adults with an average diarrhoeal duration of 2-3 days. Symptoms may include vomiting, fever, anorexia and abdominal pain (Méndez and Arias, 2007, 2009). Episodes of diarrhoea in children seldom lead to hospitalisation or significant dehydration (Méndez and Arias, 2007). Human AstV can be considered as the causative agent for encephalitis in immunocompromised patients (De Benedictis *et al*., 2011). Molecular-based assays, EM and EIA have been used in the screening of stool specimens and large-scale epidemiological studies (Guix *et al*., 2005; Le Guyader *et al*., 2008).

(iii) Epidemiology

Human AstVs have been recognised as an important aetiological agent of viral gastroenteritis in all age groups (Foley *et al*., 2000; Gofti-Laroche *et al*., 2003; Espul *et al*., 2004; Santos and Hoshino, 2005; Verma *et al*., 2010). Human AstVs are known to cause 10% of sporadic non-bacterial gastroenteritis cases in children <2 years of age, the elderly and immunocomprimised patients (Walter and Mitchell, 2003; Moser and Schultz-Cherry, 2005; Ramani and Kang, 2009). The primary route of transmission for HAstVs is through the faecal-oral route by person-to-person spread and indirectly by food and water. In SA surface water, used for irrigation, domestic and recreational purposes, and sewage effluent have tested positive for the presence of HAstV (Taylor *et al*., 2001; Nadan *et al*., 2003).

1.2.2.1 Human adenovirus

Human adenoviruses (AdVs) belong to family *Adenoviridae* in the genus *Mastadenovirus*. Adenoviruses consist of a linear, ~35 kb ds deoxyribonucleic acid

(DNA) genome that encodes for more than 30 structural and non-structural proteins (Jiang, 2006; Berk, 2007). Adenoviruses infect predominately the membrane linings resulting in conjunctivitis, gastrointestinal disease (serotypes 40 and 41, and to a lesser extent serotypes 12, 18 and 31), ocular, respiratory and neurological symptoms mainly in children and military recruits (Chen *et al.*, 2004; Jiang, 2006; Aminu *et al*., 2007; Jones *et al*., 2007; Echavarría, 2008; Maunula *et al*., 2009; Verma *et al*., 2009). Laboratory diagnosis uses direct detection methods as well as virus isolation in cell culture (except for serotypes 40 and 41), EIA and molecular based assays (Echavarría, 2008). Infections are considered endemic in the paediatric population and children under the age of 12 years. More severe epidemics and outbreaks can occur in children and adults (De Wit *et al.,* 2001, Logan *et al.,* 2006; Carraturo *et al.,* 2008; Sdiri-Loulizi *et al.,* 2008; Echavarría *et al*., 2009). Adenoviruses are highly infectious and can be transmitted by infected individuals through the respiratory route (cough or a sneeze) and the faecal-oral route with no cases of foodborne AdVs as of yet documented (Van Heerden *et al.,* 2003, 2005; Karamoko *et al.,* 2005; Verheyen *et al*., 2009).

1.2.2 Enteric viruses associated with hepatitis

Hepatitis is a liver disease caused by five well characterised viruses from a broad range of families. Hepatitis A and E viruses, both of which are small, non-enveloped ss RNA viruses, belong to the families *Picornaviridae* and *Hepeviridae*, respectively. Hepatitis E virus is morphological similar to the viruses in the family *Caliciviridae* where it was first classified, however it is clearly distinguishable from HAV when viewed by EM (Emerson and Purcell, 2007). Hepatitis A virus is a significant cause of morbidity globally whereas HEV causes enterically transmitted non-A-non-B hepatitis worldwide (FitzSimons *et al*., 2010; Khuroo, 2011). These viruses are transmitted predominantly by the faecal-oral route, directly by contact with an infected person and indirectly by faecal contaminated water and food. Hepatitis E virus is mostly transmitted by water whereas person-to-person transmission is found more often with HAV than HEV (FitzSimons *et al*., 2010; Pintó *et al*., 2010). Clinical manifestation of hepatitis A and E are indistinguishable (Carter, 2005; Emerson and Purcell, 2007).

1.2.2.1 Hepatitis A virus

Hepatitis A virus remains the most common cause of viral hepatitis, infecting millions

of people worldwide (Pintó *et al*., 2010). The majority of infections occur in developing countries despite the availability of an effective vaccine and improved sanitation and living standards (FitzSimons *et al*., 2010; Pintó *et al*., 2010; Stübgen, 2011). Hepatitis A virus is classified within the genus *Hepatovirus* in the family *Picornaviridae* (Knowles *et al*., 2012; Adams *et al*., 2013)

(i) Virology

Hepatitis A virus has a linear uncapped, positive-sense, ss RNA genome of ~7.5 kb size linked to a VPg protein and enclosed into a 27-32 nm, non-enveloped icosahedral capsid. The capsid is morphologically indistinguishable from other picornaviruses (Hollinger and Emerson, 2007; Pintó *et al*., 2010). The genome consist of a single ORF encoding the individual capsid proteins (VP1-4 and protein 2A) and non-structural proteins (2A, 2C and proteins 3A-D) necessary for RNA replication (Dotzauer *et al*., 2008). The genome is flanked by a NCR on both ends with a poly A tail present on the 3' end. Hepatitis A virus can be divided into six genotypes (I-VI) (Clark *et al*., 2012). Genotypes I, II and III (further subgenotyped into A and B (Kulkarni *et al*., 2009; Pintó *et al*., 2010)) are associated with human infection, with genotypes I and III the most prevalent in humans (Nainan *et al*., 2006; Cristina and Costa-Mattiolo, 2007). Genotypes IV, V and VI are simian related (Nainan *et al*., 2006; Pintó *et al*., 2010). Genotype I is most prevalent worldwide, with IA more common than IB (Cristina and Costa-Mattiolo, 2007).

Hepatitis A virus appears to be extremely stable in the environment with a 100-fold decline in infectivity over 4 weeks at room temperature and 3-10 mo in water (Koopmans *et al*., 2002). Hepatitis A virus can remain infectious when exposed to: i) pH conditions of pH 1 for 2-5 h, ii) 20% ether and chloroform, and iii) exposure to a temperature of 60ºC for 1 h with slight inactivation recorded after 10-12 h (Hollinger and Emerson, 2007). Hepatitis A virus is also very resistant to drying and detergents, surviving 1% sodium dodecyl sulphate as well as organic solvents like diethyl ether, chloroform and trichlorotrifluoroethane (Sprandling *et al*., 2009). Inactivation of HAV can be accomplished by: i) subjecting the virus to temperatures of 98-100ºC, reaching 50% inactivation at 61ºC for 10 min, ii) UV radiation (4 $\log_{10}$ reduction at 16-39 molecular weight/cubic centimetre), iii) formalin treatment (3% for 5 min at 25ºC), iv) iodine treatment (3 mg/ℓ for 5 min), v) potassium permanganate (30 mg/ℓ for 5 min),

vi) glutaraldehyde (2%) and vii) by free chlorine treatment (2.0-2.5 mg/ℓ for 15 min) (Hollinger and Emerson, 2007; Spradling *et al*., 2009)

(ii) Clinical

Hepatitis A has an incubation period of 28 days that ranges from 15-50 days with the onset of clinical symptoms which last for no more than 2 mo, except in 10-15% of patients where symptoms may persist for up to 6 mo (Pintó *et al*., 2010; Sharapov, 2012). Hepatitis A can manifest from a silent asymptomatic infection to icteric or fulminant hepatitis (Hollinger and Emerson, 2007). Infections among young children are often asymptomatical as opposed to adults where a symptomatic illness can be experienced with a moderate onset of symptoms such as malaise, fever, anorexia, nausea, dark urine or abdominal discomfort followed by jaundice (Hollinger and Emerson, 2007; Dotzauer *et al*., 2008; Pintó *et al*., 2010; Sharapov, 2012). In children diarrhoea, vomiting and nausea is more commonly experienced than in adults. Viral shedding peaks at the onset of symptoms and can last for a further 3-4 weeks (Polish *et al*., 1999), where viral RNA can be detected by PCR-based assays for up to 10 weeks (Robertson *et al*., 2000). Patients with a history of chronic liver diseases are at a higher risk of developing fulminant hepatitis. Laboratory diagnosis of HAV relies on the detection of immunoglobulin M, by EIA, during the acute phase of infection (Cuthbert, 2001). Infection with HAV results in a life-long immunity (Hollinger and Emerson, 2007; Pintó *et al*., 2010). Although no specific treatment for viral hepatitis exists safe and effective vaccines (Havrix® (GlaxoSmithKline Biologicals, Rixensart, BE) and Vaqta® (Merck)) have been shown to provide long-term protection (Hollinger and Emerson, 2007).

(iii) Epidemiology

Hepatitis A virus remains a significant cause of global morbidity with a low mortality rate (FitzSimons *et al*., 2010). Transmission is infrequently through blood or blood product transfusions (Nainan *et al*., 2006; Sharapov, 2012). Although in 40% of HAV infections the source remains elusive, foodborne hepatitis A outbreaks have been estimated to cause 1 566 infections, 99 hospitalisations and 7 deaths annually in the US (Pintó *et al*., 2010; Scallan *et al*., 2011b). Hepatitis A virus has a worldwide distribution (Figure 1.4), with some countries such as SA (Taylor, 1997; Venter. 2004), India (Hussain *et al*., 2005), US (Klevens *et al*., 2010) and Brazil (Villar *et al*., 2004)

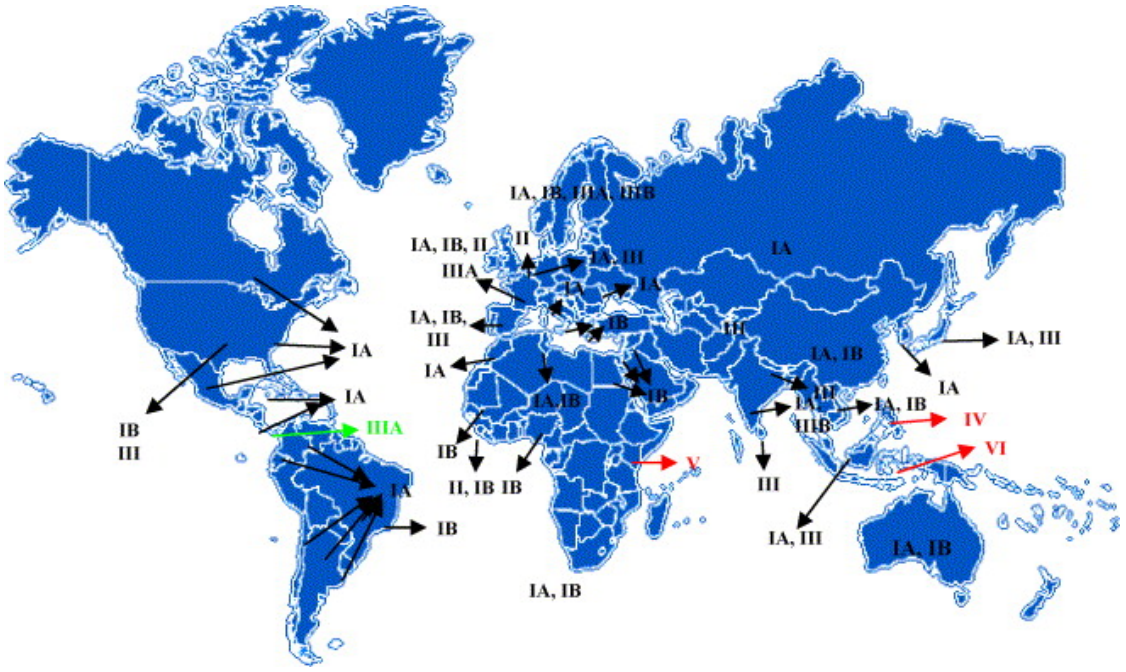

Figure 1.4: Geographic representation of HAV genotype distribution. Human HAV strains are in black, simian HAV in red and Panamanian owl monkeys HAV in green. (Cristina and Costa-Mattioli, 2007).

having multiple genotypes and/or subgenotypes co-circulating.

An epidemiological shift has been noticed in countries of Asia and America moving from high intermediate, where most children have been exposed to HAV at an early age, to a low intermediate (FitzSimons *et al*., 2010; Pintó *et al*., 2010). In countries from Southern-and Eastern Europe a shift to low endimicity was noticed (Villar *et al*., 2004), resulting in the increase of susceptibility of young individuals (FitzSimons *et al*., 2010).

1.2.2.2 Hepatitis E virus

Hepatitis E virus is a major cause of enterically transmitted non-A, non-B hepatitis worldwide causing tens of thousands of cases in endemic regions (Khuroo, 2011). Two major strains of HEV are recognised; those that infect avian species and the other mammalian species, with the last further classified into four genotypes (1-4) (Emerson and Purcell, 2007; Khuroo, 2011). Genotypes 1 and 2 are known to cause large scale waterborne epidemics whereas genotypes 3 and 4 can infect swine, wild boar and deer which serve as reservoirs species for human infections (Shuchin *et al*., 2003; Schielke *et al*., 2009; Khuroo, 2011). Hepatitis E virus is a non-enveloped, ss, positive sense, and

polyadenylated RNA virus with a genome of ~7.2 kb long. The genome is arranged into three ORFs (Emerson and Purcell, 2007). Hepatitis E is considered as an acute self-limiting disease with a high attack rate in adults (20-30 and >60 years of age) and a mortality rate of <3%, however, pregnant woman in their third trimester are more susceptible to develop fulminant hepatitis with a 20% fatality rate (Purdy and Khudyakov, 2011). Foodborne transmission, as with person-to-person transmission, is considered a relative uncommon means of transmission of HEV (Emerson and Purcell, 2007). Hepatitis E virus is prevalent in many countries around the world, including SA (Williams, 2004), principally as a zoonotic infection in industrialised countries and a waterborne infection in developing countries (Okamoto, 2011).

1.2.3 Enteric viruses associated with diverse clinical conditions

1.2.3.1 Enteroviruses

Enteroviruses cause a significant amount of morbidity and mortality worldwide (Rhoades *et al*., 2011). This group of enteric viruses belong to the genus *Enterovirus* in the family *Picornaviridae* of which >66 distinct serotypes exists (Ehlers *et al*., 2005; Van der Staten, 2011) that can cause localised and systemic infections in humans of all ages (Pallansch and Roos, 2007). These viruses are ss, positive-sense RNA viruses with a VP covalently linked to the 5'end of the genome. The virions are spherical with icosahedron symmetry measuring ~30 nm in diameter (Racaniello, 2007). Enterovirus infections are generally considered to be asymptomatic; however these viruses can cause a range of clinical distinct syndromes (Pallansch and Roos, 2007). To date, PV remains the only virus in the genus *Enterovirus* to which an effective vaccine has been developed (Shih *et al*., 2011; Van der Staten, 2011). Despite the success of the oral polio vaccine, the vaccine can cause vaccine derived PV (VDPV) in a vaccinee 7-30 days after immunisation with potential person-to-person transmission. The inactivated PV vaccine does not revert to cause VDPV (Minor, 2009). With the almost eradication of PV worldwide, a rise in EV 71 outbreaks causing hand, foot and mouth disease in children have been noticed (Solomon *et al*., 2010).

1.3 ROLE OF FOOD AND WATER IN THE TRANSMISSION OF ENTERIC VIRUSES

The global demand for food and potable drinking water poses an increasing threat for the possible occurrence of food and waterborne outbreaks (Koopmans *et al*., 2002; Hanjra and Qureshi, 2010). It is estimated that 1.1 bil people lack access to adequate water supply whereas 2.4 bil people do not have proper sanitation facilities. Environmental contamination of sewage, solid waste or land runoff poses a possible contamination threat to food and water sources and could indirectly result in the transmission of human pathogenic viruses (Koopmans and Duizer, 2004; Rodríguez-Lázaro *et al.*, 2012). Contamination of food products may occur at any stage from the farm to harvesting and processing to just before consumption (Figure 1.5) (Koopmans and Duizer, 2004).

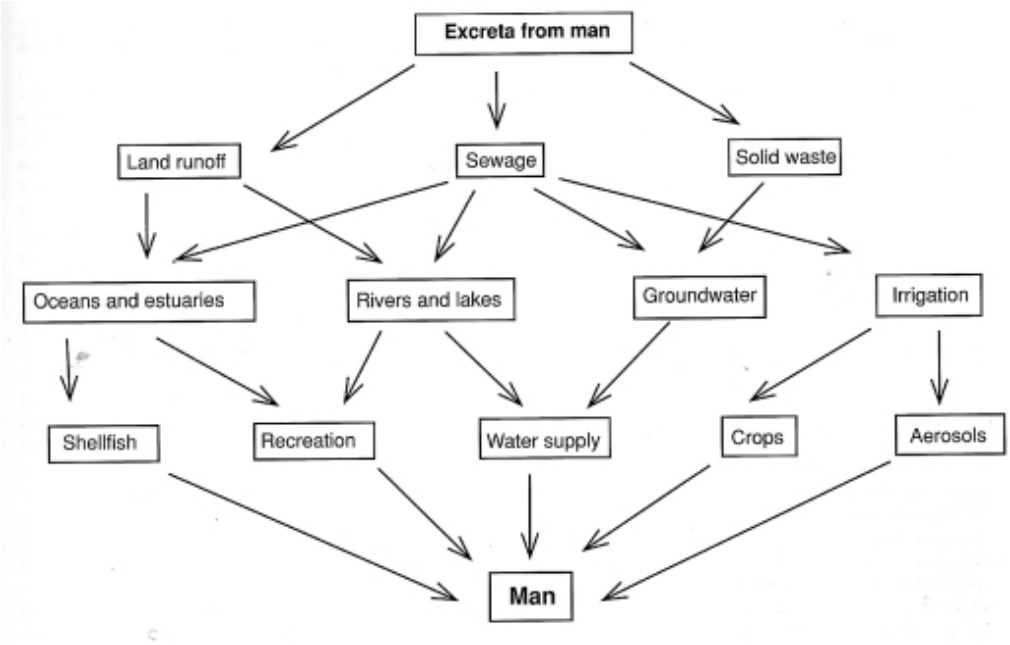

Figure 1.5: Circulation of enteric viruses in the environment (Bosch, 1998).

Human enteric viruses, such as NoVs, have a high secondary attack rate of which person-to-person transmission masks the role of food-and waterborne transmission (Carter, 2005). However, these viruses are shed in high titres and predominantly rely on the faecal-oral route for transmission. Assessing the impact of these viruses is problematical due to the difficulty experienced in their detection in food and water sources (Bosch *et al*., 2008; 2011). Sporadic and acute outbreaks of viral infections due

to the consumption of contaminated water or food is well known (Dalling, 2004; Carter, 2005; Baert *et al*., 2009b; Maunula *et al*., 2009; Ter Waarbeek *et al*., 2010). Annually the US experiences approximately 37.2 mil illnesses of which 9.4 mil illnesses are ascribed to the consumption of contaminated food (Scallan *et al*., 2011a).

1.3.1 Foodborne transmission

A foodborne outbreak is defined as an event in which two or more people fall ill after consuming a common meal or food item (Greig and Ravel, 2009). Recognising foodborne diseases is problematic and in some instances food is suspected as the vehicle whereas water or person-to-person contact might have been the route of transmission (Cliver, 2008). Classifying outbreaks as foodborne relies on the collection and screening of clinical and suspected food samples to identify the causative agent (Martinez *et al*., 2008). Epidemiological data are used in cases of suspected foodborne outbreaks to statistically identify the food source and predict possible estimates of the relevance of foodborne outbreaks (Cliver, 2008, 2010; Martinez *et al*., 2008). In the majority of cases this cannot be supported through laboratory analysis due to: i) the food item in question might not be available for analysis, ii) insufficient sensitivity of the assay used for the recovery and detection, iii) the concentration of viruses on a food source might be too low to be detected by molecular based techniques (perhaps <10 virions) and, iv) the time of sampling and storage conditions prior to analysis may have altered the sample integrity (Koopmans and Duizer, 2004; Carter, 2005; Dreyfuss, 2009; Rodríguez-Lázaro *et al*., 2012). Therefore, the role of enteric viruses in foodborne outbreaks might be underestimated.

Fresh produce irrigated with polluted surface water was identified as the primary source (Gallimore *et al*., 2005; Cheong *et al*., 2009) or as a possible source of contamination (Ethelberg *et al*., 2010; Bosch *et al*., 2011). The method applied for irrigation, be it subsurface, surface or overhead irrigation, is the single most important factor in the contamination of different parts of the plant (Alum *et al*., 2011). Polluted water used as processing/washing water postharvest may further contribute in the transfer of contaminants onto produce (Berger *et al*., 2010; Wei and Kniel, 2010a; Rodríguez-Lázaro *et al*., 2012). Besides faecal polluted water sources, sewage sludge as fertilisers and the quality of the soil used can also contribute to the contamination of fresh produce

(Santamaría and Toranzos, 2003; Koopmans and Duizer, 2004; Cheong *et al*., 2009; Berger *et al*., 2010; Wei and Kniel, 2010a). This could promote the possible intracellular uptake of viral pathogens by the roots and or leaves of these crops (Chancellor *et al*., 2006; Wei *et al*., 2011).

Food handlers in the symptomatic, asymptomatic or in the recovery phase of the infection can contaminate food sources (Koopmans and Duizer, 2004; Berger *et al*., 2010). Hand transmission of human pathogens contributes ~40% (327/816) of foodborne outbreaks either directly from person-to-person or indirectly from person to contact surface or food and back to person (Todd *et al*., 2009). The last is considered as a major contributor to foodborne outbreaks (Berger *et al*., 2010; Cliver, 2010). Enteric viruses can remain infectious on fomites for up to 90 days for HAstVs, 60 days for HRVs and HAV and 7 days for NoVs (Todd *et al*., 2009). Vendors, harvesters or any individual coming into contact or partake in the processing of food sources can be considered a potential source of contamination (Koopmans and Duizer, 2004; Carter, 2005; Todd *et al*., 2009).

Noroviruses and HAV are considered a major causes of foodborne acute gastroenteritis and hepatitis, respectively, (Cliver, 2008; Atmar, 2010; Berger *et al*., 2010; Rodríguez-Lázaro *et al*., 2012) causing significant foodborne related outbreaks worldwide (Boxman *et al*., 2006; Nenonen *et al*., 2009; Petrignani *et al*., 2010; Donnan *et al*., 2012). Other enteric viruses such as, aichi virus, HAstV, EVs, HRV and HEV (Koopmans *et al*., 2002; Gallimore *et al*., 2005; Cliver, 2008; Le Guyader *et al*., 2008; Iizuka *et al*., 2010; Mattison *et al*., 2010) have also since been detected to cause foodborne gastroenteritis and hepatitis outbreaks. During the period 1998-2006, 11, 990 foodborne outbreaks were reported in the US in which ~1% (19/1965) NoVs outbreaks were confirmed by laboratory analysis. During the same period only ~35% (274/787) of the suspected outbreaks due to food handlers could be confirmed by laboratory analysis (Dreyfuss, 2009). The role of NoVs transmission in food remains therefore a controversial subject (Dreyfuss, 2009).

Soft fruit and salad vegetables, strawberries, raspberries, green onions, raw meat and shellfish have been implicated in outbreaks of both gastroenteritis and hepatitis (Fleet *et al*., 2000; Fiore. 2004; Le Guyader *et al*., 2004; Koopmans and Duizer, 2004; Carter,

2005; Wheeler *et al*., 2005; Rutjes *et al*., 2006; De Wit *et al*., 2007; Yano *et al*., 2007; Martinez *et al*., 2008; Nenonen *et al*., 2009; Robesyn *et al*., 2009; Ethelberg *et al*., 2010; Mattison *et al*., 2010; Petrignani *et al* 2010). A multistate outbreak of hepatitis A in Australia resulted from the consumption of semidried tomatoes (Donnan *et al*., 2012). The import of shellfish into Spain caused a transcontinental hepatitis A outbreak among 184 serological confirmed cases (Sánchez *et al*., 2002). Importation of raspberries from Bosnia into Canada (Le Guyader *et al*., 2004), China to Sweden (Lysén *et al*., 2009) and from Poland to several countries in Europe (Cotterelle *et al*., 2005; Falkenhorst *et al*., 2005; Korsager *et al*., 2005; Sarvikivi *et al*., 2012) resulted in large outbreaks of enteric virus associated gastroenteritis. Strawberries imported by the US and New Zealand, respectively, have resulted in several multistate outbreaks of hepatitis A (Niu *et al*., 1992; Hutin *et al*., 1999; Calder *et al*., 2003). The recovery, detection and inactivation of virions on berry fruits is hampered by the lack of effective decontamination measures, the presence of PCR inhibitors, the decrease in the pH of the elution buffers and the uneven surface texture of the berry fruit. (Le Guyader *et al*., 2004; Sarvikivi *et al*., 2012). Foodborne transmission of infectious disease across the borders of countries has thus become a reality.

1.3.2 Waterborne transmission

“When the well is dry, we know the worth of water” (Benjamin Franklin). The global demand for water has drastically increased since the 1950s although the supply of fresh water has declined. Agriculture, consuming 80% of the global water use, is the first sector that will suffer as the water demand increase. Further challenges faced are that of the deterioration of freshwater ecosystems and the upwelling in incidence of waterborne diseases (Hanjra and Qureshi, 2010). A report by the World Health Organisation (WHO) estimated that the consumption of unsafe water contributed to the deaths of 2 mil people annually (WHO. 2010).

The quality and possible faecal contamination of water is determined by the use of model organism. Ideally, these organisms must have similar behavioural characteristics, possess the same or greater resistance to environmental stresses and does not necessarily originate from the same source as the pathogen (Jofre, 2007). Bacterial groups such as *Escherichia coli* (thermololerant coliforms), *Enterococcus* spp. (faecal

streptococci) and *Clostridium perfringens* are known model organisms and used as indicator organisms (Jiang, 2006; Jofre, 2007). The use of bacterial indicators for viral contamination of water is not sufficient since viruses can persist longer in the environment than bacteria. Viruses are also more resistant to environmental stresses and disinfection process. Incidences where bacterial indicators were not detectable in the water yet viral waterborne illnesses were contracted, exists (Sinclair *et al*., 2009). Bacteriophages and EVs have been suggested as alternatives to bacterial indicators, however, a study showed significant correlation between bacteriophages and EVs but not between bacteriophages and other viruses (Jofre, 2007; Lodder *et al*., 2010). Adenoviruses are frequently detected in human sludge/waste, are stable in the environment, able to resist most water treatment processes and are easier to detect by molecular-based methods, making them an ideal alternative to bacterial indicators (Jiang, 2006; Jofre, 2007; Silva *et al*., 2011; Rodríguez *et al*., 2012).

Environmental factors and viral morphology enables enteric viruses to persist in water for undetermined periods of time (Gerba, 2007; Pintó and Saiz, 2007; Schwab, 2007). Enteric viruses are small, highly infectious non-enveloped viruses with a negative charge on the capsid in ambient pH (pH 5-9) allowing for possible attachment to particles for transport and protection to environmental degradation and chemical inactivation (Schwab, 2007). In natural waters low temperature, salt, ambient pH, organic matter and suspended solids increase the survival of these viruses. However, sunlight (UV light), air-water interfaces (mostly for more hydrophobic viruses) and natural microflora decreases and ultimately inactivates enteric viruses in water bodies (Gerba, 2007). Preparation of surface waters for drinking water largely depends on the quality of the water source used, the approach in treatment and the nature of the pathogen itself (Springthorpe and Sattar, 2007). Processes such as filtration, sedimentation or coagulation, disinfection with free chlorine, chloramine or chlorine dioxide, UV and ozone treatment is used in the removal of contaminants from surface waters (Springthorpe and Sattar, 2007; Teunis *et al*., 2009; Rodríguez-Lázaro *et al*., 2012). Unattached viruses are more likely to escape removal from water due to their small size but are easier to remove by decontamination processes whereas the opposite is true for viruses attached to particles or organic matter (Springthorpe and Sattar, 2007).

Waterborne outbreaks as a result of enteric viruses, although not as prominent as foodborne outbreaks, have been well documented. Enteric viruses such as NoVs, HRVs, EVs, HAV, HEV, SaV, HAstVs and HAdV, to mention a few, have been detected and some directly implicated as the cause of an outbreak (Vivier *et al*., 2004; Van Zyl *et al*., 2006; Hansman *et al*., 2007; Mena, 2007; Schwab. 2007; Gibson *et al*., 2011; Rodríguez-Lázaro *et al*., 2012). Noroviruses have been documented as a waterborne pathogen, responsible for numerous waterborne-related gastroenteritis outbreaks (Maunula *et al*., 2009; Ter Waarbeek *et al*., 2010; Rodríguez-Lázaro *et al*., 2012). Waterborne transmission is less prominent in the spread of HAV, with waterborne hepatitis outbreaks mostly due to HEV and secondly to HAV (Cuthbert. 2001). Shellfish harvested from sewage-polluted areas have resulted in significant outbreaks of HAV (Pintó *et al*., 2010), masking the role of water as the origin of contamination. Furthermore, the detection of EVs, HRVs, NoVs, HAstV and HAV, to name but a few, in drinking and recreational water sources poses a substantial health risk to travellers and susceptible persons (Vivier *et al*., 2004; Maunula *et al*., 2009; Sinclair *et al*., 2009; Pintó *et al*., 2010; Gibson *et al*., 2011; Steyer *et al*., 2011).

The presence of enteric viruses in water remains a global problem and have been detected from various countries namely, Japan (Ueki *et al*., 2005; Hansman *et al*., 2007), SA (Van Zyl *et al*., 2006; Mans *et al*., 2013), Singapore (Aw *et al*., 2009), the Netherlands (Lodder *et al*., 2010), Ghana (Gibson *et al*., 2011), the US (Mena, 2007), Slovenia (Steyer *et al*., 2011) and Poland (Kozyra *et al*., 2011). Contamination of surface water by septic tanks, sewage effluent leaking from broken sewage pipes and/or infected persons may result in multiple outbreaks where thousands of people may be infected and/or co-infected with multiple microorganism ranging from enteric viruses to bacteria and protozoa (Podewils *et al*., 2007; Beaudeau *et al*., 2008; Lysén *et al*., 2009; Maunula *et al*., 2009; Dale *et al*., 2009; Sinclair *et al*., 2009; Dale, 2010; Räsänen *et al*., 2010; Ter Waarbeek *et al*., 2010; Laine *et al*., 2011; Rodríguez *et al*., 2012). The health implications can range from asymptomatic infections to gastroenteritis and other acute infections, to more serious cases such as myocarditis, hepatitis, pharyngoconjunctival fever and aseptic meningitis (Mena, 2007; Artieda *et al.*, 2009; Maunula *et al.*, 2009; Symonds *et al.*, 2009).

South Africa (SA) is a semi-arid country with freshwater being a limiting natural resource. Agriculture accounts for ~70% of the global freshwater withdrawals, yet industrial effluents, acid mine drainage, domestic and commercial sewage, agricultural runoff and litter are continuously polluting water resources (Walmsley *et al*., 1999; United Nations Educational, Science and Cultural Organization-World Water Assessment Programme, 2012). Irrigation water standards in SA make no reference to the standards of viruses present in these waters (Department of Water Affairs and Forestry, 1996) even though enteric viruses such as HAV, HAstV, HAdV, HRV, NoV and EVs have been detected in surface water used for irrigation, recreational and domestic purposes (Taylor *et al*., 2001; Nadan *et al*., 2003; Van Heerden *et al*., 2003; Barnes and Taylor, 2004; Venter, 2004; Vivier *et al*., 2004; Ehlers *et al*., 2005; Van Zyl *et al*., 2006; Venter *et al*., 2007; Netshikweta, 2012).

1.4 VIRAL RECOVERY FROM FOOD AND WATER SAMPLES

Low viral concentrations and the lack of standardised methods for the recovery and detection of viruses are some of the major obstacles experienced in determining the role food and water contributes in their transmission (Fong and Lipp, 2005; Di Pasquale *et al*., 2010; Bosch *et al*., 2011). Viruses are unable to replicate in water and food but are able to utilise these sources for their transmission (Koopmans and Duizer; 2004; Bosch *et al*., 2011). A universal method for the analysis of contaminated food and water has yet to be developed and validated based on its efficiency, consistency of performance, robustness, cost effectiveness and complexity. However, such a method may not be suitable for all fruits and vegetables (Croci *et al*., 2008; Bosch *et al*., 2011). Viral analysis of water and food requires that a representative sample be used based on size, weight, volume, number of the specific item(s) and the quality of the sample analysed. However, economical and logistical boundaries limit this process (Bosch *et al*., 2011). Analysis of enteric viruses in food and water samples rely on methods that utilise a primary recovery step followed by a secondary concentration step prior to nucleic acid extraction and detection by molecular-based methods (Dubois *et al*., 2007; Croci *et al*., 2008).

1.4.1 Water samples

Viral analysis of water is mostly considered as a multistage process where viruses are first recovered from water, ranging from 10-100 ℓ, further concentrated to about 5-10 millilitre (mℓ) followed by nucleic acid extraction and detection by molecular-based methods (Wyn-Jones, 2007). Consideration must be given to the volume and the quality of the water sampled in order to obtain a representative sample (Bosch *et al*., 2011). The volume of water needed largely depends on the number of virions that is likely to be present in the water (Wyn-Jones, 2007). Sewage effluent contains a high concentration of enteric viruses with a lower concentration present in the receiving waters. Treated drinking water virtually contains no viruses and therefore a representative sample from each source will differ (Carter, 2005; Wyn-Jones, 2007).

1.4.1.1 Primary recovery

Viral properties and characteristics such as ionic charge, particle size, density and sedimentation coefficient have been used to recover viruses using techniques such as absorption-elution, ultrafiltration and ultracentrifugation, respectively (Wyn-Jones, 2007). In addition to these techniques, immune-affinity columns or broad-based antibody capture techniques, including magnetic beads, utilises antibodies directed towards the epitopes present on the viruses. Antibody-based techniques can be used further for the purification of a sample from inhibitory substances (Wyn-Jones, 2007; Bosch *et al*., 2011).

Ultrafiltration relies on membranes, capillaries or hollow fibres, with an approximate pore size between 30-100 kilo Daltons (Wyn-Jones, 2007; Liu *et al*., 2012). Tangenital flow allows for larger volumes of water (10-100 ℓ) to be processed. Pre-treatment of water is not a requirement and based on pore size various microbes can be recovered when eluted from the filters with an organic based buffer, e.g. beef extract. An efficiency of more than 50% can be achieved for the recovery of HAV and NoV GI, however, this may be variable (Wyn-Jones, 2007; Liu *et al*., 2012). Despite the improvements made to reduce the high running and cost of equipment, it remains unlikely to be implemented for routine analysis of water samples for pathogenic microorganisms (Wyn-Jones, 2007).

Ultracentrifugation, consider as a secondary concentration method, can also be used as an alternative method in the analysis of environmental water samples. This method requires minimal manipulation of the water, no pH adjustment or the need for an elution step (Prata *et al*., 2012). The method relies on sedimentation to concentrate viruses in a solution by applying sufficient g-force and adequate time (Wyn-Jones, 2007). An efficiency of 69% and 76% of HAdV and HRV in wastewater and recreational water, compared to 38% and 22% respectively using a flocculation technique (skimmed milk) was recorded (Prata *et al*., 2012). Although the technique allows the concentration of viruses to fewer millilitres with a higher efficiency of detection than absorption-elution and flocculation methods, a considerably reduced volume of environmental water (≤1 ℓ) can been processed (Prata *et al*., 2012). Furthermore, the technique remains inadequate for routine viral screening of water samples due to the lack of portability of the equipment and the high capital cost involved (Wyn-Jones, 2007; Bosch *et al*., 2011).

Absorption-elution methods are based on the electrostatic charges on the viral capsids introduced when subjected to different pH levels. Electronegative-and positive membranes, glass wool and/or glass powder utilises these charges to attract and absorb the viral particles from the water. An organic buffer (e.g. beef extract) of a pH ≥9 are used to elute the viruses (Wyn-Jones, 2007). Electronegative membranes require the pre-adjustment of the water sample to pH 3.5 in order to confer a positive charge on the virus. However, electropositive membranes rely on the negative based charge that the viral particles possess at an ambient pH. The efficiency in recovery of both electronegative-and positive membranes is reported to be similar (~22.5% for PV in drinking water). Large volumes of water, up to 1000 ℓ, can be processed and the capital cost is much lower than the above mentioned methods (Wyn-Jones, 2007).

A modified glass wool absorption-elution method has been applied to screen large volumes of surface waters on a routine bases for enteric viruses in SA (Taylor *et al*., 2001; Vivier *et al*., 2004; Ehlers *et al*., 2005; Mans *et al*., 2013). Glass wool has both electrostatic and hydrophobic properties and refers to very fine glass fibres that when compacted, forms a mass resembling wool (Grabow, 2001; Lunn *et al*., 2009). Glass wool has been reported to be very effective (80-95% efficiency) in the routine detection of enteric viruses as opposed to phages (28%) (Grabow, 2001). The method does not require the pre-treatment of the water since the viruses can absorb to the glass wool at

an ambient pH. Different quality water samples such as treated drinking water (100-1000 ℓ), surface water (30 ℓ) and wastewater (10 ℓ) can be analysed with variability in efficiency seen (Grabow, 2001; Wyn-Jones, 2007).

1.4.2 Food samples

Viral recovery from food samples relies on the rupture of the electrostatic and hydrophobic bonds that exists between the virions and food items (Mattison and Bidawid, 2009; Bosch *et al*., 2011). An ideal method is one that can concentrate low levels of virions but at the same time eliminate inhibitory substance. (Mattison and Bidawid, 2009). Several published elution buffer systems have been developed (Croci *et al*., 2008; Kim *et al*., 2008; Bosch et al., 2011) for the recovery of viruses from fruits and vegetables, however, a single standardised method may not be suitable for all food types (Croci *et al*., 2008). This is due to differences in morphology and hydrophobic interactions, tissue composition and processing conditions among each of the different food items. Therefore, it was proposed that various protocols be developed for each food type rather than a single standardised protocol for all food types (Croci *et al*., 2008; Mattison and Bidawid, 2009). The basics of these methods rely on the elution buffer used to recover the virus(es) from the food item(s). The suspension may be further concentrated using polyethylene glycol (PEG)/sodium chloride (NaCl) precipitation, ultrafiltration or ultracentrifugation methods prior to detection with molecular-based techniques (Mattison and Bidawid, 2009).

1.4.2.1 Primary recovery

A general strategy for the detection of foodborne viruses in food samples consists of; i) viral recovery, ii) extraction of viral RNA, and iii) molecular-based detection of the purified RNA (Stals *et al*., 2012). During the recovery phase viruses are recovered from the food samples and the eluent further concentrated to a smaller volume (Stals *et al*., 2012). Primary recovery relies on the development of a suitable buffer system, although this largely depends on the food type and the associated virus(es) (Croci *et al*., 2008). Beef extract and glycine are frequently used in elution buffers, since they are able to reduce non-specific virus adsorption (Baert *et al*., 2008; Kim *et al*., 2008; Stals *et al*., 2012). An alkaline pH (9-10.5) in combination with a tris (hydroxymethyl) amino methane (TRIS)-based buffer has been reported to facilitate in the recovery of

viruses from food and further reduce the effect of the acidic substances present in the fruits and vegetables (Stals *et al*., 2012). The addition of pectinase to the eluate of soft fruits is used to prevent the formation of gelatinous substance (Bosch *et al*., 2011).

1.4.3 Secondary concentration of viruses recovered from food and water samples

Secondary concentration of eluents may in some cases be necessary and involves the use of flocculants or techniques such as; ultrafiltration, ultracentrifugation or immunoconcentration (Wyn-Jones, 2007; Bosch *et al*., 2011; Lui *et al*., 2012, Prata *et al*., 2012). Flocculants such as PEG efficiently precipitates viruses at neutral pH, reduces the volume to be extracted and does not precipitate other organic material (Baert *et al*., 2008; Kim *et al*., 2008; Stals *et al*., 2012). The use of a high protein concentration in buffer solutions facilitates the flocculation of NoV on PEG molecules (Stals *et al*., 2012). Huang *et al*. (2000) reported that by dissolving the NaCl in the solution prior to the addition of PEG further increases the recovery of NoVs efficiency by 14-16%. However, a drastic loss of >50% was recorded when a PEG/NaCl method was applied in the recovery of HAV virus during the evaluation of different absorption-elution methods (Venter, 2004).

The use of ultracentrifugation- and/or filtration of food, however, is limited due to the co-concentration of inhibitors present in the eluents (Bosch *et al*., 2011). Ultracentrifugation gives consistent and reproducible results, however, fruit and vegetable matter must be eliminated from the virus elutes and pellets may be difficult to dissolve. Other methods, such as immunoconcentration with antibodies attached to magnetic beads have been used quite successfully with the added advantage of eliminating PCR inhibitors. The high specificity of this technique may limit detection of all the possible strains of the target virus (Bosch *et al*., 2011).

1.5 VIRAL NUCLEIC ACID EXTRACTION FROM FOOD AND WATER SAMPLES

The isolation of DNA or RNA is a critical step for many biochemical and diagnostic processes (Berensmeier, 2006; Petrich *et al*., 2006; Croci *et al*., 2008; Stals *et al*., 2012). A method developed by Boom *et al*. (1990) exploited the binding of nucleic acid to

glass or silica particles in the presence of a high chaotropic agent concentration such as guanidinium thiocyanate. This method has since been applied for the isolation of nucleic acid form water (Jothikumar *et al*., 2010; Schultz *et al*., 2011), food (Butot *et al*., 2007; Croci *et al*., 2008; Lees, 2010), stool and urine samples (Tang *et al*., 2005; Petrich *et al*., 2006). The majority of these methods rely on the capture of nucleic acid on a column or bead of silica with differences seen based on the virus and/or matrix analysed by the particular kit (Bosch *et al*., 2011).

A variety of classical methods are known for the isolation and purification of nucleic acid that are generally based on a complex series of precipitation and washing steps which are time consuming and laborious to perform. Alternative separation techniques such as hydrogen-binding interaction with an underivatised hydrophilic matrix under chaotropic conditions, ionic exchange under aqueous conditions by means of an anion exchanger, affinity and size exclusion mechanisms have been developed for the purification of nucleic acid (Berensmeier, 2006). In these methods bonding of the nucleic acid to the carries, such as silica, is achieved in the presence of a chaotropic agent or PEG and salts at high concentrations or the presence of a detergent. Such methods are relatively time consuming, requires a number of steps which increases the risk of degradation, sample loss or cross-contamination of samples (Berensmeier, 2006).

Automated purification methods using specifically functionalised magnetic particles allow for quick and efficient purification of nucleic acid directly after extraction from crude cell extracts. Magnetic particles are produced from different synthetic polymers, biopolymers, porous glass, or inorganic magnetic material such as surface-modified iron oxide. The nucleic acid binding process to these particles is facilitated by the presence of example, a chaotropic agent, in which case the nucleic acid “wraps around” these particles. These particles experience a magnetic moment when placed in a magnetic field, thus removing/isolating the nucleic acid from the rest of the solution (Berensmeier, 2006).

The European Committee for Standardization has considered the use of the NucliSens® miniMAG™ extraction procedure (Biomérieux, Marcy l’Etoile, France) as a possible standard method for the detection of enteropathogenic viruses in food and water samples. A less labour intensive version of the NucliSens® miniMAG™ system

(Biomérieux), the NucliSens® easyMAG™ system (Biomérieux), has since been developed (Perelle *et al*., 2009). Viral RNA extraction is accomplished by denaturation of viral coat proteins and the binding of the nucleic acid to the silica-coated magnetic beads (Schultz *et al*., 2011). The NucliSens® easyMAG™ system (Biomérieux) could detect 1 plaque forming unit (PFU)/1.5 ℓ of HAV in bottled water samples (Perelle *et al*., 2009).

Similarly, the MagNa Pure LC system (Roche Diagnostics GmbH, Mannheim, Germany), relies on the purification of nucleic acid with the aid of silica coated magnetic beads. This method was found to be less efficient in extracting RNA from stool samples when compared to the NucliSens miniMAG method (Biomérieux). This was due to the presence of amplification inhibitors (Petrich *et al*., 2006). The MagNa Pure LC system (Roche Diagnostics) has been successfully applied in the detection of enteric viruses from river and wastewater samples from Kenya (Kiulia *et al*., 2010) and in stool samples from paediatric children in SA (Mans *et al*., 2010).

1.6 VIRAL DETECTION AND CHARACTERISATION IN FOOD AND WATER SAMPLES

Viral detection in food and water remains problematic despite the advances made in the field (Koopmans and Duizer, 2004). Historically, EM and EIAs have been applied in screening stool and serum samples respectively to obtain a positive diagnosis of gastroenteritis and hepatitis A- associated viruses (Koopmans and Duizer, 2004; Stals *et al*., 2012). The isolation of viruses through the use of cell culture assays allows for low concentrates of infectious virions to be detected. Despite the technique's high sensitivity (<10 infectious virions can be detected), adequate cell culture systems are not available for all viruses (NoVs and HEV) (Croci *et al*., 2008) while other viruses (HAV) grow too slow for routine detection (Koopmans and Diuzer, 2004; Bosch *et al*., 2011). Molecular amplification of viral genomes lack the constraints of cell culture assays by being able to detect all viruses on a routine basis at a faster pace, increased efficiency and specificity but at a lower detection rate (10-1000 virions) and increased cost (Koopmans and Duizer, 2004; Croci *et al*., 2008; Bosch *et al*., 2011). In the past decade, *rt* PCR have revolutionised the detection of viral contaminants by allowing qualitative and quantitative detection to assess the degree of contamination (Bosch *et al*., 2011).

1.6.1 Viral isolation

Cell culture assays remain the gold standard in the confirmation of viral infectivity (Leland and Ginocchio, 2007). Viruses can be considered infectious when they can penetrate and express at least one viral gene within a susceptible cell (Hamza *et al*., 2011). Cell types that can be used for the isolation of HAdV, HAV, HRV and EV from environmental samples are; buffalo green monkey (BGM) kidney cells, African green monkey kidney (Vero) cells and primary liver carcinoma (PLC/PRF/5) cells to name but a few (Croci *et al*., 2008; Hamza *et al*., 2011). Indeed, no single cell line can be used to propagate all viruses and therefore a combination of cell lines can be used (Hamza *et al*., 2011). Cytopathic effect (CPE) is monitored by microscopic examination with some viruses being able to produce a visible CPE in a single day while others may take up to three weeks. Morphological changes in infected cells such as shrinking, swelling, rounding of cells, syncytium formation, clustering or complete destruction of cells may be noted (Leland and Ginocchio, 2007). Plaque assays and immunofluorescence or neutralisation assays are used in the quantification and typing of viruses' respectively (Bosch *et al*., 2011). The need for persons with cell culture expertise, the long incubation period for some viruses to produce CPE, the lack of some viruses to proliferate in cell culture, the cytotoxicity of compounds in environmental samples and the expense in equipment and maintenance of cell cultures are some of the drawbacks to this age old technique (Leland and Ginocchio, 2007; Hamza *et al*., 2011). Integrated cell culture techniques combine cell culture assays with PCR to detect slow growing and non-CPE producing viruses with the added advantage of being less time consuming and increases the detection sensitivity (Hamza *et al*., 2011)

1.6.2 Viral detection

In the detection of viral contaminants in water, food and environmental samples, various techniques based on difference in viral morphology, antigen and genome sequences have been developed and applied. Methods such as EM, EIA, hybridization assays, PCR and *rt* PCR's role in viral detection will be briefly discussed.

1.6.2.1 Electron microscopy

Electron microscopy remains the gold standard in viral diagnosis since most gastroenteritis viruses do not grow in tissue cultures while for others immunological reagents do not exist (Goldsmith and Miller, 2009). Electron microscopy remains the one diagnostic method that is able to detect all the agents presents when in sufficient numbers (10^5-10^6 virions) (Koopmans and Duizer, 2004; Goldsmith and Miller, 2009). The method is ideal for screening faecal samples for viral contaminants; however, it remains insufficient for the detection of low viral contaminants in food, water and environmental samples (Seymour and Appleton, 2001). In today's day and age, EM remains on the forefront for virus identification, surveillance of emerging diseases and potential bioterrorism agents despite its lack in sensitivity, high maintenance cost and labour-intensiveness (Koopmans and Duizer, 2004; Richards, 2005; Goldsmith and Miller, 2009).

1.6.2.2 Viral antigen detection

Enzyme immunoassays utilises hyper-immune antisera raised against a recombinant capsid of a specific virus and are predominantly type specific, being able to detect only strains of the same or genetically similar genotypes (Jiang *et al*., 1995; Jiang *et al*., 2000). Viral capsid proteins are expressed using baculovirus expression techniques, purified and antisera obtained by immunising rabbits and/or guinea pigs (Hansman *et al*., 2006). Enzyme immunoassays have been developed for several enteric viruses, e.g. NoV, HAstV, HRV, SaV and HAdV with the advantage of rapid results, high specificity and onsite diagnosis in outbreak situations (Koopmans and Duizer, 2004; Hansman *et al*., 2006; Rahouma *et al*., 2011). The method has been shown to be inappropriate for the detection of NoV in sporadic cases as appose to outbreaks with a sensitivity ranging from 31.58 – 46.32% when compared to >2, 000 PCR confirmed cases (Gray *et al*., 2007). With the low titres and diversity of viruses in water, food and environmental samples, EIA techniques are not considered sensitive enough for use (Koopmans and Diuzer, 2004).

1.6.2.3 Molecular-based techniques

Probe hybridization techniques were used for viral contamination analysis from food and water samples prior to the advent of PCR based techniques (Jiang *et al*., 1986; De Leon *et al*., 1992; Atmar *et al*., 1995; Wong and Medrano, 2005). Jiang and colleagues

developed a complementary DNA (cDNA) hybridization assay for the detection of HAV in seeded estuarine samples in 1986 (Jiang *et al*., 1986). A major limitation to this technique was the low sensitivity (10^4 virions), short shelf life of the probe and the radioactive waste (Jiang *et al*., 1986). An advancement made was to amplify the extracted nucleic acid using PCR-based techniques with an oligonucleotide probe hybridization assay to detect and confirm the identity of the amplified fragments (De Leon *et al*., 1992; Atmar *et al*., 1995; Jaykus *et al*., 1996). The probes were end-labelled with, e.g. digoxigen or biotin which could be detected using streptavidin or antibody conjugates and a chemiluminescent or colour reaction (Hoeltke *et al*., 1992). These assays showed increase sensitivity as low concentrations of virions (e.g. 10 PFU of PV and 2000 HAV particles) could be detected; however, PCR inhibition by possible environmental inhibitors remains a recurrent problem (Atmar *et al*., 1995; Bosch *et al*., 2011).

Despite the inability of PCR-based methods to distinguish between infectious and non-infectious viruses, the possibility of false negative test results and the lack of standardised methods, the method remains the gold standard in the detection of viruses in food and water (Bosch *et al*., 2008; Havelaar and Rutjes, 2008; Girones *et al*., 2010; Bosch *et al*., 2011). During PCR, a conserved region of the viral genome is amplified using specific primer pairs. Primers can be design to differentiate between either whole virus orders or a single type of virus or specific viral serotypes, making PCR-based methods very specific and sensitive (Fong and Lipp, 2005; Croci *et al*., 2008). Molecular based analysis of viruses with a RNA genome is made possible by the use of a reverse transcriptase enzyme which converts the RNA into DNA using randomised, polythymine or specific primers, a method known RT-PCR. As with conventional PCR and RT-PCR, the final product is analysed by gel electrophoresis (Rodríguez *et al*., 2009).

Real-time RT-PCR assays utilise internal oligonucleotides, also known as probes that are equipped with a fluorescent marker, relinquishing the need for analysis by gel electrophoresis (Rodríguez *et al*., 2009; Bosch *et al*., 2011). The addition of specific probes into PCR assays that can emit fluorescence and allow the real-time monitoring of the cycles confirms the specificity of the amplicon. Several different probe formats have been designed such as; hybridisation-and hydrolysis probes, molecular beacons,

scorpion primers and locked nucleic acid (LNA) probes. Hybridisation probes utilise a pair of fluorogenic hybridisation oligos whereas molecular beacons uses a single hairpin-shaped oligoprobe terminally labelled with a reporter and a quencher flourophore (Hoffmann *et al*., 2009). Scorpion primers contains a similar design to molecular beacons and are believed to work in a unimolecular manner, probing by an intra-rather than intermolecular manner (Thelwell *et al*., 2000). Locked nucleic acid probes consists of one or more LNA monomers or nucleic acid analogues, used to increase the melting temperature of the probe allowing shorter probe sequences to be used (Vester and Wengel, 2004; Hoffmann *et al*., 2009).

Hydrolysis probes, which consist of dual-flourophore-labelled oligonucleotides have been widely exploited by various authors for the use in *rt* RT-PCR assays (Hoffmann *et al*., 2009; Stals *et al*., 2009; Blaise-Boisseau *et al*., 2010; Wolf *et al*., 2010; Waters *et al*., 2011). During amplification, the probe sequence is cleaved by the 5'exonuclease activity of the DNA polymerase separating the quencher from the reporter and allowing the detection of fluorescence from the flourophore (Hoffmann *et al*., 2009). The addition of a 3' minor groove binding ligand to the probe sequence drastically increases the sequence-specificity and reduces the length of the probe while maintaining the desired melting temperature (De Kok *et al*., 2002; Hoffmann *et al*., 2009). Minor groove binding probes can be used for the detection of single-nucleotide polymorphisms since they are easily destabilised by nucleotide changes within the hybridisation site (Hoffmann *et al*., 2009).

Real-time instruments can differentiate between different fluorescent emissions from each probe, allowing for multiple viral targets to be detected simultaneously in a single reaction, a method known as multiplex *rt* RT-PCR. This method offers a reduction in time, cost and contamination with an increase in speed, sensitivity, reproducibility and specificity over conventional and RT-PCR methods (Rodríguez *et al*., 2009; Bosch *et al*., 2011). In addition, a two-step approached in combination with a multiplex *rt* RT-PCR assay further reduces reagent and labour cost while improving the turnaround time (Svraka *et al*., 2009). A two-step approach, as appose to a one-step approach, separates the RT-reaction from the *rt* PCR assay, allowing several different *rt* PCR assays to be performed from a single cDNA reaction (Wong and Medrano, 2005). In addition, no

difference in the absolute sensitivity between multiplex and singleplex *rt* RT-PCR assay existed (Jansen *et al*., 2011).

Multiplex *rt* RT-PCR assays can and have been used to distinguish between false negative or failed reactions by the incorporation of an amplification and/or process control (further discussed under quality control/quality assurance) (Stals *et al*., 2009; Blaise-Boisseau *et al*., 2010; Wolf *et al*., 2010; Bosch *et al*., 2011). The co-amplification of an amplification control (AC) increases the reliability of the *rt* RT-PCR reaction while validating negative test results (Hoffmann *et al*., 2009).

1.6.3 Viral characterisation

Molecular characterisation plays a vital role in studying the epidemiology of enteric viruses, providing an overview of the circulating or prevalent strains and genotypes in a specific geographical region (Bosch *et al*., 2008; Aw and Gin, 2010; Trang *et al*., 2012). Screening acute gastroenteritis and hepatitis cases using molecular based techniques followed by genotyping and phylogenetic confirmation provides aetiological identification and genetic characterisation of enteric viruses. This contributes to a more comprehensive investigation of the disease burden caused by these viruses (Kittigul *et al*., 2009; Park *et al*., 2009; Sdiri-Loulizi *et al*., 2009; Kokkinos *et al*., 2010; Trang *et al*., 2012). Furthermore, by analysing the viral sequence data obtained from an outbreak and that of the suspected source, it may confirm or increase the suspicion of the item as the source of the outbreak. Typing of the polymerase region of NoV GI.4 confirmed that frozen raspberries was the source of multiple gastroenteritis outbreaks in Finland during the period of 2009 (Sarvikivi *et al*., 2012). In a similar study, NoV GII.4 detected on spinach showed a 99% similarity to the GII.4 strain detected from infant patients in South Korea (Cheong *et al*., 2009). The study, however failed to confirm that irrigation water was the source of contamination but did show that enteric viruses were present in the water; therefore it was considered a potential source of contamination.

1.7 QUALITY CONTROL/QUALITY ASSURANCE

Quality control methods for the detection of enteric viruses necessitate the use of

adequate controls throughout the different steps (Stals *et al*., 2012). Achieving reliable and sensitive results, requires the use of controls to evaluate the extraction efficiency (process control), optimal RT-PCR conditions (amplification controls), contamination of samples and reagents (negative controls) and adequate primer sets (positive controls) (Croci *et al*., 2008; Bosch *et al*., 2011). Bosch *et al*. (2011) further outlines a number of controls that should be considered applicable for such validation of results.

Analytical methods for food and environmental virology remain limited by the quality of the sample processing step due to poor sample quality or untested matrix effects (Mattison and Bidawid, 2009). These factors are overcome by the implementation of a sample process control which is added to the sample, co-extracted and co-concentrated with the target of interest and detected from the same extract. Ideally, a process control should have similarities in size, behaviour and genetic build up, be non-pathogenic to humans, be easily cultivated and detected but should not be normally associated with the sample tested (Baert *et al*., 2011; Mattison *et al*., 2009). The incorporation of such a control will verify that pre-amplification sample treatment was successful and identify samples in which it was not (Diez-Valcarce *et al*., 2011). Feline calicivirus (Mattison *et al*., 2009), Murine norovirus 1 (Stals *et al*., 2011) and mengovirus (Costafreda *et al*., 2006; Da Silva *et al*., 2007) have been used as sample process controls for methods aimed at detecting RNA viruses from food and water samples (Baert *et al*., 2011). Mengovirus has been used (Costafreda *et al*., 2006; Da Silva *et al*., 2007) and been suggested as a process control for the use in shellfish, produce and bottled water by the European Committee for Standardization (Stals *et al*., 2012). Qualitative and quantitative data of the process control can be used to determine and analyse the recovery success rate and the efficiency of recovery, respectively. However, the data cannot be used to analyse the individual efficiencies of the RT and PCR steps and therefore additional controls are required (Stals *et al*., 2011).

The implementation of PCR based methods for the routine analysis of enteric viruses' remains challenging and various obstacles hinder the detection of these viruses (Bosch *et al*., 2011). The European Committee for Standardization in collaboration with the International Standard Organization requires the use of specific controls, including the use of an internal AC (IAC), for the validation of foodborne pathogens by PCR (Anonymous, 2005). According to these standards, an IAC is defined as the addition of

a known copy number of DNA to each reaction which serves as an internal control for amplification. In addition, an external AC (EAC) is identical to an IAC but detected in a separate reaction to that of the target. The need for such controls was required to rule out inhibition, malfunctioning thermal cycler, incorrect PCR mixture, poor DNA polymerase activity and the possible presence of inhibitory substances in a negative test result (Hoorfar *et al*., 2004). As a result of the lack of standardised methods for the development of an IAC, different types of IAC can be used (Hoorfar *et al*., 2004).

Internal AC can occur naturally within the specimen, referred to as an endogenous IAC, or has to be added to the specimen prior to amplification and is referred to as an exogenous IAC (Croci *et al*., 2008). Housekeeping genes have been used as endogenous IAC, targeting a specific organism that naturally occurs in the sample, however their use in water and food is unrealistic due to the different food sources used and unknown composition of water (Jain *et al*., 2006; Croci *et al*., 2008; Bosch *et al*., 2011). To overcome these obstacles, different approaches have been developed on the design and use of an exogenous IAC, especially in deciding whether to use a competitive or non-competitive IAC (Hoorfar *et al*., 2004). Competitive exogenous IAC are non-target DNA or RNA sequences that are amplified with one common set of primers in the same reaction and under the same conditions as the target. In addition, the concentration of the IAC and its size are the two major factors that must be considered to reduce the competition during amplification. Conversely, a PCR containing an IAC should always produce a signal or be detected; unless a competitive exogenous IAC is used where the possibility exists that the IAC can be outcompeted by the target, abolishing any signal production from the IAC (Hoorfar *et al*., 2004). The IAC can be distinguished from the target either by size differentiation or by an IAC specific probe that can be continuously monitored by a *rt* PCR assay (Croci *et al*., 2008; Hoffmann *et al*., 2009). In contrast to an endogenous IAC, an exogenous IAC is amplified using a different set of primers, eliminating the competition between the IAC and target and allowing the IAC amplification to be regulated based on the initial primer concentration. The downside to an exogenous IAC is the lack in amplification accuracy of the primary target that is obtained due to primer sequence differences (Hoorfar *et al*., 2004).

Further controls required by the European Standardization Committee are process, negative and positive controls (Anonymous, 2005). Quality control and quality assurance measures include the use of both positive and negative controls to validate true positive and negative results, excluding any false results respectively (Bosch *et al*., 2011). Negative controls include the following; i) a negative process control (a target pathogen-free sample of the matrix under investigation which is run through all stages of the analytical process), ii) a negative extraction control (a control carried through all steps of nucleic acid extraction procedure in the absence of a test sample) and iii) a negative PCR control, a reaction performed with nuclease-free water without any PCR inhibitors (Anonymous, 2005). Positive controls include a positive PCR control (a reaction containing the target DNA in a defined amount or copy number and could also be replaced by an endogenous IAC) or the use of a positive process control, a sample that has been spiked with the target organism and processed in parallel with the test samples (Hoorfar *et al*., 2004; Anonymous, 2005).

1.8 MOTIVATION FOR THIS INVESTIGATION

The SA production of strawberries was estimated at about 350 hectares producing about 10, 500 tons of strawberries annually (South African Fruit Farms, 2012). With the global trade increasing, outbreaks of gastroenteritis and hepatitis due to the consumption of berry fruits have resulted in numerous outbreaks worldwide (Calder *et al*., 2003; Lysén *et al*., 2009; Sarvikivi *et al*., 2012), yet no standardised method for the routine surveillance of enteric viruses on berry fruit exists in SA. In the last decade, *rt* PCR assays made a remarkable impact in the detection of enteric viruses with the further advent of multiplex *rt* RT-PCR assays. Implementation of multiplex *rt* RT-PCR assays and an optimised method for the recovery and detection of enteric viruses from berry fruits reduces the cost, labour and turnaround time in the surveillance of berry crops for the presence of enteric viruses in SA.

1.8.1 Hypothesis of this investigation

The hypothesis for this study is:

1. In SA berry fruits irrigated with faecal contaminated water or handled and processed under poor hygiene conditions are a potential source of foodborne gastroenteritis or hepatitis;
2. Faecal contaminated irrigation or washing water maybe a possible source of enteric viruses leading to the contamination of fresh produce.

1.9 AIMS OF THIS INVESTIGATION

The aim of this investigation is to develop and apply a cost effective multiplex *rt* RT-PCR assays for the detection of selected enteric viruses on berry fruits and in associated irrigation and processing waters.

1.10 SPECIFIC OBJECTIVES

1. To develop and optimise sensitive multiplex *rt* RT-PCR assays for the simultaneous detection of combinations of selected enteric viruses namely: NoV GI, NoV GII, SaV, HAV, HAstV, HRV and mengovirus;
2. The optimised multiplex assays will be compared to the monoplex assays currently in use to assess the levels of sensitivity and specificity;
3. To optimise methods for the recovery of enteric viruses from berry fruits;
4. To apply the optimised methods to analyse irrigation and processing water and berry fruits for the presence of enteric viruses;
5. To characterise, by sequence analysis, NoVs and HAV isolates from the berry fruits and irrigation or processing water.

CHAPTER 2

AMPLIFICATION CONTROLS FOR THE REAL-TIME RT-PCR ANALYSIS OF NOROVIRUS GII IN WATER AND FOOD

2.1 INTRODUCTION

Noroviruses, formerly called “Norwalk-like viruses” or small round-structured viruses, was first discovered in 1972 during an acute infectious nonbacterial gastroenteritis outbreak in Norwalk, Ohio (Kapikian *et al.*, 1972). Attempts to propagate NoVs in cell culture have been unsuccessful (Duizer *et al*., 2004b; Teunis *et al*., 2008; CDC, 2011). The detection of NoVs in water, food and environmental samples largely relies on the use of molecular-based techniques. Currently, *rt* RT-PCR assays are considered the method of choice for the detection of NoVs, either individually or as part of a multiplex assay (Butot *et al*., 2010; Van Maarseveen *et al*., 2010; Wolf *et al*., 2010). Limitations often experienced by the use of molecular techniques are the presence of low viral titres, sensitivity to inhibitors, possibly resulting in false negative results and the inability to distinguish between infectious and non-infectious viral particles (Rolfe *et al*., 2007; Hoffmann *et al*., 2009; Bosch *et al*., 2011).

Implementation and acceptance of molecular techniques, such as *rt* RT-PCR, for the routine detection of viruses from environmental samples requires adequate quality control measures before declaring samples pathogen free (Hoorfar *et al*., 2003; Pintó and Bosch, 2008; Bosch *et al*., 2011). The use of internal controls (Hoffmann *et al*., 2009; Deer *et al*., 2010) or IAC (Hoorfar *et al*., 2003; 2004; Rip and Gouws, 2009), referred to in this study as an AC, are becoming the more popular choice of control method for the detection of reaction failure due to inhibition (Deer *et al*., 2010; Hata *et al*., 2011; Zahra *et al*., 2011). Amplification controls can be distinguished either as an endogenous AC system, where the AC occurs naturally in the test specimen (Barker *et al*., 2010; Amer and Almajhdi. 2011), or an exogenous AC system, where the AC has to be added to the reaction (Oikonomou *et al*., 2008; Deer *et al*., 2010; Kirchner *et al*., 2010). The last mentioned AC can be added to the sample before nucleic acid extraction, such as the use of encapsulated RNA (Pintó and Bosch. 2008) and may

therefore require the addition of an extra primer pair (non-competitive AC) (Hoorfar *et al*., 2004). Exogenous AC can also be engineered to contain identical target sequences as that of the target (competitive AC) (Hoorfar *et al*., 2004; Rip and Gouws, 2009) which can be added to the reaction mix prior to the amplification step (Hoffmann *et al*., 2009). Detection and or quantification of the AC can be done in a single reaction along with the target or in a separate reaction to that of the target (Costafreda *et al*., 2006).

For the purpose of this study a competitive exogenous AC was developed for NoV GII for the detection of false negative test results. Furthermore, the AC will be applied as either an exogenous AC (EAC, where NoV GII and the EAC will be amplified and detected in separate identical singleplex one-step *rt* RT-PCR) or as an internal AC (IAC, where NoV GII and the IAC will be amplified and detected in a duplex one-step *rt* RT-PCR). The aim of this study was to develop and determine the accuracy of an AC, applied as either an EAC or IAC, in a duplex *rt* RT-PCR assay for the detection of NoV GII in food and water samples.

2.2 MATERIALS AND METHODS

2.2.1 Viral and nucleic acid stocks

***Ureaplasma parvum*:** The nucleic acid was obtained from clinical specimens kindly provided by Dr. E. Muller of the National Institute for Communicable Disease, Sandringham, Johannesburg.

***Norovirus GII*:** The NoV GII stock (2.85 x 10^{10} copies/mℓ) originated from a clinical strain detected in the stool specimen from a patient after an outbreak of gastroenteritis on a cruise ship.

***Mengovirus*:** The virus was kindly provided by Prof A Bosch of the Department of Microbiology, Faculty of Biology, University of Barcelona, Barcelona, Spain. The virus was further propagated and titrated in the Vero African green monkey kidney cell line (1.3 x 10^6 $TCID_{50}$/mℓ) and used in this study as a process control.

2.2.2 Oligonucleotide primers and probes

The primers and probes used in this study are listed in Table 2.1. The forward

oligonucleotide primer (gg2UpIACF2) was designed to contain the forward primer sequences of NoV GII and *U. parvum* as well as an SP6 RNA polymerase promoter sequence. The reverse oligonucleotide (gg2UpIACR) was designed to contain the reverse NoV GII and *U. parvum* set of primer sequences. The molecular beacon FP6 was made into an LNA probe and labelled with a HEX fluorophore.

2.2.3 Acquisition of the internal non-target DNA

U. parvum DNA was used as a template for the internal non-target DNA. Three samples of DNA isolated from *Ureaplasma* cultures obtained from clinical specimens were screened for the presence of *U. parvum* sequences in a PCR with primers UPF and UPR. The reaction was performed using an EXPRESS qPCR SuperMix Universal kit (Invitrogen, Carlsbad, California [CA]); containing 0.5 µM of each primer and 5 µl of template DNA solution. The PCR reaction was amplified in a LightCycler® 2.0 instrument (Roche Diagnostics GmbH, Mannheim, Germany), using the following cycling conditions: an initial denaturation and enzyme activation step of 95°C for 2 min was followed by 40 cycles of 94°C for 15 sec, 52°C for 1 min, and 65°C for 1 min, with a final extension step of 65°C for 5 min and cooling step of 40°C for 1 min. The amplified PCR fragments were analysed by 2% agarose gel (SeaKem® LE Agarose, Lonza, Rockland, NY) electrophoresis and visualised by ethidium bromide (EtBr) staining and UV illumination using a 100 base pair (bp) marker (O'GeneRuler, Fermentas Life Sciences, Burlington, Ontario, Canada) to determine the amplicon size. The amplified PCR fragments were purified using the DNA Clean and Concentrator™-25 kit (Zymo Research Corporation [Corp.], Orange, CA) according to manufacturer's recommendations and used in a second round of amplification with the primer pair designed for this study (Table 2.1). The reactions conditions of both rounds of PCR were identical to that described above. The amplified PCR fragment size was determined relative to a 100 bp marker (Fermentas Life Sciences, Burlington, Ontario, Canada).

2.2.4 Construction of the AC amplicon

The AC was constructed following the principles of the method of Rodriguez-Lazaro *et al*. (2004), but using the bacteriophage SP6 RNA polymerase promoter sequence

Table 2.1 Nucleic acid sequences of the primers and probes used in this study

Name	Type	Sequence	Reference
QNIF2	Norovirus GII forward primer	5'-ATGTTCAGRTGGATGAGRTTCTCWGA-3'	Loisy *et al*. (2005).
COG2R	Norovirus GII reverse primer	5'-TCGACGCCATCTTCATTCACA-3'	Kageyama *et al*. (2003).
QNIFS	Norovirus probe	5'-FAM-AGCACGTGGGAGGGCGATCG- TAMRA-3'	Loisy *et al*. (2005).
Gg2UpIACF2	AC forward primer	5'-ATTTAGGTGACACTATAGGATGTTCAGATGGATGAGATTCTCTG AGTATTTGCAATCTTTATATGTTTTCG-3'	This study
gg2UpIACR	AC reverse primer 1	5'-TCGACGCCATCTTCATTCACATCCAGCTCCAACTAAGGTAAC-3'	This study
FP6	*Ureaplasma parvum* probe (serovar 6)	5'-HEX-AGTGTTCATATTTTTTACTAG-BBQ-3'	Cao *et al*. (2007).
UP F	*Ureaplasma parvum* forward primer	5'-GTATTTGCAATCTTTATATGTTTTCG-3'	Cao *et al*. (2007).
UP R	*Ureaplasma parvum* reverse primer	5'-TCCAGCTCCAACTAAGGTAAC-3'	Cao *et al*. (2007).

(Brown *et al*., 1986). *U. parvum* DNA fragments (refer section 2.2.3) were amplified, by PCR, using the IAC primer pair gg2IACF1 and gg2IACR in an EXPRESS qPCR SuperMix Universal kit (Invitrogen). A 0.5 µM concentration of each primer pair were used and the reaction was performed on a Px2 thermal cycler (Thermo Electron Corp., Waltham, Massachusetts [MA]) using the following cycling conditions: 50°C for 2 min, 95°C for 2 min, followed by 50 cycles of 94°C for 15 sec, 52°C for 1 min, and 65°C for 1 min, followed by a final cooling step of 40°C for 1 min. The amplified PCR fragments were analysed by 2% agarose gel (SeaKem® LE Agarose) electrophoresis and visualised by EtBr staining and UV illumination. The size of the fragments was determined using a 100 bp marker (O'GeneRuler). The amplified PCR fragments were purified using a DNA Clean and Concentrator™-25 kit (Zymo Research Corp.) and stored at -20^{o}C.

2.2.5 Cloning of an exogenous AC

The AC DNA amplicons (section 2.2.4) were ligated into the pGEM®-T Easy Vector System (Promega Corp. Madison, Wisconsin [WI]) according to the manufacturer's recommendations with the exception that the ligation reaction was incubated overnight at 4°C using the T4 DNA ligase (Promega Corp.). *Escherichia coli* competent cells (20 microliter [µℓ]) (Lucigen® Corp., Middleton, WI) were transformed with four microliters of the ligation reaction following the manufacturer's recommendations. The *E. coli* solution was then plated on Luria-Bertani (LB) enriched agar containing 100 µg/mℓ ampicillin (Sigma-Aldrich Company [Co.], St. Louis, Missouri [MO]) and incubated overnight at 37°C. Transformed colonies were randomly selected and screened for the specific insert by PCR amplification using GoTaq® Flexi DNA Polymerase (5 U/µℓ) (Promega Corp.), (deoxynucleotide triphosphates) dNTPs (2.5 mM) (Roche Diagnostics), NoV GII primers (4 micromole (µM) each). The reaction was amplified using a Px2 thermal cycler (Thermo Electron Corp.) with the following cycling conditions: 95°C for 3 min followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min with a final extension step of 72°C for 7 min with a final holding step of 4°C. The amplified PCR fragments were analysed by 2% agarose gel (SeaKem® LE Agarose) electrophoresis. A recombinant colony containing the approximate sized fragment (282 bp) was isolated and propagated at 37°C overnight in 100 mℓ LB media with 100 µg/mℓ ampicillin (Sigma-Aldrich Co.). Nucleic acid was

extracted using the PureYield™Plasmid Midiprep System 25 preps (Promega Corp.), according to the manufacturer's recommendation, and along with the remainder of the *E. coli* suspension was stored at -70°C

2.2.6 Sequence validation of the exogenous AC

The amplified PCR AC fragments (section 2.2.4) were sequenced with the NoV GII primers (Table 2.1) using the ABI PRISM BigDye® Terminator v3.1 Cycle Sequencing Kit on a Px2 thermal cycler (Thermo Electron Corp.). The cycling conditions used were as follows: 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec, 50°C for 10 sec, and 60°C for 4 min, followed by a final holding step of 4°C. The amplified PCR fragments were precipitated as follows: 2 µℓ of ethylenediaminetetraacetic acid (Merck, Darmstadt, Germany) and sodium acetate (Merck) pH 5.2 was added to the PCR product followed by an additional 50 µℓ of molecular grade absolute ethanol (EtOH) (Merck). The samples were incubated overnight at -20°C and centrifuged for 20 min at 14 000 rpm at 4°C using an Eppendorf 5402 benchtop refrigerated centrifuge (DJB Labcare Ltd, Buckinghamshire, England). One hundred microliters of 70% EtOH (Merck) was added and centrifuged again for 10 min as mentioned above. The supernatant was removed and the samples dried at 95°C for 3 min in the Px2 thermal cycler (Thermo Electron Corp.) and stored at -20°C. The nucleotide sequencing reaction was run on an ABI 3130 automated analyser (Applied Biosystems, Foster City, CA). The software MEGA version 4 (Tamura *et al*., 2007) was used for the analysis of the sequences.

2.2.7 In vitro transcription of the AC

To apply the AC more effectively in the detection of PCR inhibitors, the AC was transcribed into RNA so as to validate the efficiency of the cDNA synthesis and PCR amplification steps. The AC DNA fragments (section 2.2.4) were transcribed to RNA using the Riboprobe® in vitro Transcription Systems kit (Promega Corp.) according to the manufacturer's recommendations with the following modifications. The reaction volume was doubled and the incubation time extended to 90 min for optimal RNA synthesis. A single ten-fold dilution of the newly synthesised RNA suspensions was made to which 110 units (U) DNase (Promega Corp.) was added and incubated at 37°C

overnight. The presence of contaminating DNA was determined by PCR amplification on a Px2 thermal cycler (Thermo Electron Corp.) according to the protocol described in section 2.2.5. Once the remainder of DNA was successfully removed, the total RNA was quantified with the Quant-iT™ RiboGreen® RNA Assay Kit (Invitrogen) on an ABI 7300 real-time PCR system (Applied Biosystems) according to the manufacturer's recommendations. The concentration of the AC RNA fragments was determined as described by Waters *et al*. (2011).

2.2.8 Determination of competition between the AC and NoV GII

In order to reduce the possible competition during the simultaneous amplification of the AC- and NoV GII fragments, the lowest detectable concentration of the AC was determined by agarose gel (SeaKem® LE Agarose) electrophoresis. A ten-fold serial dilution series of the AC RNA was prepared in diethylpyrocarbonate-treated water (Fermentas Life Sciences) and amplified in duplicate using the Quantitect® Probe RT-PCR kit (Qiagen, Hilden, Germany) with the NoV GII primer set (4 µM) according to the manufacturers recommendations. The LightCycler® v2.0 platform (Roche Diagnostics) was used with the following cycling conditions: 50°C for 60 min followed by an activation step of 95°C for 15 min, followed by 50 cycles of 94°C for 15 sec, 60°C for 1 min, and 65°C for 1 min, with a cooling step of 40°C for 1 min. The amplified PCR fragments were analysed by 2% agarose gel (SeaKem® LE Agarose) electrophoresis and the lowest detectable concentration determined.

Five independent serial ten-fold dilution series of NoV GII were made and the mean Ct value at each of the ten-fold dilution factors (10^{-1} - 10^{-5}) from each of the five dilution series were determined to evaluate the possible competition between the AC and NoV GII during *rt* RT-PCR analysis. The lowest detectable concentration of the AC was used. The Quantitect® Probe RT-PCR kit (Qiagen) was used with a primer and probe concentration of 4 µM and 8 µM respectively. The LightCycler® v2.0 platform (Roche Diagnostics) was used with the cycling conditions described in the above paragraph. The singleplex *rt* RT-PCR assay was optimised to a duplex *rt* RT-PCR assay for the simultaneous detection of the AC and NoV GII by adding 2 µℓ of the AC RNA and 1 µℓ of the AC probe (8 µM) to the reaction mix. Amplification was identical to that of the above singleplex *rt* RT-PCR assay, with the AC being detected in the 560 nm

channel (HEX) and NoV GII in the 530 nm channel (FAM) of the LightCycler® v2.0 platform. The Ct-values generated by the LightCycler® v2.0 (Roche Diagnostics) was used for statistical evaluation.

2.2.9 Evaluation of the sensitivity of NoV GII and mengovirus *rt* RT-PCR assays to possible inhibitors

In order to evaluate the sensitivity of NoV GII and mengovirus *rt* RT-PCR assays, five river and two drinking water samples, previously identified to contain possible PCR inhibitors were used. The waters were submitted for nucleic acid extraction prior to the artificial contamination of 12 µℓ of the extracted nucleic acid of each of the waters with 1.5 µℓ of previously extracted genomic RNA of NoV GII (4.6×10^5 copies/mℓ) and mengovirus (6.5×10^3 tissue culture infectious dose 50 ($TCID_{50}$)/mℓ) respectively. Five microliters of the artificially contaminated nucleic acid was used in one-step *rt* RT-PCR assays (Quantitect® Probe RT-PCR kit [Qiagen]) for the individual detection of NoV GII and mengovirus individually. The LightCycler® v2.0 platform (Roche Diagnostics) was used with the cycling conditions described in section 2.2.8. In the event that either NoV GII or mengovirus could not be amplified or detected in a reaction, a single ten-fold dilution of the artificially contaminated nucleic acid was made and the analysis repeated.

2.2.10 Application of the AC in environmental samples

Environmental samples consisting of water and vegetable samples were used for the use of the AC in the detection of false negative test results. The samples were analysed for the presence of NoV GII with an AC present in each *rt* RT-PCR assay. Two methods were implemented for the addition of the AC in the PCR reaction. In the first method, the IAC was added directly to the *rt* RT-PCR reaction master mix and simultaneously amplified and detected along with NoV GII. In the second method the EAC was added individually to each *rt* RT-PCR reaction mix and were amplified and detected in a separate reaction to that of NoV GII.

Briefly, nucleic acid was extracted from previously processed water samples (10 river, five irrigation and five drinking water samples) using the MagNa Pure LC Total Nucleic

Acid Isolation Kit (large volume) (Roche Diagnostics) on an automated MagNa Pure LC platform (Roche Diagnostics) according to the manufacturer's recommendations. The extracted nucleic acid suspensions were ten-fold diluted to a dilution factor of 10^{-2} of which all dilutions (10^{0} to 10^{-2}) were screened for the presence of NoV GII. The sensitivity of each of the ACs to the presence of NoV GII in a water sample were determined by artificially contaminating a 100 mℓ aliquot of each of the irrigation water samples with a 100 µℓ of NoV GII (1.4 x 10^{9} copies/mℓ). The contaminated water samples were concentrated using a modified PEG_{6000} (Merck)/NaCl (Merck) precipitation method described by Vilaginès *et al*. (1997) as modified by Minor (1985) and re-suspended in 10 mℓ phosphate buffered saline (PBS) (Sigma-Aldrich Co.). From the suspension 1 mℓ was used for nucleic acid extraction as described earlier. A duplex one-step assay was used for the screening of NoV GII and the IAC whereas two identical singleplex one-step *rt* RT-PCR assays were used for the separate detection of NoV GII and the EAC. The reactions were set up using the Quantitect® Probe RT-PCR kit (Qiagen) according to the manufacturer's recommendations with a primer and probe concentration of 4 µM and 8 µM respectively. The reactions were amplified on a LightCycler® v2.0 platform (Roche Diagnostics) using the cycling conditions described in section 2.2.8.

For the application of the AC for vegetable samples, five tomatoes were each seeded with 100 µℓ and 10 µℓ aliquots of the artificially contaminated irrigation water and mengovirus (1.5 x 10^{7} $TCID_{50}$/mℓ), respectively. For this study, two controls were introduced, a positive control where an additional tomato was seeded with a 10 µℓ aliquot of NoV GII (2.85 x 10^{10} copies/mℓ) and the mengovirus (1.5 x 10^{7} $TCID_{50}$/mℓ) and a negative control with a tomato that was not seeded. The tomato samples were washed and processed further following the protocol outlined by Netshikweta (2012). One millilitre of the recovered viral suspension (2 mℓ) were used for nucleic acid extraction as described above and screened, in the same manner as the water samples, for the presence of NoV GII and mengovirus

2.2.11 Statistical analysis

Analysis of the viral presence rate in the samples tested in combination with the IAC and EAC was carried out using Fisher's exact test using StatView software (ver. 5.0;

SAS Institute Incorporated, Cary, North Carolina [NC], US). A 95% confidence interval was used with *P*-values exceeding 0.05 considered as not significant.

2.3 RESULTS

2.3.1 Acquisition of an exogenous AC

Amplification of the *U. parvum* DNA resulted in the detection of a ~216 bp PCR fragment (Figure 2.1A). A ~282 bp AC fragment was obtained after individual screening of randomly selected recombinant *E. coli* colonies (Figure 2.1B).

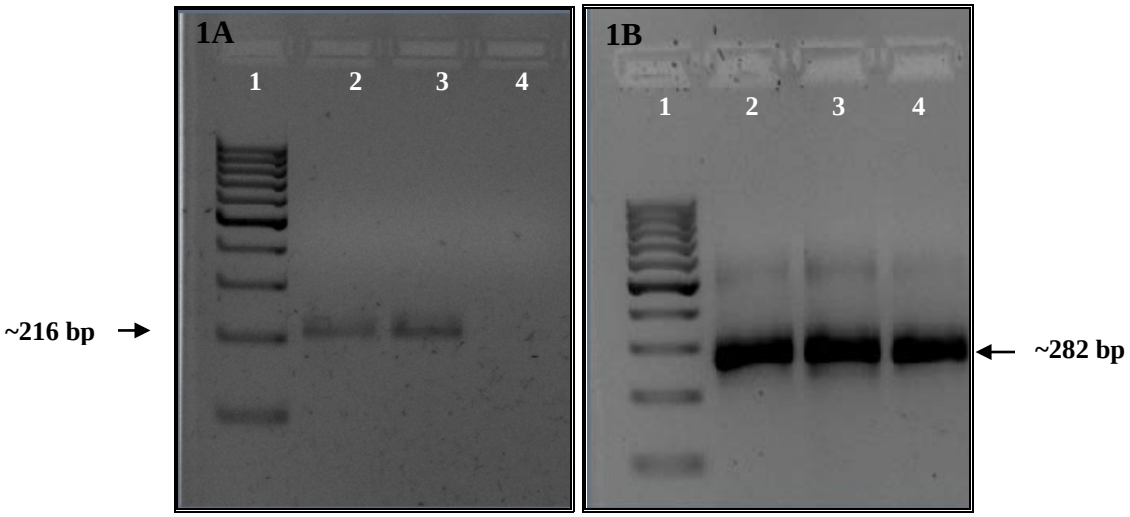

Figure 2.1: Agarose gel picture of the *U. parvum* DNA fragment from the first round of amplification (1A) and from the cloned DNA fragment after the second round of amplification (1B). Internal region amplicons of the MBA gene region of *U. parvum* are seen in lanes 2 and 3 (1A). Cloned amplification control fragments amplified from the recombinant *E. coli* hosts with the gg2UpIACF2 and gg2UpIACR primers are seen in lanes 2, 3 and 4 (1B). A 100 bp molecular marker is present in lane 1.

The concentration of the transcribed AC RNA was determined to be 2.1 x 10^{14} copies/mℓ. The lowest detectable concentration was calculated as 4 x 10^{6} copies/mℓ or 4 x 10^{2} copies/PCR reaction (5 µℓ).

2.3.2 Determination of competition between the AC and NoV GII

The crossing threshold (Ct) values obtained from the individual dilutions was used in

determining the effect that the AC might have on the amplification and thus detection of NoV GII. The data was summarised in Table 2.2 and used for statistical analyses. The data indicated that at high concentrations of NoV GII (concentrations of 10^4 copies/reaction and more) the AC was not detectable, however, in the absence or at lower concentrations of NoV GII (concentrations of 10^3 copies/reaction and lower) it was possible to amplify and detect the AC (Table 2.2).

Furthermore, the AC was only amplified when NoV GII was either not detected in the reaction or present in very low, almost undetectable concentrations. Based on statistical analysis, the AC had no inhibitory effect on the detection of NoV GII although the presence of the AC slightly improved the detection of NoV GII. This was however, not supported statistically. The data was not without variation but this was more prominent between the five individual dilution series than between the dilution factors where the AC was detected or not detected.

2.3.3 Statistical evaluation of NoV GII dilutions with or without IAC present

Analysis of the data, excluding all the replicates that were not detected, with a probability (p) ≤ 0.05 was considered significant. Differences were found between the series (with or without AC), however, significant differences ($p = 0.0336$) were observed between “with” versus “without” as well as differences between dilutions. Furthermore, no evidence existed to show that these differences were due to the presence of the AC ($p = 0.1835$) [Figure 2.2].

2.3.4 Application of the AC in environmental samples

The results of two application methods developed for the AC in the evaluation of false negative test results were summarised in Table 2.3. All five of the artificially contaminated irrigation water samples tested positive for NoV GII and negative for both the IAC and EAC (Table 2.3). Serial ten-fold dilutions of water samples which tested negative for the ACs were made and the analysis repeated. False negative test results were found in 70% of the river water samples tested with the IAC and 90% tested with the EAC upon the first round of screening (Table 2.3). Subsequent dilutions reduce the

Table 2.2: Summary of results showing the effect of the internal (IAC) - and external amplification control (EAC) on the detection and amplification of NoV GII in a duplex one-step *rt* RT-PCR assay.

Dilution series	NoV GII Copies/5 µℓ	Dilution factor	Without IAC (Ct)	With IAC (Ct)	IAC Detected/ Not detected	IAC (Ct)
1	3.326E7	1/10	25.89	25.35	Not detected	-
	3.326E6	1/100	30.05	29.91	Not detected	-
	3.326E5	1/1000	34.09	33.54	Not detected	-
	3.326E4	1/10000	36.22	36.15	Not detected	-
	3.326E3	1/100000	36.45	36.65	Detected	33.84
2	3.326E7	1/10	24.97	25.26	Not detected	-
	3.326E6	1/100	29.14	29.44	Not detected	-
	3.326E5	1/1000	32.86	32.63	Not detected	-
	3.326E4	1/10000	36.08	34.75	Not detected	-
	3.326E3	1/100000	-	36.37	Detected	32.63
3	3.326E7	1/10	25.37	25.88	Not detected	-
	3.326E6	1/100	29.87	29.91	Not detected	-
	3.326E5	1/1000	34.01	33.52	Not detected	-
	3.326E4	1/10000	35.27	35.97	Detected	32.73
	3.326E3	1/100000	39.09	-	Detected	33.28
4	3.326E7	1/10	24.95	25.02	Not detected	-
	3.326E6	1/100	29.67	29.99	Not detected	-
	3.326E5	1/1000	32.59	31.82	Not detected	-
	3.326E4	1/10000	34.78	34.08	Not detected	-
	3.326E3	1/100000	-	-	Detected	33.23
5	3.326E7	1/10	25.46	25.74	Not detected	-
	3.326E6	1/100	28.31	28.27	Not detected	-
	3.326E5	1/1000	31.12	31.44	Not detected	-
	3.326E4	1/10000	35.31	33.79	Not detected	-
	3.326E3	1/100000	37.83	35.30	Not detected	-

Ct = Crossing threshold

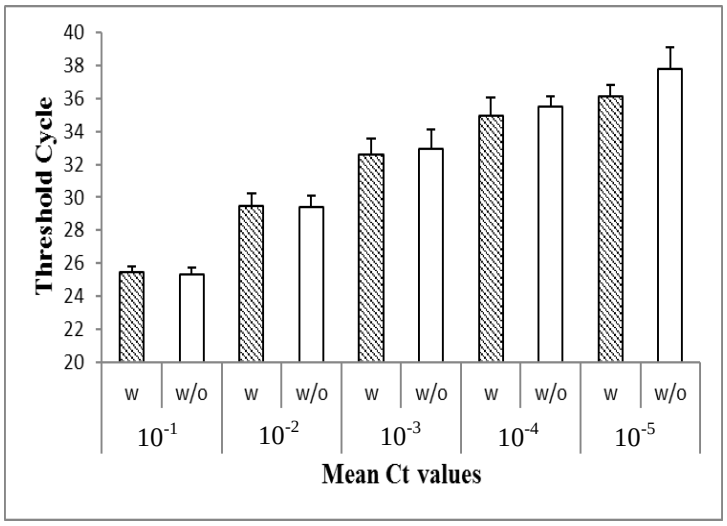

Figure 2.2: Summary of the mean Ct values of NoV GII at each dilution with (w) and without (w/o) the presence of the AC in the PCR reaction. The error bars indicate the standard deviation between the meant Ct value and the individual Ct values.

number of false negative tests; however, 20% of the IAC and 50% of the EAC river water samples remained inconclusive (Table 2.3). Further dilutions for these water samples were not done and reasons for this will be addressed in the discussion below. All false negative test results were resolved for the drinking water samples with the IAC method, however, 40% of the drinking water samples tested with the EAC method remained inconclusive (Table 2.3). A major limitation of the EAC is the inability to distinguish between a false negative test result, where NoV GII was present in the reaction and masked the EACs detection, and true negative test result due to the presence of inhibitory samples. Gel electrophoresis could resolve this problem; however the aim of the investigation was to determine, by using *rt* RT-PCR assays,

Table 2.3: Results of the comparison of an internal (IAC) - and external amplification control (EAC) using different water samples.

Samples (No.)	**NoV GII / IAC**			**Total**	**NoV GII / EAC**			**Total**
	1	**1/10**	**1/100**		**1**	**1/10**	**1/100**	
Irrigation water (5)	5/0	ND	ND	5/0	5/0	ND	ND	5/0
River water (10)	2/1	1/0	0/4	3/5	1/0	1/1	2/0	4/1
Drinking water (5)	0/3	0/0	0/2	0/5	0/2	0/1	0/0	0/3

ND = not done, No. = number

which method, either the IAC or EAC, would be more efficient as an indicator for false negative test results.

Analysis of the artificially contaminated river and drinking water samples that showed to contain inhibitory substances (Table 2.4) tested negative for NoV GII upon first round of analysis but improved by 60% and 50%, respectively, upon ten-fold dilution.

Table 2.4: Summary of the assessment of NoV GII and the mengovirus (process control) to inhibitors by artificially contaminating river and drinking water samples respectively.

Samples (No.)	**NoV GII**		**Total**	**Mengovirus**		**Total**
	1	**1/10**		**1**	**1/10**	
River water (5)	0	3	3	4	1	5
Drinking water (2)	0	1	1	2	0	2

No. = number

Furthermore, 80% and 100% of the river and drinking water samples, respectively, tested positive for mengovirus yet negative for NoV GII upon first round analysis. Mengovirus was therefore less sensitive to the presence of inhibitory substances than NoV GII, giving a false indication of the presence of inhibitory substances in the sample as appose to the IAC and possible the EAC. Similar results were obtained with the tomato samples where a 100% of the samples tested positive for mengovirus, 60% and 40% of the tomatoes tested positive for NoV GII and the IAC, respectively, and 60% of the samples testing positive for the EAC only (Table 2.5).

In two instances both the IAC and EAC were detected but not NoV GII. In another instance NoV GII were detected with the IAC method whereas NoV GII and the AC could not be detected with the EAC method (results not shown). It remains unclear why two tomato samples would test negative for NoV GII and positive for both ACs and mengovirus when the tomatoes were seeded with NoV GII, however, the possibility remains that NoV GII were lost or present in too a concentration.

Table 2.5: Summary of the detection of NoV GII and mengovirus (process control) on seeded tomato samples with the internal (IAC) - and external amplification control (EAC) detection methods respectively.

Samples (No.)	NoV GII / IAC	NoV GII / EAC	Mengovirus
Tomato (5)	3/2	0/3	5
Positive control (1)	1/0	1/0	1
Negative control (1)	0/0	0/1	0

No. = number

2.4 DISCUSSION

In this study a competitive AC was developed for the control of false negative *rt* RT-PCR results for the detection of NoV GII. Following the guidelines by Rodríguez-Lázaro *et al*, (2004), the AC was designed as a single-stranded RNA fragment flanked by the targets primers, in this case NoV GII, and contains a separate molecular beacon hybridisation site to that of the target. Since the AC utilised the same primer set as the target, a working concentration that resulted in the least amount of inhibition to the amplification and detection of the target but remained detectable by gel electrophoresis was determined.

The competition the AC imposed on the target or vice versa was determined by comparing the presence and absence of the AC in five independent serial ten-fold dilution series of NoV GII. Statistical analysis indicated that there was no significant difference when the AC was present or absent (Figure 2.2). However, detection of the AC was only seen when NoV GII could not be detected or present in lower, almost undetectable concentrations. A study by Oikonomou *et al*. (2008) found similar results with an AC that was larger than the amplified product. During the amplification of the AC fragment the reaction kinetics favoured the amplification of the smaller target (274 bp) rather than the larger AC fragment (3196 bp) (Oikonomou *et al*., 2008). In this study a size difference of 174 bp between the 262 bp AC fragment and the 88 bp NoV GII PCR product was introduced with similar results.

The study also evaluated two methods, the IAC method and EAC method that have been used in the application of the AC in the indication of inhibitory substances. In samples where inhibition of the *rt* RT-PCR assay was detected due to the lack of amplification of either NoV GII or the ACs, a ten-fold serial dilution of the samples were made and the analysis repeated. However, caution must be taken when working with environmental samples due to the presence of low viral titres in these samples (Katayama *et al*, 2002). The use of dilutions works well for the removal of inhibitory substance (Albinana-Gimenez *et al*., 2006), however, this also decreases the concentration of the RNA present and lowers the probability of detecting the target. For the evaluation of the two methods, *rt* RT-PCR assays were developed and water samples were screened for the presence of NoV GII simultaneously with the IAC or in a separate reaction for the EAC method. In the seeded water samples (Table 2.3), NoV GII was found in all the samples leaving both the ACs undetectable. However, in the river water samples, 20% of the samples remained negative in all of the dilutions when tested with the IAC method but were positive for NoV GII by the EAC method. The detection of NoV GII in these samples varied upon the method applied with only two samples positive for NoV GII by both methods. Such results can be contributed to the lack of reproducibility of the RT-PCR, the variable quality of the RNA or the interference of background nucleic acids. Noticeable improvements in the detection of the IAC, EAC and NoV GII in the majority of the samples were detected when subjected to a ten-fold dilution. Samples found to be negative for NoV GII but IAC positive at a dilution of 10^{-2}, raised the question that, can an IAC positive result at such a low dilution be considered a true indication of the absence of the target or not and what risk does this have to the consumer?

A lack of reproducibility and variability observed in RT-PCR experiments can be attributed to the inconsistent conversion of RNA to cDNA (Bustin *et al*, 2005). Therefore, a reaction can be presumed negative whereas the possibility remains that the reaction may in actual fact be positive. When using the EAC method the possibility exists that the results may vary between the separate reactions. In the case of an EAC positive test result, the reaction might be considered as a true negative test result free of inhibitors and the target unless a target positive test result is obtained in the other reaction in which case the reaction will be considered positive. However, if a negative test result is obtained for both the EAC and target in their separate reactions, the

question arises if indeed this result may be considered as a false negative or a target positive. Initially such test results will indicate the presence of an inhibitor and be presumed to be a false negative. However, taking into account that the AC was designed to be detectable in the absence or at very low concentrations of the target and the variability of the cDNA synthesis, the possibility arises that amplification of the target may have been present in the EAC reaction and absent in the target reaction thus resulting in a false negative tests result. To prevent this, test can be performed in triplicate; however, cost and time will be the main limitations making it an impossible task for routine surveillance.

Using five river and two drinking water samples an assessment was made to determine the sensitivity and to what extent NoV GII and mengovirus are affected by the presence of possible inhibitory substances in the samples. Upon initial testing of the waters, NoV GII could not be amplified in the water samples whereas 80% of the river- and a 100% of the drinking water samples tested positive for mengovirus. A ten-fold dilution of these samples resulted in 60% of the waters testing positive for NoV GII and a 100% detection rate for mengovirus (Table 2.4). The remainder of the river water samples (40%) were clear false negative test results even though these samples tested positive for mengovirus. Analysis of the seeded mengovirus Ct values indicated a 1 – 2 $\log_{10}$ (3 cycles) difference when compared to the concentrated Ct value of the positive control. Although mengovirus indicated the presence of inhibitory substances, detection of NoV GII were completely impeded and only slightly for mengovirus. Consequently, mengovirus serves as a poor control for the indication of inhibitory substance in a sample and therefore the detection of mengovirus in a sample does not guarantee the detection of NoV GII.

In addition, the detection of viruses from food samples is a major problem due to the presence of inhibitory substance in these items. In Sweden, over 400 workers became ill with gastroenteritis from the consumption of contaminated tomatoes after being handled by infected food handlers (Zomer *et al*, 2010). In this study, tomatoes which had been artificially seeded with NoV GII and mengovirus were used to compare the IAC and EAC. The results showed that with the IAC method, 60% of the samples tested positive for NoV GII and 40% for the IAC whereas with the EAC method, NoV GII could not be detected in any of the samples with 60% of the samples testing positive

for the EAC (Table 2.5). False negative results were not excluded by the EAC method with 40% of the samples testing positive for both the ACs. Clearly NoV GII was absent in these samples although all the samples tested positive for mengovirus. Furthermore, inhibitors present in the negative control for the IAC method completely inhibited the detection of the IAC; however, a very weak Ct value (40.67) was detected for the EAC.

2.5 CONCLUSION

In conclusion, an AC was developed in this study that did not impede the detection of NoV GII and was only amplified when NoV GII were not detected or present in low, almost undetectable concentrations, and in the absence of inhibiting substances. The application of the IAC method overall was more efficient in detecting samples containing inhibitory substances and was overall more cost effective and less time consuming when compared to the EAC method. In contrast to the detection of mengovirus, the IAC showed to have equal sensitivity to inhibiting substances as NoV GII and therefore serves as a better indicator of inhibiting substance in a sample.

CHAPTER 3

OPTIMISATION OF MULTIPLEX REAL-TIME REVERSE TRANSCRIPTASE-PCR ASSAYS FOR THE SIMULTANEOUS DETECTION OF SELECTED ENTERIC VIRUSES

3.1 INTRODUCTION

Enteric viruses have been recognised as a cause of water- and foodborne diseases worldwide (Carter, 2005; Ikner *et al*., 2012). In view of the global increase of diseases due to viral contamination of food and water matrices, the European Committee of Standardisation aimed to establish standardised methods for the detection of viruses from these matrices (Bosch *et al*., 2011). Currently, viral detection relies on: i) the isolation of viruses using appropriate cell culture assays, and/or ii) the use of molecular-based techniques. Cell culture-based assays have been implemented to isolate infectious viral agents, but with the lack of efficient cell culture systems for all food- and waterborne enteric viruses, detection of viral genomes by molecular-based techniques have proven to be an alternative method for the detection of viruses in food and water (Bosch *et al*., 2011).

Advancements made over the past decade in molecular biology include the development of rapid, sensitive and reproducible *rt* RT-PCR assays for the detection of viruses (Bosch *et al*., 2011). These assays apply highly specific fluorescent probes to detect the amplified molecular markers through the increase in fluorescence emitted by each probe (Hoffmann *et al*., 2009). This method has been proposed to be the gold standard for viral quantification and the method of choice for the detection of enteric viruses in the environment (El-Sensousy *et al*., 2007; Rodríguez-Lázaro *et al*., 2012). Using the ability of real-time platforms to differentiate between individual fluorophores, multiple targets can simultaneously be detected and distinguished from each other by the use of fluorescent probes fluorescing at different wavelengths, also known as multiplex *rt* RT-PCR assays (Rodríguez *et al*., 2009; Bosch *et al*., 2011). A major advancement in using these assays is the reduction in cost, turnaround time and increased sensitivity and specificity (Coiras *et al*., 2004).

The aim of this study was to develop individual multiplex assays for the detection of selected enteric viruses known to be present on food matrices and in water sources. Previous singleplex assays have proven to be reliable and highly sensitive but are costly, time consuming and laborious. Using these assays as a basis, multiplex *rt* RT-PCR assays for combinations of NoV GI, NoV GII, HAV, SaV, HRV and HAstV were proposed to reduce the cost, labour intensity and improve the turnaround time. The introduction of an IAC for NoV GII and a process control (mengovirus) for the validation of the recovery and extraction efficiency in the multiplex *rt* RT-PCR assays will be a further improvement on the singleplex assays.

3.2 MATERIALS AND METHODS

3.2.1 Viral stock

***Hepatitis A virus*:** The cell culture-adapted strain HM-175 43c ($TCID_{50}$ 1 x 10^8 copies/mℓ) used was supplied by Prof A Bosch of the Department of Microbiology, Faculty of Biology, University of Barcelona, Barcelona, Spain

***Human AstV*:** The virus stock (Lab No.: 1918761) was obtained from a clinical specimen referred to the Virology Diagnostic Laboratory, National Health Laboratory Service (NHLS) Tshwane Academic Division (TAD) for the analysis of gastroenteritis viruses.

***Human SaV*:** The virus stock (Lab number (No.): 1383767) was obtained from a clinical specimen referred to the Virology Diagnostic Laboratory, NHLS TAD for the analysis of gastroenteritis viruses.

***Mengovirus*:** The viral stock culture (passage 4; 1.5 x 10^7 $TCID_{50}$/mℓ) was supplied by Prof A Bosch of the Department of Microbiology, Faculty of Biology, University of Barcelona, Barcelona, Spain and further propagated and titrated in the Vero African green monkey kidney cell line and stored at -70ºC.

***Norovirus GI*:** Two different stocks of virus were available. Both stocks originated from clinical specimens, the first from an outbreak of waterborne gastroenteritis and the second was obtained from a stool specimen referred to the Virology Diagnostic Laboratory, NHLS TAD for the analysis of gastroenteritis viruses.

***Norovirus GII*:** 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. The virus was typed by Dr M Wolfaardt from the Enteric Virus and Environmental Research Group, Department of Medical Virology, University of Pretoria and quantified by Prof A Bosch and co-workers, Department of Microbiology, Faculty of Biology, University of Barcelona, Barcelona, Spain.

***Simian RV*:** A simian RV SA 11 (RV-SA11) (ATCC VR-899) stock that was propagated in an African green monkey kidney cell line MA104 by Prof MB Taylor was used. This was used as a surrogate for HRV.

3.2.2 Primers and probes

Published sets of highly specific and sensitive primers and probes were used in this study (Table 3.1). Fluorophore combinations for each of the probes used were selected based on recommended fluorescent dyes used for the ABI 7300 (Applied Biosystems) real-time platform. Two sets of fluorescent probes were selected for each viral target, except for mengovirus, and were used to optimise single as well as duplex and triplex assays.

3.2.3 Nucleic acid extraction

The QIAmp® Viral RNA Mini Kit (Qiagen) was used for the nucleic acid extraction from each of 100 µℓ of the individual viral stocks. The nucleic acid was eluted in a 100 µℓ elution buffer, aliquoted and stored at -70ºC.

3.2.4 Evaluation of a multiplex assay for NoV GI and NoV GII

Primer and probe sequences (Table 3.1) for both NoV GI and NoV GII were subject to pairwise comparison against the complete genome sequences of NoV GI (AF093797.1) and NoV GII (FJ514242.1) in Genbank. This was done to determine the cross reactivity of the individual probes to the amplified products. Extracted nucleic acid of NoV GI and NoV GII was used in a series of singleplex reactions containing different combinations of probes and nucleic acid. Individual reactions were setup to a final volume of 20 µℓ which included 5 µℓ of nucleic acid using the QuantiTect® Multiplex

Table 3.1: Summary of the primers and probes used in this study for the development and optimisation of singleplex and multiplex *rt RT-PCR* assays.

Virus	**Forward primer**	**Reverse primer**	**Probe**	**Dye**	**Reference**
Hepatitis A virus	HAV 68	HAV 240	HAV 150	FAM* NED**	Bosch *et al.* (2001) and Costafreda *et al.* (2006)
Human astrovirus	AV2	AV1	AVs	FAM* NED**	Le Cann *et al.*(2004)
Human rotavirus	RotaF	RotaR	Rota Pr	FAM#	Zeng *et al.* (2008)
Human sapovirus	CU-SV-F1 CU-SV-F2	CU-SV-R	CU-SV-Pr	FAM** NED*	Chan *et al.* (2006)
Mengovirus	Mengo110	Mengo 209	Mengo 147	FAM#	Pintó *et al.* (2009)
Norovirus GII	QNIF2	COG2R	QNIFS	ROX** FAM#	Loisy *et al.* (2005) Kageyama *et al.*(2003)
Norovirus GI	QNIF4	NV1LCR	NV1LCpr	FAM* VIC**	Svraka *et al.* (2007) Da Silva *et al.* (2007)
IAC	QNIF2	COG2R	FP6	JOE* VIC*	This study Cao *et al.* (2007).
M13 cloning primers	M13 (-21)	M13 Rev			Messing (1983)
Primers developed during this study					
Virus	Primer name	Sequence			
Norovirus GI	SP6-QNIF4	ATTTAGGTGACACTATAGAACGCTGGATGCGIT TCCAT			
Astrovirus	SP6AV1	ATTTAGGTGACACTATAGCCGAGTAGGATCGAGGGT			

* Probes used in singleplex reactions

** Probes used in multiplex reactions

Probes used for both single-and multiplex reactions

RT-PCR NoRox kit (Qiagen) with a 4 µM and 8 µM primer and probe concentrations, respectively. The Transcriptor First Strand cDNA synthesis kit (Roche Diagnostics) was used for complementary DNA (cDNA) synthesis according to manufacturer's recommendations. Amplification was performed on a LightCycler® v2.0 *rt* platform (Roche Diagnostics) under the following cycling conditions 50°C for 2 min followed by 95°C for 2 min followed by 50 cycles of 94°C for 15 sec, 60°C for 1 min, and 65°C for 1 min with a final cooling step of 40°C for 1 min.

3.2.5 Assessment of *rt* RT-PCR and cDNA synthesis kits

For the development of individual multiplex assays, the following four *rt* RT-PCR kits were assessed for their sensitivity, specificity and cost effectiveness:

i) the one-step QuantiTect® Multiplex RT-PCR NR kit (Qiagen),
ii) the one-step EXPRESS One-Step Superscript® qRT-PCR kit (Invitrogen)
iii) the two-step kit QuantiTect® Multiplex PCR NR kit (Qiagen) and
iv) the two-step EXPRESS qPCR Super Mix Universal kit (Invitrogen).

In addition, two cDNA synthesis kits were assessed on their efficiency and cost effectiveness in conjunction with each of the two-step *rt* RT-PCR kits:

i) the Transcriptor First Strand cDNA synthesis kit (Roche Diagnostics), and
ii) the RevertAid Premium™ First Strand cDNA synthesis kit (Fermentas Life Sciences).

The efficiency of each of the kits was evaluated upon the successful amplification and detection of the lowest concentration of NoV GII (10^0 = 5 x 10^8 copies/mℓ), HAV (10^0 = 1 x 10^8 copies/mℓ) and mengovirus (10^0 = 2 x 10^{10} copies/mℓ) by serial ten-fold dilution. The manufacturer's recommendations for the setup of each of the cDNA synthesis and PCR reactions for the individual kits were followed. The primer and probe (Table 3.1) concentrations, cycling conditions and real-time platform used for each of the PCR reactions was described in section 3.2.4. For all reactions a final volume of 20 µℓ which included 5 µℓ nucleic acid was used. The amplified RT-PCR fragments from individual one-step *rt* RT-PCR assays were analysed by 2% agarose gel (SeaKem® LE Agarose) electrophoresis and visualised by EtBr staining under UV illumination using a 100 bp marker (O'GeneRuler) to determine amplicon size. In contrast, fluorescent probes were used to evaluate the sensitivity of the two-step *rt* RT-PCR kits for each of the viruses.

3.2.5.1 Analysis of a field sample to determine the optimal *rt* RT-PCR kit combination

In order to determine the optimal *rt* RT-PCR assay for the amplification of multiple targets in a single reaction, the one-step QuantiTect® Multiplex RT-PCR NR kit (Qiagen) and the two-step EXPRESS qPCR Super Mix Universal kit (Invitrogen) in combination with the RevertAid Premium™ First Strand cDNA synthesis kit (Fermentas Life Sciences) was assessed. A surface water sample, which previously

tested positive for NoV GII, HRV and HAV was used. Mengovirus (10 µℓ of a 2 x 10^{10} copies/mℓ stock) was added to 1 mℓ of the water sample and served as a process control. Nucleic acid extraction (section 3.2.3) was followed by amplification with the individual *rt* RT-PCR assays according to the manufacturer's recommendations with a 4 µM primer concentration (Table 3.1). The cycling conditions and real-time platform used for each of the PCR reactions was identical to what was described in section 3.2.4. The amplified PCR fragments were analysed by electrophoresis on a 20-25% polyacrylamide gel (40% Acrylamide/Bis Solution, 19:1) (Bio-Rad Laboratories, Hercules, CA) and visualised by EtBr staining and UV illumination using a 10 bp marker (O'GeneRuler) to determine the fragment size.

3.2.6 RevertAid™ Premium Reverse Transcriptase optimisation

The RevertAid™ Premium Reverse Transcriptase (Fermentas Life Sciences) enzyme was evaluated on its ability to synthesise cDNA at the recommended concentration (200 U/µℓ) and at two dilutions concentrations (100 U/µℓ and 50 U/µℓ). Hepatitis A virus nucleic acid at two different dilutions (3 x 10^6 and 3 x 10^4 copies/mℓ) was each combined with fixed concentrations of the IAC (2 x 10^6 copies/mℓ) and mengovirus (1.5 x 10^6 copies/mℓ) nucleic acid. The incubation period of RT-enzyme (30 min), as recommended by the manufacturer, was extended to 60 min at 50°C. The EXPRESS qPCR Super Mix Universal kit (Invitrogen) with 4 µM and 3 µM primer and probe concentrations, respectively, was used for the amplification of the separate reactions. An ABI 7300 real-time platform (Applied Biosystems), with cycling conditions of 50°C for 2 min followed by 95°C for 2 min prior to 50 cycles of 94°C for 15 sec, 60°C for 1 min, and 65°C for 1 min with a final cooling step of 40°C for 1 min, was applied.

3.2.7 Proposed virus groupings and preparation of viral stocks

3.2.7.1 Reaction A

Noroviruses, especially NoV GII, and HAV were grouped together as they are considered to be the leading causes of food- and waterborne gastroenteritis and hepatitis, respectively. The IAC (Chapter 2) and mengovirus (process control) served as quality control measures in the proposed tetraplex assay containing NoV GII, HAV,

IAC and mengovirus. This assay was designed to allow for the rapid analysis of specimens in the event of an outbreak.

3.2.7.1.1 *Preparation of viral stock of NoV GII, HAV, IAC and mengovirus*

In this aspect of this study the IAC (2 x 10^6 copies/mℓ) was used in a two-step reaction as opposed to a one-step reaction. This was to obtain a concentration of 5 x 10^5 copies/mℓ of IAC in each of the two-step *rt* RT-PCR reactions. Viral stocks used were: HAV (1 x 10^8 copies/mℓ), NoV GII (2.85 x 10^9 copies/mℓ) and mengovirus (2 x 10^{10} copies/mℓ). A combined working stock suspension of all three viruses with a final working concentration of 1 x 10^8 copies/mℓ for each virus was made of which a 100 µℓ was extracted using the QIAmp® Viral RNA Mini Kit (Qiagen) according to the manufacturer's recommendations. The nucleic acid was eluted in 100 µℓ elution buffer, aliquoted and stored at -70ºC.

3.2.7.2 Reaction B

Norovirus GI and HRV which are leading causes of gastroenteritis were grouped together with mengovirus. The initial proposed multiplex assay of NoV GI, HRV and mengovirus was changed with SaV substituting for mengovirus after encountering problems during the optimisation of a combination of NoV GI and mengovirus.

3.2.7.2.1 *Preparation of stock nucleic acid for NoV GI, RV-SA11 and SaV*

Amplified PCR fragments of RV-SA11, SaV and NoV GI were generated to allow for quantification by the use of DNA standard curves.

i) Preparation of RV-SA11 and SaV PCR fragments

Sapovirus PCR fragments were generated by cDNA synthesis and amplification using the RevertAid Premium™ First Strand cDNA synthesis kit (Fermentas Life Sciences) and EXPRESS qPCR Super Mix Universal kit (Invitrogen), respectively. The reaction setup was according to the manufacturer's recommendation with a 4 µM primer concentration. Amplification was performed on an ABI 7300 (Applied Biosystems) real-time platform using the following cycling conditions 50°C for 2 min followed by 95°C for 2 min prior to50 cycles of 94°C for 15 sec, 60°C for 1 min, and 65°C for 1 min with a final cooling step of 50°C for 1 min. The PCR fragments were analysed by 2% agarose gel (SeaKem® LE Agarose) electrophoresis and visualised by EtBr staining

under UV illumination using a 100 bp marker (O'GeneRuler) to verify the amplicon size. The expected band size (80 bp) was excised from the gel and purified using the Zymoclean™ Gel DNA Recovery kit (Zymoclean Research Corp.).

Simian RV-SA11 PCR fragments were generated after cDNA synthesis using the RevertAid Premium™ First Strand cDNA synthesis kit (Fermentas Life Sciences) and PCR amplification using GoTaq® Flexi DNA Polymerase (5 U/µℓ) (Promega Corp.), dNTPs (2.5 mM) (Roche Diagnostics) and 4 µM primer concentrations. Amplification was performed on a Little Genius thermocycler (BIOER Technology Co., Hangzhou, China) using the following conditions: 95°C for 3 min followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec followed by an extension at 72°C for 7 min and held at 4°C. The PCR fragments were analysed by 2% agarose gel (SeaKem® LE Agarose) electrophoresis and visualised by EtBr staining and UV illumination using a 100 bp marker (O'GeneRuler). The PCR fragments were purified using the DNA Clean and Concentrator™-25 kit (Zymoclean Research).

Purified PCR fragments of both RV-SA11 and SaV were ligated into the pGEM®-T Easy Vector System (Promega Corp.). Twenty microliters of *E. cloni*® 5-alpha Chemically Competent Cells (Lucigen® Corp.) were transformed according to the manufacturer's recommendations with the exception that 400 µℓ of SOC media (Sigma-Aldrich Co.) was used. The transformed cells were plated on LB media containing 100 µg/mℓ ampicillin (Sigma-Aldrich Co.) and incubated overnight at 37°C. Colonies were randomly selected and screened for the specific viral insert by PCR amplification using the GoTaq® Flexi DNA Polymerase (Promega Corp.) with the M13 cloning primer set (4 µM) (Table 3.1). Amplification was performed on a Little Genius thermocycler (BIOER Technology Co., Hangzhou, China) using the following conditions: 95°C for 3 min followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec followed by an extension at 72°C for 7 min and held at 4°C. The PCR products were analysed by 2% agarose gel (SeaKem® LE Agarose) electrophoresis and visualised by EtBr staining under UV illumination using a 100 bp marker (O'GeneRuler) to verify the amplicon size.

A recombinant colony of both RV-SA11 and SaV, containing the correct size insert, was selected and propagated in 100 mℓ LB-media supplemented with 100 µg/mℓ ampicillin (Sigma-Aldrich Co.) and incubated at 37^{o}C overnight. A second PCR

reaction, identical to the first PCR reaction, was performed to confirm the presence of PCR fragments. The nucleic acid was extracted from the plasmid containing the relevant virus PCR fragment using the Pure Yield™ Plasmid Midiprep System 25 preps (Promega Corp.) according to the manufacturer's recommendation. The nucleic acid was used to amplify larger RV-SA11 and SaV PCR fragments using the M13 reverse primer with both of the virus forward primers and analysed by 2% agarose gel (SeaKem® LE Agarose) electrophoresis. The PCR fragments were purified using the DNA Clean and Concentrator™-25 kit (Zymoclean Research) and quantified using the NanoDrop™ 1000 Spectrophotometer (NanoDrop Technologies, LLC, Wilmington, DE). The copy number/µℓ was calculated using formula 3.1:

Formula 3.1: Copies/µℓ = [PCR fragment concentration (g/µℓ)] / [(product length, bp)*(1.096 x 10^{-21} g/bp)] (Waters *et al*., 2011).

ii) Preparation of NoV GI RNA fragments

Norovirus GI nucleic acid were transcribed into cDNA using the RevertAid Premium™ First Strand cDNA synthesis kit (Fermentas Life Sciences) and amplified with the EXPRESS qPCR Super Mix Universal kit (Invitrogen) using the SP6-QNIF4 forward primer with the NV1LCR reverse primer (Table 3.1). Amplification setup and cycling conditions used was identical to that of RV-SA11 and SaV. The PCR fragments were analysed by 2% agarose gel (SeaKem® LE Agarose) electrophoresis and visualised by EtBr staining under UV illumination using a 100 bp marker (O'GeneRuler). Purification of the PCR fragments was done using the DNA Clean and Concentrator™-25 kit (Zymoclean Research). The Riboprobe® *in vitro* Transcription Systems kit (Promega Corp.) was used in the synthesis of RNA from the PCR products according to the manufacturer's recommendations with 2.5 millimole (mM) rNTP stock with rCTP included. The removal of contaminating DNA from the RNA solutions involved the dilution of the samples a thousand-fold and the addition of 3 µℓ RQ1 RNase-Free DNase (Promega Corp) followed by an incubation period of 37°C for 1 hr and overnight incubation at room temperature (23°C). The removal of the DNA from the RNA solution was validated by PCR.

The RNA fragments were serially diluted followed by cDNA synthesis using the RevertAid Premium™ First Strand cDNA synthesis kit (Fermentas Life Sciences) and

amplified with the EXPRESS qPCR Super Mix Universal kit (Invitrogen), with the NoV GI primer and probe concentration of 4 µM. The lowest detectable concentration of NoV GI was determined by re-testing the detected concentration and immediate upper and lower concentrations in triplicate reactions.

3.2.7.3 Reaction C

Human SaV, a member of the *Caliciviridae* family and recognised as an important human pathogen, along with HAstV was initially combined for the final multiplex assay together with mengovirus. As a result of amplification difficulties with NoV GI and mengovirus, SaV was moved to reaction B. The final combination for reaction C included HAstV, the IAC and mengovirus.

3.2.7.3.1 *Preparation of HAstV RNA fragments*

As insufficient clinically-derived HAstV stock was available, RNA transcripts was made from DNA PCR fragments as described for NoV GI (section 3.2.7.2.1). Briefly, HAstV genomic RNA was transcribed into cDNA using the RevertAid Premium™ First Strand cDNA synthesis kit (Fermentas Life Sciences) and amplified using the EXPRESS qPCR Super Mix Universal kit (Invitrogen) with the SP6AV1/AV2 primer (Table 3.1) combination. The PCR fragments were purified using the DNA Clean and Concentrator™-25 kit (Zymoclean Research) and quantified using the NanoDrop™ 1000 Spectrophotometer (NanoDrop Technologies). The copy number/µℓ was calculated using formula 3.1. The DNA PCR fragments were in vitro transcribed to RNA, purified and the lowest copy number determined.

3.2.8 Construction of standard curves and quantification of the RNA transcripts

3.2.8.1 Construction of DNA standard curves

Ten-fold serial dilutions of the quantified PCR fragments of NoV GI, SaV, RV-SA11 (section 3.2.7.2.1) and HAstV (section 3.2.7.3.1) were prepared to the lowest PCR concentration detected. Five consecutive serial ten-fold dilutions were used in the construction of the standard curves. The dilutions consisted of the lowest PCR concentration detected and the four preceding dilutions and were assessed in triplicate. The EXPRESS qPCR Super Mix Universal kit (Invitrogen) with 4 µM and 3 µM primer and probe (Table 3.1) concentrations, respectively, was used. The final volume of each

of the PCR reactions was made up to 20 µℓ and included 5 µℓ of nucleic acid. Amplification was on the ABI 7300 real-time platform (Applied Biosystems) using the following cycling conditions: 50°C for 2 min followed by 95°C for 2 min prior to 50 cycles of 94°C for 15 sec, 60°C for 1 min, and 65°C for 1 min with a final cooling step of 50°C for 1 min. The slope and the Pearson co-efficient of determination value (R^2) were determined by the real-time platform software and the efficiency (E) of each standard curves was calculated.

3.2.8.2 Quantification of viral stocks

Serial ten-fold dilutions were made from the nucleic acid (section 3.2.3) of NoV GI, SaV and RV-SA11 (section 3.2.1) along with the HAstV *in vitro* transcribed RNA fragments (section 3.2.7.3.1). The dilutions consisted of the lowest PCR concentration detected and the three preceding dilutions and were assessed in triplicate by cDNA synthesis using 50 U/µℓ of the RT enzyme from the RevertAid Premium™ First Strand cDNA synthesis kit (Fermentas Life Sciences) as described in section 3.2.6, and the EXPRESS qPCR Super Mix Universal kit (Invitrogen) as described in section 3.2.8.1. The final volume of each of the PCR reactions was made up to 20 µℓ and included 5 µℓ of nucleic acid per reaction. The mean Ct-value for the highest dilution was determined for each of the viruses and the quantity of nucleic acid present at this specific dilution was quantified using Formula 3.2 with the aid of their respective DNA standard curves.

Formula 3.2: $y = x\,(1 + e)^n$

y = final quantification or yield of the amount of starting material
x = starting material concentration
n = the Ct-value generated
e or E = the efficiency of the PCR.

In brief, a Ct-value of the DNA standard curve (example 30.24) corresponding to a RNA dilution Ct-value (example 30.57) or where the Ct-value for the RNA dilution (example 30.62) were between two DNA standard curve values (eaxample 29.72 and 31.52) was determined. The concentration of nucleic acid present at the respective Ct-value(s) for the DNA standard curve dilutions were known and could be substituted along with the efficiency into Formula 3.2 to determine the “y” or the mean “y” value.

To quantify the RNA nucleic acid at the specified dilution (example 30.57), the "y" or mean "y" value from the DNA standard curve with the corresponding RNA Ct-value and the efficiency of the DNA standard curve value could be used to determine the "x" value using th Formula 3.2.

3.2.8.3 Construction of RNA standard curves

The RNA dilutions of the nucleic acid of NoV GI, SaV, simian RV and HAstV, prepared for the quantification of viral stocks (section 3.2.8.2) were used for constructing the respective RNA standard curves by making further serial ten-fold dilutions and testing each dilution on triplicate. The "x" value generated in section 3.2.8.2 was considered as the starting concentration of nucleic acid/virus at a particular dilution. In the case of NoV GII and HAV, of which the viral stock concentrations were known, serial ten-fold dilutions of the extracted nucleic acid (section 3.2.3) was made and the lowest three consecutive *rt* RT-PCR detectable concentrations were used to generate their respective RNA standard curves. The cDNA synthesis and amplification reactions were as described previously (section 3.2.8.2).

3.2.9 Optimisation of reaction A

3.2.9.1 Triplex assay for HAV, IAC and mengovirus

Serial ten-fold dilutions made for the construction of the RNA standard curve (section 3.2.8.3) was used to evaluate the competition and primer-probe interactions with the additional amplification of; i) the IAC (2 x 10^6 copies/mℓ) and ii) mengovirus (3 x 10^6 copies/mℓ). Duplex assays were individually optimised with HAV dilutions containing either the IAC or mengovirus, respectively. The IAC was added to the cDNA synthesis reaction by substituting 1 µℓ of nuclease free water (Promega Corp.) with the IAC or mengovirus nucleic acid. In the triplex reaction, 2 µℓ of PCR grade water in the cDNA synthesis reaction was substituted for 1 µℓ of the IAC and 1 µℓ mengovirus nucleic acid. The RevertAid Premium™ First Strand cDNA synthesis kit (Fermentas Life Sciences) (section 3.2.6) with an enzyme concentration of 50 U/µℓ and the EXPRESS qPCR Super Mix Universal kit (Invitrogen) (section 3.2.8.1) were used. The individual PCR reactions were performed in triplicate and the mean Ct-value and standard deviation at each dilution determined. A primer and probe concentration of 4 µM and 3 µM was used for each of the respective targets. The final volume of each of the PCR

reactions was 20 µℓ which included 5 µℓ of nucleic acid. Amplification was performed on the ABI 7300 real-time platform (Applied Biosystems) using the following cycling conditions; 50°C for 2 min followed by 95°C for 2 min prior to 50 cycles of 94°C for 15 sec, 60°C for 1 min, and 65°C for 1 min with a final cooling step of 50°C for 1 min.

3.2.9.2 Triplex assay for NoV GII, IAC and mengovirus

The serial ten-fold dilutions of NoV GII (section 3.2.8.3) were used in the optimising of duplex assays for the IAC and mengovirus in a two-step reaction. The PCR reaction setup, addition of the IAC and mengovirus, respectively, and cycling conditions was identical to that described in section 3.2.9.1. The individual PCR reactions were performed in triplicate and the mean Ct-value and standard deviation at each dilution determined and used for statistical analysis. In the optimisation of the duplex assay for NoV GII and mengovirus, different concentrations of mengovirus nucleic acid (3 x 10^6 and 1.5 x 10^6 copies/mℓ) and primer concentrations (4 µM and 3 µM) was used.

3.2.9.3 Triplex assay for NoV GII, HAV and IAC

The duplex NoV GII/IAC assay (section 3.2.9.2) was used for the optimisation of the triplex assay by the addition of HAV nucleic acid (section 3.2.9.1) at similar concentrations to that of NoV GII nucleic acid (section 3.2.9.2) while keeping the IAC concentration constant. The different nucleic acid concentrations were made by combining 10 µℓ of a specific concentration of the two viruses in a single tube and made up to a final volume of 100 µℓ using nuclease free water (Promega Corp.). A total of 10 µℓ of the 100 µℓ volume was used for cDNA synthesis. The cDNA synthesis, PCR reaction setup and cycling conditions were identical to that described in section 3.2.9.1. The individual PCR reactions were performed in triplicate and the mean Ct-value and standard deviation at each dilution determined.

3.2.10 Optimisation of reaction B

3.2.10.1 Triplex assay for NoV GI, HRV and SaV

The RNA standard curve dilutions and concentrations (3.2.8.3) were used to optimise each of the duplex assay combinations and the triplex assay following the same procedure as described in reaction A (section 3.2.9). Individual nucleic acid concentrations were made to a final volume of 100 µℓ by combining 10 µℓ of the

specific standard curve concentration from two viruses and nuclease free water (Promega Corp.). A total of 10 µℓ of the final 100 µℓ volume was used for cDNA synthesis. The cDNA synthesis, PCR reaction setup and cycling conditions were identical to that described in section 3.2.9.1. The individual PCR reactions were performed in triplicate and the mean Ct-value and standard deviation at each dilution determined.

3.2.11 Optimisation of reaction C

3.2.11.1 Triplex assay for HAstV, IAC and mengovirus

The HAstV RNA transcript (section 3.2.8.3) was combined with a standard concentration of the NoV GII, the IAC and mengovirus (section 3.2.9.1). A primer and probe concentration of 4 µM and 3 µM was used for each of the respective targets. In the optimisation of the assay, the mengovirus primer pair concentrations were decreased to 2 µM, and later to 1 µM. The HAstV primer pair and probe concentrations were increased to 10 µM and 4 µM, respectively. The cDNA synthesis, PCR reaction setup and cycling conditions were identical to that described in section 3.2.9.1. The individual PCR reactions were performed in triplicate and the mean Ct-value and standard deviation at each dilution determined and used.

3.2.12 Statistical analysis

The data (Ct-values) obtained from the analysis of reaction A, B and C were used to determine if the differences between and among dilutions and different viruses were statistically or analytically significant or not. The Ct-values were used to calculate the mean, their respective standard deviations, probability and 95% confidence intervals. Analysis was carried out using Fisher's exact test using StatView software (ver 5.0; SAS Institute Inc, Cary, NC).

3.2.13 Evaluation of multiplex assays using environmental samples

3.2.13.1 Environmental samples

Water samples (10 ℓ) comprising river, dam, borehole and irrigation water from pivots from a commercial farm were collected from the Limpopo province, SA. The samples

were collected over a period of 5 mo.

3.2.13.2 Processing of water samples

Viruses were recovered from the water samples using a glass wool adsorption-elution method. The glass wool adsorption-elution method was based on the method described by Vilaginès *et al*. (1997) and improved by Venter (2004). Viruses having a negative charge absorbed to the positively charged glass wool and were recovered by eluting twice from the column with 50 mℓ of 0.5% glycine-beef extract buffer (pH 9.5) (3.754 g/ℓ glycine (Merck), 5 g/ℓ beef extract powder (BBLTM Becton Dickinson and Co., Sparks, MD). The pH was adjusted afterwards to a neutral pH (pH 7) with 1 mole (M) hydrochloric acid (HCl) (pH 1) (Merck) and sodium hydroxide (NaOH) (Merck). Further concentration of the viral suspension was done using the PEG_{6000} (Merck)/NaCl (Merck) precipitation method described by Vilaginès *et al*. (1997) as modified by Minor (1985). The viruses were re-suspended in a final volume of 10 mℓ PBS (Sigma-Aldrich Co.) and stored at -20°C prior to further analysis.

3.2.13.3 Nucleic acid extraction

Nucleic acid was extracted from a 1 mℓ aliquot of the viral suspension as described in section 3.2.3. The nucleic acid was aliquoted into 10 µℓ volumes and stored at -70°C until further analysis.

3.2.13.4 Viral analysis by *rt* RT-PCR assays

The nucleic acid samples were screened in a previous study for HAV, NoV GI, NoV GII and mengovirus using individual environmental one-step *rt* RT-PCR ceeramTools™ Detection Kits (Ceeram s.a.s, La Chappelle-SurErdre, France). In this study the three optimised multiplex assays A, B and C detecting NoV GII/HAV/IAC, NoV GI/HRV/SaV and HAstV/IAC/mengovirus, respectively, were applied. The sensitivity of the triplex assays was compared to the individual commercial assays.

3.3 RESULTS

3.3.1 Evaluation of a multiplex assay for NoV GI and NoV GII

From Table 3.2 it is evident that the NoV GII probe could detect both the amplified product of NoV GI and NoV GII indicating that the NoV GII probe cross-reacted with the PCR fragments of NoV GI resulting in a false positive test result. A sequence alignment of the probe sequence of NoV GII to that of the genome sequence of NoV GI confirmed the results, i.e. the NoV GII probe sequence aligned in the amplified region of NoV GI (results not shown).

Table 3.2: Summary of the specificity of NoV GI and NoV GII probes in their ability to distinguish between the PCR amplicons of NoV GI and NoV GII.

Primers combination (forward/reverse)	Probe	Nucleic acid	Amplification
GI/GII	GI	GI	+
GI/GII	GI	GII	-
GI/GII	GII	GI	+
GI/GII	GII	GII	+
Negative control (GI/GII)	GI/GII	-	-

3.3.2 Assessment of *rt* RT-PCR and cDNA synthesis kits

The results of the assessment of the combination of the different *rt* RT-PCR kits and cDNA synthesis kits are summarised in Table 3.3. When comparing the one-step *rt* RT-PCR kits no difference was noted in the limit of detection for NoV GII as 5 x 10^3 copies/mℓ using both kits. However, a $\log_{10}$ difference was noted for HAV and mengovirus. In the analysis of the two-step assays, differences in detection were noted between the cDNA synthesis kits rather than the *rt* RT-PCR kits. When the Transcriptor First Strand cDNA synthesis kit (Roche Diagnostics) in combination with either of the amplification kits the detection limit for all three viruses was one $\log_{10}$ higher than when the RevertAid Premium™ First Strand cDNA synthesis kit (Fermentas Life Sciences) was used.

3.3.2.1 Analysis of a field sample to determine the optimal *rt* RT-PCR assay

When using a water sample it was conclusively shown that the EXPRESS qPCR Super

Table 3.3: Results, expressed as copies/mℓ of the detection limit of NoV GII, HAV and mengovirus by independent one-step multiplex *rt* RT-PCR kits and two-step multiplex *rt* RT-PCR kits in combination with two cDNA synthesis kits.

	One-step *rt RT-PCR* kits		**Two-step *rt RT-PCR* kits**			
Virus stock	**QuantiTect Multiplex RT-PCR NR kit (copies/mℓ)**	**EXPRESS One-Step Superscript® qRT-PCR kit (copies/mℓ)**	**QuantiTect Multiplex PCR NR kit / Transcriptor kit (copies/mℓ)**	**QuantiTect Multiplex PCR NR kit / RevertAid Premium™ kit (copies/mℓ)**	**EXPRESS qPCR Super Mix Universal kit / Transcriptor kit (copies/mℓ)**	**EXPRESS qPCR Super Mix Universal kit / RevertAid Premium™ kit (copies/mℓ)**
NoV GII	5×10^3	5×10^3	5×10^4	5×10^3	5×10^4	5×10^3
HAV	1×10^3	1×10^2	1×10^4	1×10^3	1×10^4	1×10^3
Mengovirus	2×10^4	2×10^5	2×10^5	2×10^2	2×10^5	2×10^2

Mix Universal kit (Invitrogen) in combination with the RevertAid Premium™ First Strand cDNA synthesis kit (Fermentas Life Sciences) was the optimal choice (Figure 3.1). This was evident as HAV (~172 bp) and mengovirus (~99 bp) could not be amplified using the one-step QuantiTect® Multiplex RT-PCR NR kit (Qiagen) (Figure 3.1, lane 2) while being successfully amplified by the two-step assay (Figure 3.1, lane 1).

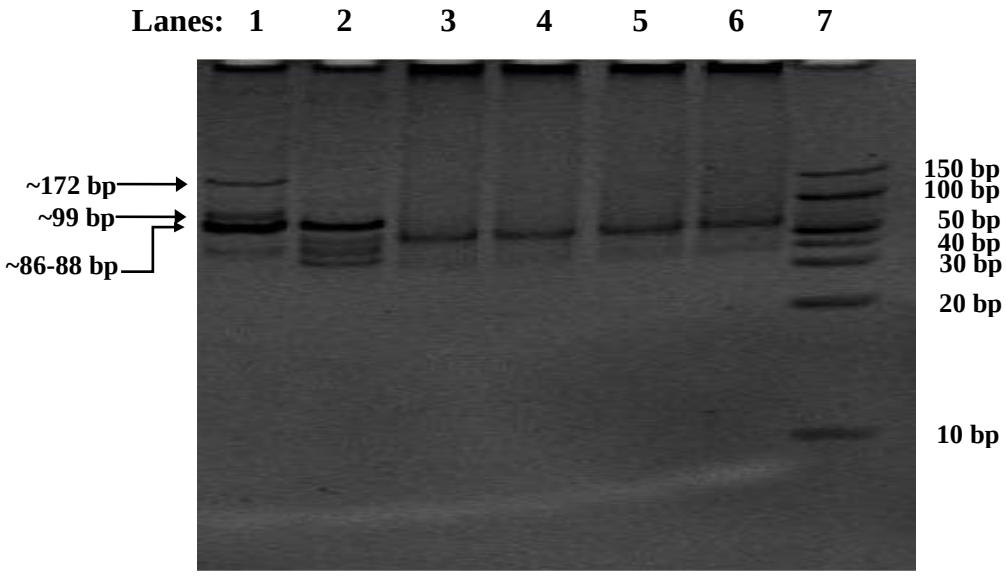

Figure 3.1: Polyacrylamide gel analysis summarising the results of the amplification of HAV (~172 bp), NoV GII (~88 bp), RV (~86 bp) and mengovirus (~99 bp) by the two-step assay (lane 1) compared to the one-step assay (lane 2).

3.3.3 RevertAid™ Premium Reverse Transcriptase optimisation

In the optimisation of the RT-enzyme concentrations used in the RevertAid Premium™ First Strand cDNA synthesis kit (Fermentas Life Sciences) in the multiplex assay deviations between the Ct-values of HAV, the IAC and mengovirus were noted at different HAV concentrations (Figure 3.2). A smaller insignificant deviation was noted between the 200 U/µℓ and 100 U/µℓ RT-enzyme concentrations than between the 200 U/µℓ and 50 U/µℓ concentrations (Figure 3.2). The 50 U/µℓ concentration gave consistently similar results compared to the recommended 200 U/µℓ concentration.

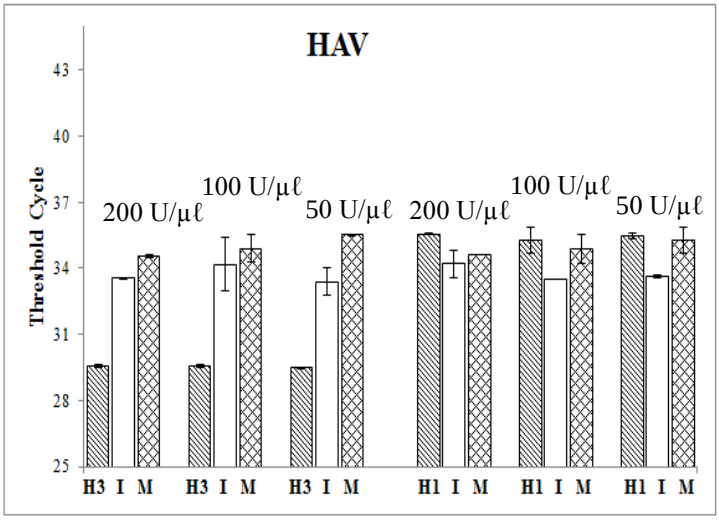

Figure 3.2: Summary of cDNA synthesis by the RevertAid Premium™ Enzyme (Fermentas Life Sciences) at three concentrations (50 U/µℓ, 100 U/µℓ and 200 U/µℓ). The error bars indicate the standard deviation for the triplicate reactions H3 (HAV @ 3 x 10^4 copies/mℓ); H1 (HAV @ 3 x 10^6 copies/mℓ); internal amplification control (I) and process control (mengovirus) (M).

3.3.4 Construction of standard curves and quantification of viral stocks

3.3.4.1 Construction of DNA standard curves

The amplified PCR fragments generated for each of the respective viruses were quantified by spectrophotometrically and the concentrations determined using Formula 3.1 (results not shown). Each of the standard curves generated had an efficiency (E) and R^2 value close or equal to 1 (Figure 3.3). There existed very little deviation among the replicates of each dilution for each of the respective virus, with the slope of each of the standard curves aligning at an approximate 45° angle to the x-axis.

3.3.4.2 Quantification of viral stocks

The quantification of the viral RNA nucleic acid (NoV GI, SaV and RV-SA11) and RNA fragments of HAstV was determined at a specific dilution and the data, equations and results were summarised in Table 3.4 for each of the respective viruses. The Ct-values used to quantify the nucleic acid concntration at the individual DNA and RNA dilutions are highlighted in bold (Table 3.4). The RNA concentrations for the different viruses were calculated as follows: HAstV (3.1 x 10^6 copies/mℓ), NoV GI (6.8 x 10^5 copies/mℓ), SaV (3.4 x 10^6 copies/mℓ) and RV-SA11 (1.7 x 10^6 copies/mℓ).

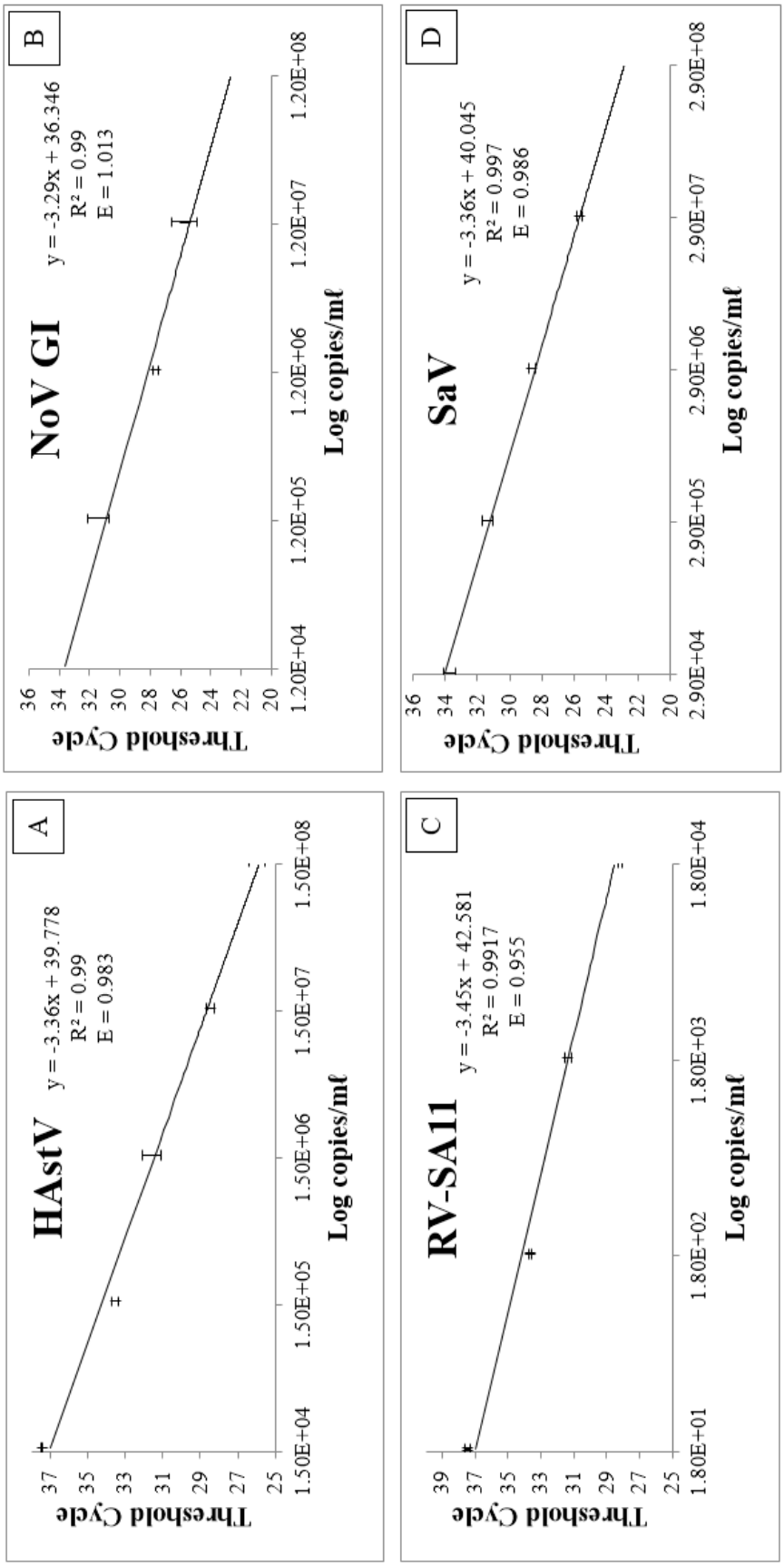

Figure 3.3: Summary of the DNA standard curves for HAstV (A), NoV GI (B), Sa11 (C) and SaV(D). The error bars depict the standard deviation. The slope, Pearson co-efficient of determination value (R^2) and efficiency (E) was determined.

Table 3.4: Summary of the quantification of viral RNA fragments (HAstV) and genomic RNA (NoV GI, SaV and RV-SA11) at specific dilutions using DNA standard curves and the formula "$y = x(1+E)^n$"

HAstV (DNA)				HAstV (RNA)		
Mean Ct-value	**DNA Copies/5 µℓ**	**Determining y**	**Mean y-value**	**Mean Ct-mean**	**Determining x**	**RNA Copies/mℓ**
25.98	7.9×10^5			**30.72**	$x = 1.6 \times 10^4$	3.1×10^6
28.16	**7.9×10^4**	$y = 2.3 \times 10^{13}$		34.43		
31.36	**7.9×10^3**	$y = 1.9 \times 10^{13}$	2.1×10^{13}	36.67		
33.65	7.9×10^2			41.06		
37.44	7.9×10^1					

NoV GI (DNA)				NoV GI (RNA)		
Mean Ct-value	**DNA Copies/5 µℓ**	**Determining y**	**Mean y-value**	**Mean Ct-mean**	**Determining x**	**RNA Copies/mℓ**
22.48	6.2×10^5			**28.73**	$x = 3.4 \times 10^4$	6.8×10^5
25.73	6.2×10^4	$y = 1.3 \times 10^{12}$		31.62		
27.64	**6.2×10^3**	$y = 1.8 \times 10^{12}$	1.53×10^{12}	35.18		
31.42	**6.2×10^2**			37.48		
33.34	6.2×10^1					

Table 3.4 (continued): Summary of the quantification of viral RNA fragments (HAstV) and genomic RNA (NoV GI, SaV and RV-SA11) at specific dilutions using DNA standard curves and the formula "$y = x(1+E)^n$"

SaV (DNA)			SaV (RNA)		
Mean Ct-value	**DNA Copies/5 µℓ**	**Determining y**	**Mean Ct-mean**	**Determining x**	**RNA Copies/mℓ**
22.58	1.5×10^6		**28.40**	$x = 1.7 \times 10^4$	3.4×10^6
25.8	1.5×10^5		31.49		
28.62	**1.5×10^4**	$y = 5 \times 10^{12}$	34.98		
31.36	1.5×10^3		37.43		
33.73	1.5×10^2				

RV-SA11 (DNA)			RV-SA11 (RNA)		
Mean Ct-value	**DNA Copies/5 µℓ**	**Determining y**	**Mean Ct-mean**	**Determining x**	**RNA Copies/mℓ**
25.98	9.2×10^5		**31.47**	$x = 8.5 \times 10^3$	1.7×10^6
28.16	9.2×10^4		35.05		
31.36	**9.2×10^3**	$y = 1.2 \times 10^{13}$	37.64		
33.65	9.2×10^2		40.67		
37.44	9.2×10^1				

3.3.4.3 Construction of RNA standard curves

The quantified RNA nucleic acid (Table 3.4) for each of the viruses served as the starting material in the making of the RNA standard curves (Figure 3.4). For all the standard curves made, the E and R^2 values were as close to 1 with the slope having an approximate angle of 45° to the x-axis. Four dilutions as appose to five dilutions were used for plotting the RNA standard curves as the fifth point was to low to be detected by the assays (results not shown).

3.3.5 Optimisation of reaction A

3.3.5.1 Triplex assay for HAV, IAC and mengovirus

The results for the optimisation of the triplex assay for the simultaneous detection of HAV, the IAC and mengovirus are presented in Figure 3.5. Little to no competition was noted in the detection of HAV when simultaneously amplified with the IAC and mengovirus, i.e. less than three Ct-values (Figure 3.5). However, a deviation of less than a $\log_{10}$-factor was detected between the single- and duplex assays during the amplification of HAV (H1 = 3 x 10^4 copies/mℓ) with mengovirus. Hepatitis A virus could be detected in 2/3 replicates at the dilution H0 = 3 x 10^3 copies/mℓ in the duplex reaction with either of the controls but not in the HAV singleplex assay and thus not statistical data exists for HAV at this dilution.

3.3.5.1.1 *Statistical analysis for the triplex assay HAV, IAC and mengovirus*

Based on the results statistical significant differences ($p \leq 0.05$ considered significant) between these combinations for each of the dilutions were noted (overall p-factor for HAV at dilutions H3: p = 0.0047, H2: p = 0.4457 and H1: p = 0.1044), however, analytical differences of less than three cycles that are more applicable to this study were not detected. Based on the statistical analysis, the triplex assay for HAV, IAC and mengovirus was equally as sensitive as the singleplex assay for HAV, except at the dilution H0 = 3 x 10^3 copies/mℓ where the duplex assays was more sensitive than the single- or triplex assays.

3.3.5.2 Triplex assay for NoV GII, IAC and mengovirus

In the proposed triplex assay for the detection of NoV GII, IAC and mengovirus, mengovirus could be detected but not NoV GII (results not shown). This proposed combination of viruses was as a result abandoned.

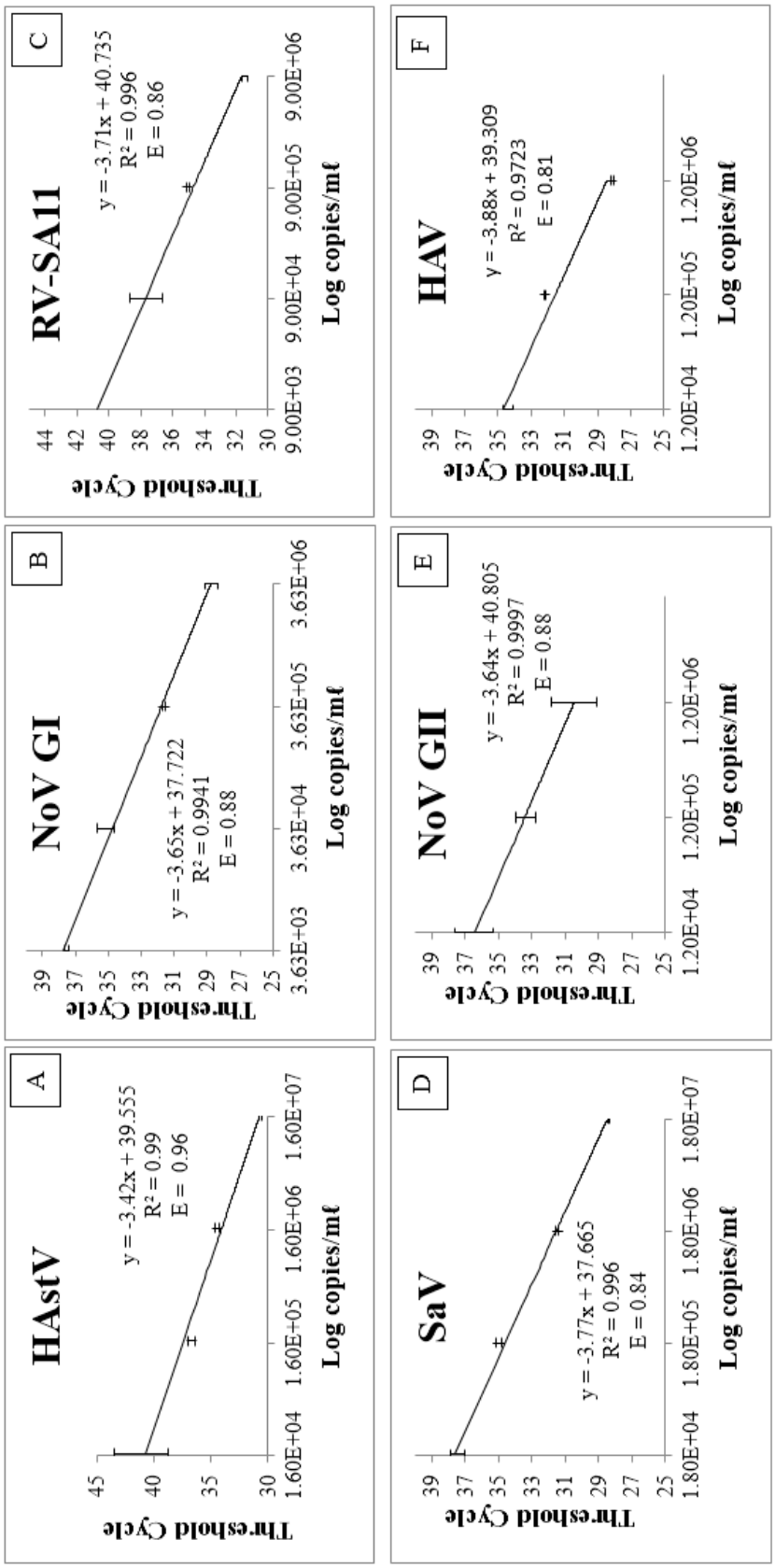

Figure 3.4: Summary of the RNA standard curves for HAstV (A), NoV GI (B), Sa11 (C), SaV(D), NoV GII (E) and HAV (F). The error bars depict the standard deviation at each dilution. The slope, Pearson co-efficient of determination value (R^2) and efficiency (E) was determined.

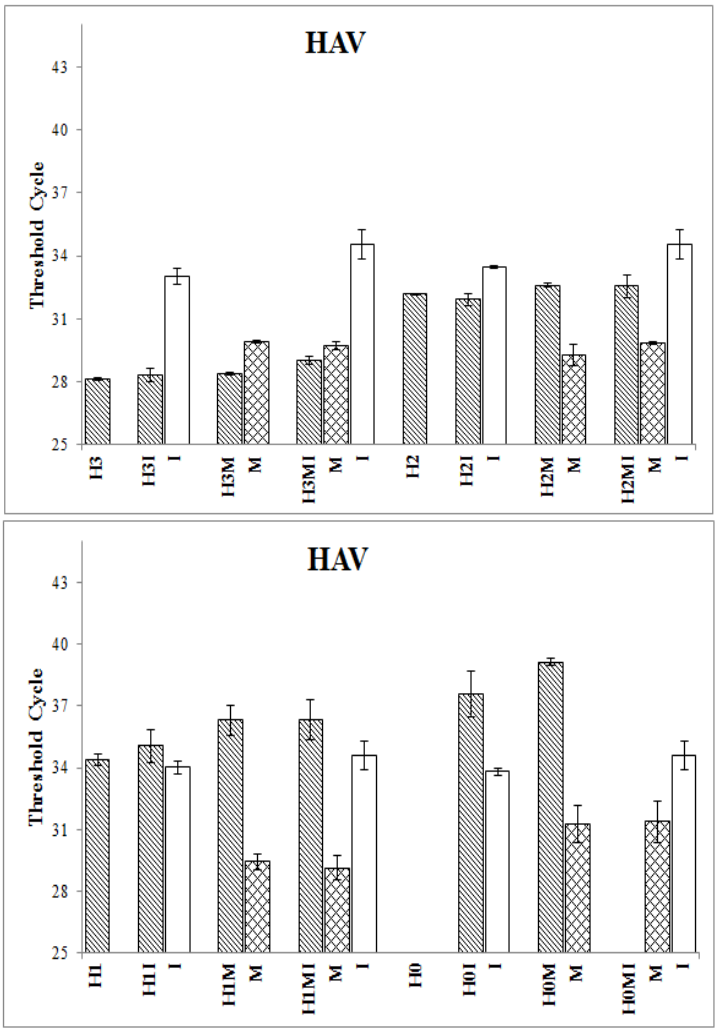

Figure 3.5: Summary of the results for the amplification of HAV at different dilutions in a singleplex, duplex and triplex assay with the internal amplification control (IAC) and/or mengovirus. The error bars indicate the standard deviation between the individual replicates at each dilution. The abbreviation H for HAV, I for IAC and M for mengovirus was used and a numerical value was specified to each target according to the respective dilution used (H3 = 3 x 10^6 copies/mℓ, H2 = 3 x 10^5 copies/mℓ, H1 = 3 x 10^4 copies/mℓ, H0: 3 x 10^3 copies/mℓ, I: 2 x 10^6 copies/mℓ and M: 3 x 10^6 copies/mℓ)

3.3.5.3 Triplex assay for NoV GII, HAV and IAC

It was evident from the results that HAV (Figure 3.6) had no significant effect on the detection of NoV GII (Figure 3.7) and visa versa for all the dilutions and combinations tested based on the analysis of the Ct-value means. Deviations were noted in the detection of the IAC with NoV GII and HAV, however, these deviations were less than a $\log_{10}$ factor and were not considered significant.

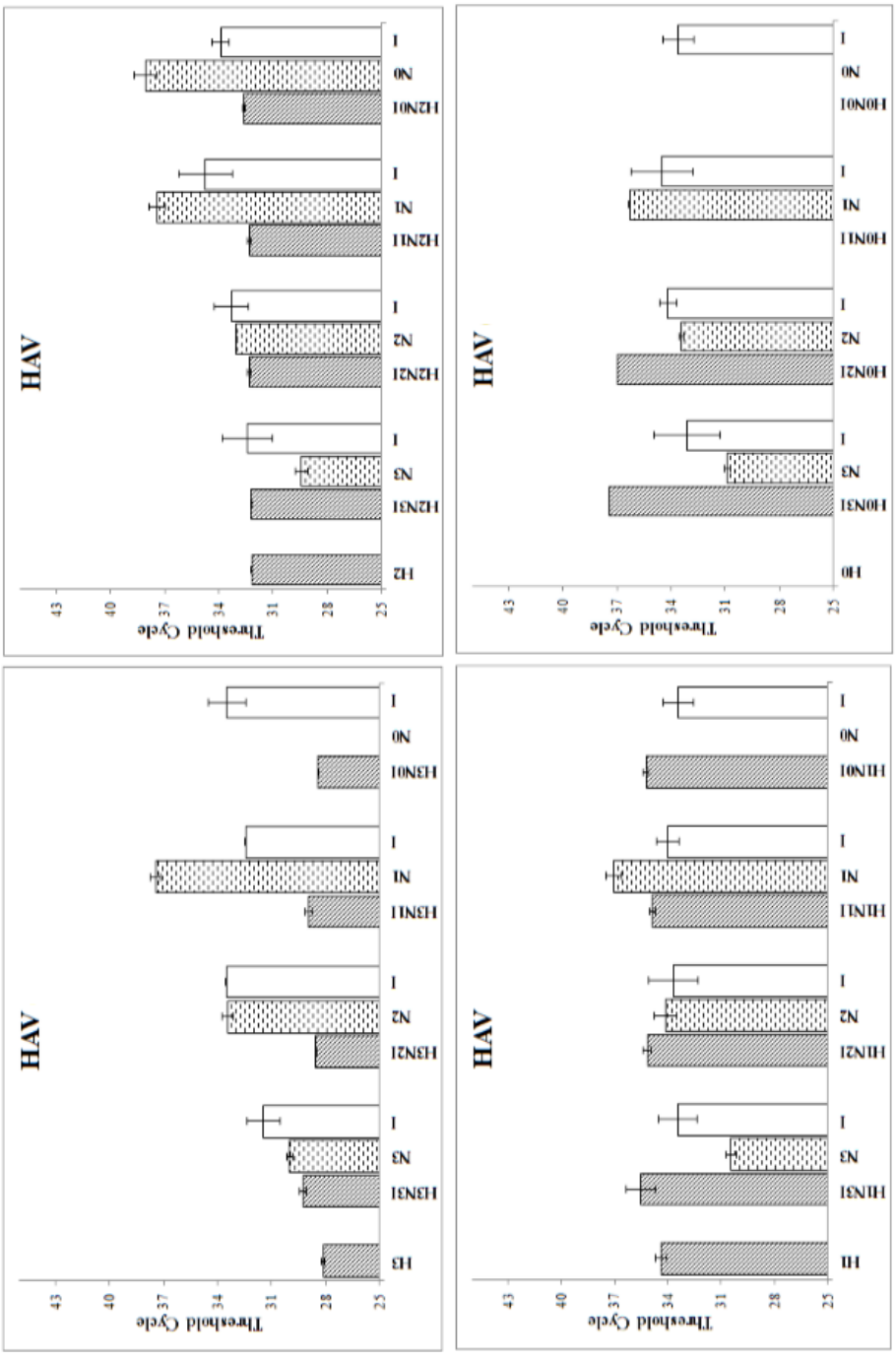

Figure 3.6: Results of HAV (H) triplex assays in combination with NoV GII (N) and the IAC (I), respectively, tested with four different serial ten-fold dilutions of HAV. The error bards indicate the standard deviation between the respective triplicate reactions.

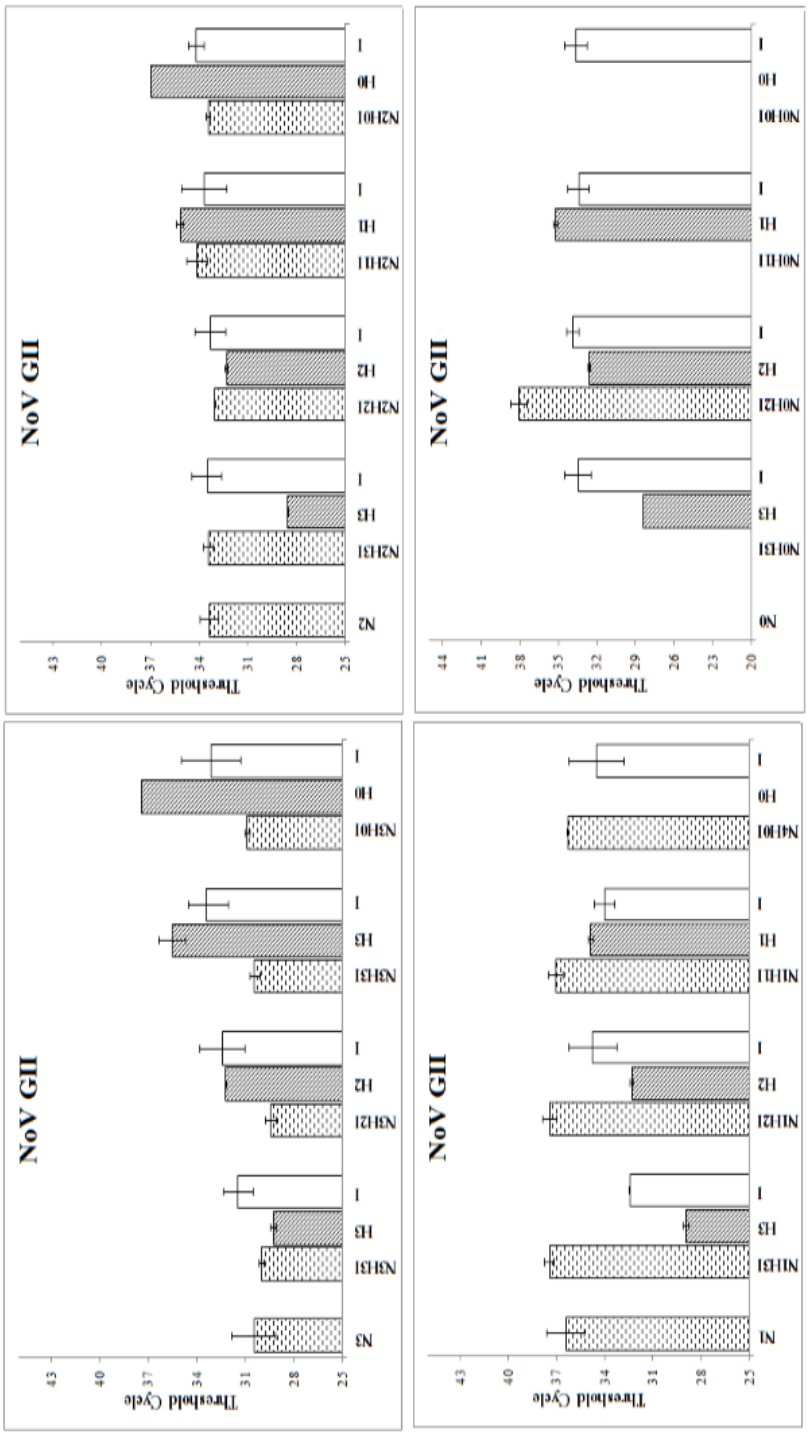

Figure 3.7: Results of NoV GII (N) triplex assays in combination with HAV (H) and the IAC (I), respectively, tested with four different serial ten-fold dilutions of NoV GII. The error bards indicate the standard deviation between the respective triplicate reactions.

3.3.5.3.1 *Statistical analysis for the multiplex assays of NoV GII, HAV and the IAC*

In the optimisation of the multiplex *rt* RT-PCR assay form the individual singleplex assays, deviations of less than a $\log_{10}$ factor were noticed and found to be statistical significant (overall p-factor for HAV at dilution H3: $p = 0.0047$) but for the purpose of this study it was considered irrelevant. In the detection of HAV at H3 = 3 x 10^6 copies/mℓ, a Ct-value mean of Ct 28.12 for the singleplex assay, Ct 29.02 for the triplex assay HAV, IAC and mengovirus, Ct 29.23 for the triplex assay HAV, NoV GII and the IAC and 28.33 for the duplex assay HAC/IAC were statistical significant deviations it was not considered analytical significant. Furthermore, no statistical data was available for the dilution H0/N0 = 3 x 10^3 copies/mℓ for either HAV or NoV GII since these viruses were not detected in the singleplex assays but rather in the duplex and triplex assays.

In the case of the IAC, statistical ($p = 0.000$) and analytical significant deviations were noticed, however, NoV GII was detected in each of the incidences where an analytical significant difference were noted. For the statistical significant deviations a 95% confidence interval of approximately 19.77-45.05 and 16.513-49.68 were noticed which deviated from the overall confidence interval of 33.10-33.5. These differences were only noted in the event where the IAC was detected in 2/3 of the replicates. The differences were noticed in the combinations: i) N3H2I and ii) N3H0I where NoV GII was present at an increased concentration (N3 = 3 x 10^6 copies/mℓ) and where an increased amount of competition for primers and nucleotides existed.

3.3.6 Optimisation of reaction B

3.3.6.1 Triplex assay for NoV GI, HRV and SaV

A sequence alignment of the NoV GI and mengovirus primer and probe set with the respective PCR fragments was done. The analysis indicated a 72% (13/18), 54% (14/26) and 65% (13/20) sequence similarity between the mengovirus forward (18 bp)- and reverse (26 bp) primer and probe (20 bp) sequence, respectively, to that of the NoV GI PCR fragment sequence. Mengovirus was therefore removed from the triplex assay and replaced by SaV. The abbreviation n for NoV GI, h for RV-SA11 and S for SaV was used and a numerical value was specified to NoV GI (n3 = 3.4 x 10^6 - n0 = 3 x 10^3

copies/mℓ), RV-SA11 (h3: 8.5 x 10^6 - h0 = 8.5 x 10^3 copies/mℓ) and SaV (S3 = 1.7 x 10^7 – S0 = 1.7 x 10^5 copies/mℓ) for each of the respective dilutions used. The mean Ct-value and standard deviation was determined for the singleplex assays and compared to that of the duplex and triplex assays and summarised in Figure 3.8. No analytical significant deviations were noted, however, a Ct-value mean deviation of +/- 2.7 was detected for SaV at a dilution S0n3h3 (S0 = 1.7 x 10^5 copies/mℓ) when compared to the singleplex assay. It was further recognized that RV-SA11 could not be detected at the lower concentration, h0 = 8.5 x 10^3 copies/mℓ, when i) SaV at dilution S0 = 1.7 x 10^5 copies/mℓ and S3 = 1.7 x 10^7 copies/mℓ and ii) NoV GI at dilution n3 = 3.4 x 10^6 copies/mℓ was present, however, RV-SA11 at the h0 dilution was detected at other combinations (h0n0, h0S3n0 and h0n3S0) for reasons that could not be explained.

3.3.6.1.1 *Statistical analysis for the multiplex assays of NoV GI, RV-SA11 and SaV*

Statistical data for NoV GI (n3: p = 0.0078, n1: p = 0.0625 and n0: p = 0.0217) indicated significant differences for two of the dilutions tested, however, for the purpose of this study these differences were not considered important as the overall and individual standard deviations were not analytically significant. Furthermore, the statistical differences noted for NoV GI at the dilution n0 were due to the inability to detect the virus at all three the replicates where in some instances NoV GI could only be detected in 0/3, 1/3 or 2/3 replicates (data not shown).

In the case of RV-SA11, the statistical data for the different dilutions tested (h3: p = 0.000, h1: p = 0.3116 and h0: p = 0.1993) indicated the existence of significant statistical differences between the Ct-value means of the singleplex assays compared to that of the individual duplex and triplex assays. No analytical significant deviations were noted for all the dilutions and combinations of the multiplex *rt* RT-PCR assays when compared to the singleplex *rt* RT-PCR assay. Furthermore, RV-SA11 at the h0 = 8.5 x 10^3 copies/mℓ dilution could be detected in 1/3, 2/3 and once in all three replicates (data not shown).

In the statistical analysis of SaV at the different dilutions tested (S3: p = 0.4821, S1: p = 0.1611 and S0: p = 0.1138) little statistical significant differences were noted among the different assays evaluated. The only exception was noted for the dilution S0 = 1.7 x 10^5 copies/mℓ with a 95% confidence interval of 30.56 - 31.708, however, with an overall

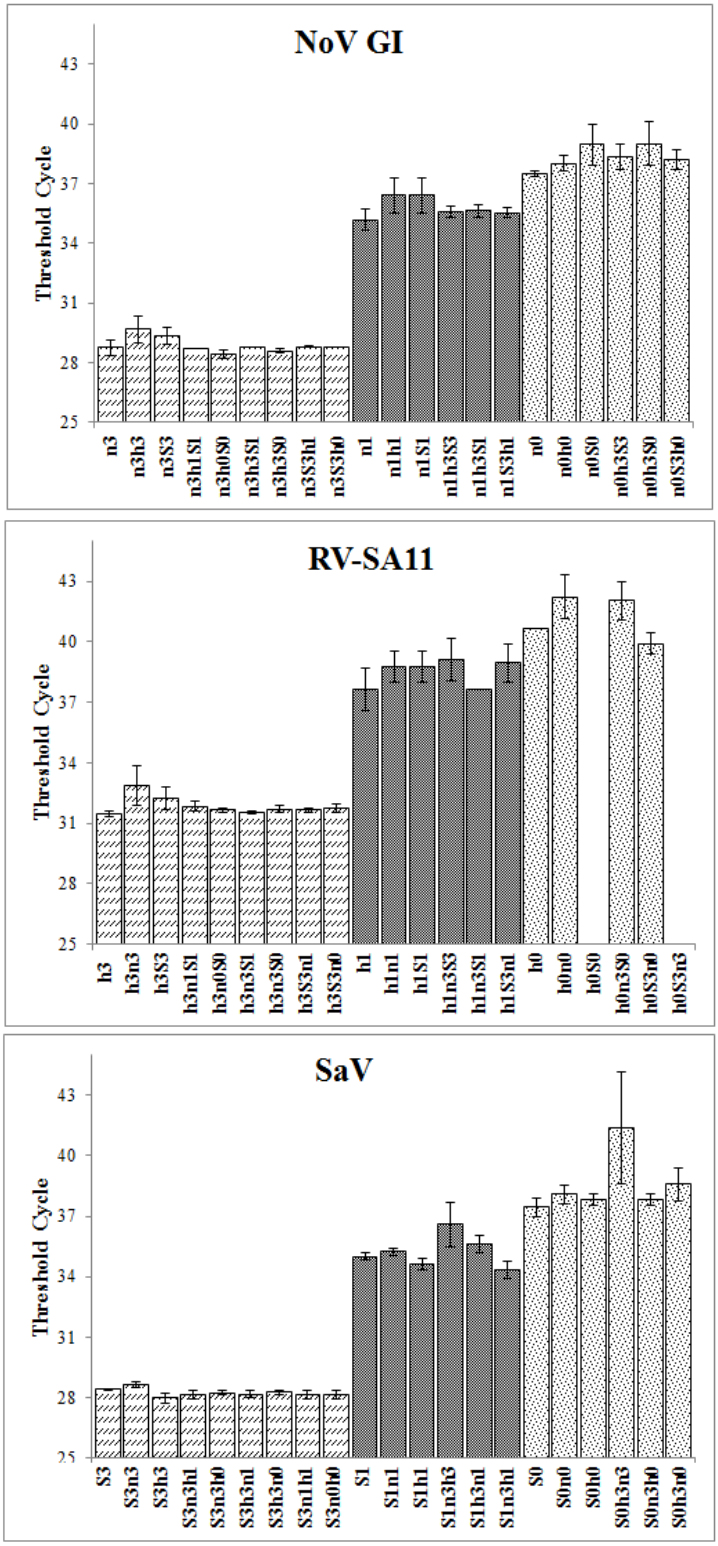

Figure 3.8: Summary of results from the amplification of NoV GI (n), simian RV (SA11) (h) and SaV (S) in singleplex assays and then amplified simultaneously in duplex and triplex assays at set concentrations.

standard deviation of 0.211 between the Ct-value means, the difference were not considered analytically significant. Furthermore, the data for SaV at a dilution S0 = 1.7

x 10^5 copies/mℓ, showed no statistical significant deviations but were considered analytically significant. The detection of SaV at the S0 = 1.7 x 10^5 copies/mℓ dilution (Ct-value mean: 37.433 (95% confidence interval of 36.328 - 38.538) in a triplex assay with RV-SA11 (h3 = 8.5 x 10^6 copies/mℓ) and NoV GI (n3 = 3.4 x 10^6 copies/mℓ) an analytical significant difference (Ct-value mean: 41.36 (95% confidence interval of 34.38 - 48.34)) were noted. Furthermore, when NoV GI (S0n3h0: 95% confidence interval of 36.95 - 38.69) or RV-SA11 (S0h3n0: 95% confidence interval of 29.31 - 47.87) were present with SaV, no analytical significant differences were noted with individual mean Ct-values for SaV of 37.82 and 38.59 recorded, respectively.

3.3.7 Optimisation of reaction C

3.3.7.1 Triplex assay for HAstV, the IAC and mengovirus

In the optimisation of the triplex assay for HAstV, the IAC and mengovirus the abbreviation A for HAstV. I for IAC and M for mengovirus was used and a numerical value was specified to each target according to the respective dilution used (A3 = 1.7 x 10^7 - A0 = 1.7 x 10^4 copies/mℓ) (Figure 3.9). Analytical significant differences were noted for HAstV at the dilutions A1 and A0 with the simultaneous amplification of the IAC and mengovirus. An increased HAstV primer and probe concentration (10 µM and 4 µM, respectively) with a decreased mengovirus primer concentration (4 µM to 2 µM) was used in the further optimisation of the triplex assay, however, HAstV remained undetected at the A0 dilution. A $\log_{10}$-factor deviation for HAstV at the dilution A1 = 1.7 x 10^5 copies/mℓ was noted. Analytical significant deviations remained after the mengovirus primer concentration decreased to 1 µM per reaction, although HAstV could be detected at the dilution A0 = 1.7 x 10^4 copies/mℓ (Figure 3.10). Sequence analysis showed a 58% (15/26 bp) sequence identity between the mengovirus reverse primer and HAstV PCR fragment, overlapping the region to which the HAstV probe anneals.

3.3.7.1.1 *Statistical analysis for the triplex assay for HAstV, the IAC and mengovirus*

Analysis of the statistical data for HAstV at the different dilutions (A3: p = 0.6825, A2: p = 0.0238, A1: p = 0.0683 and A0: p = 0.6273) indicated both significant and

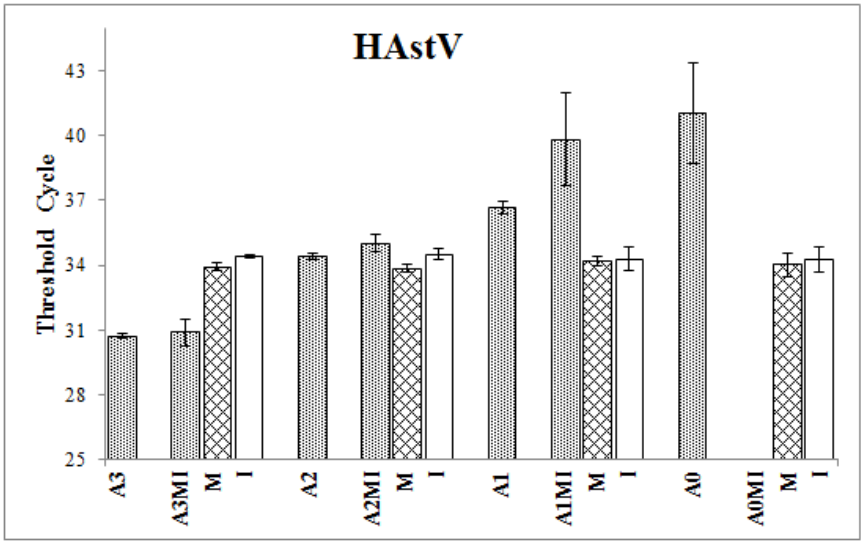

Figure 3.9: Summary of the amplification results of HAstV (A) in a singleplex assay and then in combination with the internal amplification control (I) and mengovirus (M) in a triplex assay. The error bars indicate the standard deviation present among the individual three replicates per dilution tested.

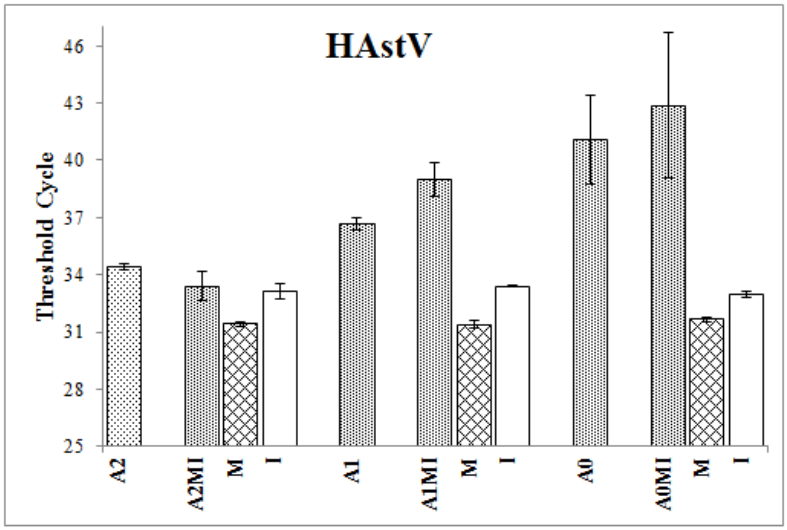

Figure 3.10: Summary of the amplification results for HAstV (A) in a singleplex assay and compared to the triplex assay with the internal amplification control (I) and mengovirus (P), with a reduced primer concentration for mengovirus used. The error bars indicate the standard deviation present among the individual three replicates per dilution tested.

analytical deviations were noted. For the purpose of the study, the statistical significant deviation noted for the HAstV dilution A2 was not considered important as there were no analytical differences among the data at this dilution. Analytical differences were, however, noted for the dilutions A1 = 1.7 x 10^5 copies/mℓ and A0 = 1.7 x 10^4 copies/mℓ

where the A1 mean Ct-value: 36.67 (singleplex assay) deviated by more than three cycles when compared to the Ct-value means of the triplex assays (A1M1I mean Ct-value: 38.997 and A1M2I mean Ct-value: 39.827). Furthermore, for the dilution A0 = 1.7 x 10^4 copies/mℓ the analytical difference was noted between the individual replicates (A0 standard deviation: 2.333; A0M1I standard deviation: 3.811).

In the analysis of the IAC with an overall mean Ct-value of 33.305, no analytical significant deviations of the means (Ct-values of ~31 and ~34) were noticed. Analysis of the data for mengovirus with HAV and/or the IAC showed no analytical significant deviations with a 95% confidence interval of the means ranging from 27.3826 - 32.5949. Mengovirus amplified simultaneously with HAstV at the different dilutions (Figure 3.9) with the IAC had an overall Ct-value mean of 33.998 with an overall standard deviation of 0.16834 and a 95% confidence interval of 33.2740 - 34.7226. However, when compared to the lowered 1 µM primer concentration of mengovirus (Figure 3.10), an almost analytically significant difference was noted between the overall Ct-value means of 31.499, the overall standard deviation of 0.1449 and a 95% confidence interval of 30.8754 - 32.1224.

3.3.8 Evaluation of multiplex assays using environmental samples

3.3.8.1 Environmental samples

A total of 19 water samples from eight surface- and three ground water sources were collected during the 5 mo period from the Mpumalanga province in SA (Table 3.5).

3.3.8.2 Viral analysis by separate *rt* RT-PCR assays

Analysis of the water samples was based on the absence or presence of the viruses in the water samples and the results of both the one-step *rt* RT-PCR and triplex assays were summarised in Table 3.6. Based on the results, the one-step *rt* RT-PCR assay was more sensitive in the detection of HAV and mengovirus than that of the NoV GII/HAV/IAC and HAstV/IAC/mengovirus triplex assays, respectively. The one-step *rt* RT-PCR assay could detect the presence of HAV in three of the water samples which could not be detected by the triplex assay. However, the triplex assay did manage to detect HAV in a single water sample which was not detected by the one-step *rt* RT-PCR assay. In the case of NoV GI and NoV GII, the triplex assay was more sensitive in their detection

Table 3.5: Surface waters collected from the Mpumalanga province, SA. Different sample points were used over a 5 month period. Indicated below is the site number allocated, time of collection and the source of the sample.

Site No.	Date	Sample type
1	2012/08/13	Bloed river
5	2012/08/13	River water before Sandriver
7	2012/03/27 2012/05/14 2012/06/26 2012/08/13	Sandriver
9	2012/04/24 2012/08/13	Borehole water: Cement dam
16B	2012/08/13	Kareebosch dam
17B	2012/03/27 2012/05/14 2012/06/26 2012/08/13	KallieSnyman dam
23	2012/08/13	River water Seshego final
Pivit	2012/03/27 2012/05/14	Irrigation pivot
KBBE	2012/04/24	Borehole water
KPL2	2012/04/24	Borehole water
KPL1	2012/06/26	Irrigation water

being able to detect these viruses in a single and six additional water samples, respectively, as appose to the one-step *rt* RT-PCR assay.

3.4 DISCUSSION

Difficulties in the detection of enteric viruses in food and water is a well-recognised problem due to the large variety and complexity of samples, the low copy numbers of the viral particles and the presence of inhibiting substances (Rodríguez-Lázaro *et al*., 2012). Real-time RT-PCR based assays have become the method of choice for the routine viral detection and surveillance and have been reinforced by the European Committee of Standardisation to serve as the basis for the detection of NoVs and HAVs (Rodríguez-Lázaro *et al*., 2012). Molecular-based assays such as multiplex assays have the advantage over singleplex assays that if optimised right, provides the information needed quickly and inexpensively to identify the viral agent(s) in an outbreak situation to in order to take the necessary steps in managing the further spread of the disease.

Table 3.6: Results of the comparative analysis of the Limpopo water samples using the triplex assays and commercial singleplex one-step *rt* RT-PCR assays. Differences between the singleplex and multiplex assays are indicated in red.

Sample Name/No.	Sampling Date	ceeramTools™ *rt* RT-PCR assay				Multiplex *rt* RT-PCR assays						
		HAV	NoV GI	NoV GII	Mengovirus	HAV	HAstV	HRV	NoV GI	NoV GII	SaV	Mengovirus
1	2012/08/13	-	-	+	+	+	+	+	+	+	+	-
5	2012/08/13	+	+	+	+	+	+	+	+	+	+	-
7	2012/03/27	+	+	+	+	+	+	+	+	+	+	+
	2012/05/14	+	+	+	-	+	+	+	+	+	+	-
	2012/06/26	+	+	+	-	+	+	+	+	+	+	-
	2012/08/13	+	+	+	-	+	+	+	+	+	+	-
9	2012/04/24	+	+	+	+	+	+	+	+	+	+	+
	2012/08/13	-	-	-	-	-	-	-	-	-	-	+
16B	2012/08/13	+	+	+	-	-	+	+	+	+	+	-
17B	2012/03/27	+	+	+	+	+	+	+	+	+	+	+
	2012/05/14	+	+	+	+	+	+	+	+	+	+	-
	2012/06/26	-	-	-	-	-	-	-	+	+	+	-
	2012/08/13	-	-	+	-	-	+	+	+	+	-	-
23	2012/08/13	+	+	+	-	+	+	+	+	+	+	-
Pivot	2012/03/27	+	-	+	+	+	+	-	+	+	+	+
	2012/05/14	+	+	+	+	-	+	+	+	+	+	-
KBBE	2012/04/24	-	-	-	+	-	-	-	-	-	-	+
KPL2	2012/04/24	+	-	+	+	-	+	+	+	+	-	-
KPL1	2012/06/26	+	-	+	-	+	+	+	+	+	+	-

In the design of a multiplex assays, commercially available kits may provide some advantages over "in-house" protocols (De Paula *et al*., 2004) with the added advantage of being available to the public. Commercially available *rt* RT-PCR multiplex kits were evaluated on their sensitivity for the detection of mengovirus, NoV GII and HAV, with the two last viruses mentioned considered worldwide as the leading causes of viral gastroenteritis and hepatitis, respectively (Bull *et al*., 2006; Okada *et al*., 2007; Siebenga *et al*., 2009). The results obtained were similar to that reported by De Paula *et al*. (2004) where the one-step *rt* RT-PCR assays were found to be more sensitive, despite the detection method used, than the two-step *rt* RT-PCR assays (Table 3.3). The true detection limit of the one-step *rt* RT-PCR kits might have been masked by the method used in the analysis of the results, however, the assays remained sensitive in the detection of NoV GII and HAV in the singleplex assays. Contrasting results were noted in the sensitivity of the two one-step *rt* RT-PCR in the detection of mengovirus and HAV and thus no conclusion could be made as to which kit was more sensitive.

In the analysis of the two-step *rt* RT-PCR assays, clear differences were noted between the cDNA synthesis kits used as appose to the two-step *rt* RT-PCR kits where no differences in the detection of NoV GII, HAV and mengovirus were noted. An environmental sample was used to determine the difference in the use of random primers (two-step assay) as compared to specific primers (one-step assay) for cDNA synthesis to determine which PCR kits were better optimised for a multiplex analysis. The use of random primers for cDNA synthesis with the EXPRESS qPCR Super Mix Universal kit (Invitrogen) proved to be more efficient in the detection of the viral targets than the use of specific primers. It was evident that the EXPRESS qPCR Super Mix Universal kit (Invitrogen) in combination with the RevertAid Premium™ First Strand cDNA synthesis kit (Fermentas Life Sciences) was the optimal assay for the use in the design of the multiplex assays to follow. With the further reduction in the RT-enzyme concentration (50 U/µℓ) (Figure 3.2), the costs of the multiplex *rt* RT-PCR assays were further reduced.

A multiplex assay designed by Stals *et al*. (2009) presented the opportunity to simultaneously detect NoV GI and GII in a single reaction using the same sets of

primers and probes as used in this study. Attempts to optimise such a duplex assay failed as the NoV GII probe could also detect the amplified NoV GI PCR fragment, resulting in a false positive test result. The reaction was repeated and similar results were obtained. Sequence analysis of the NoV GI probe aligned to the NoV GII.4 full genome sequence (Genbank accession No: KF712497.1) showed an 80% (16/20) sequence similarity in the amplified region. These results were in contrast to that presented by Stals *et al*. (2009) and resulted in the optimisation of separate multiplex assays for NoV GI and NoV GII.

Norovirus GII.4 is renowned as the causative agent in outbreaks of gastroenteritis worldwide and was proposed together with HAV, the IAC (Chapter 2) and mengovirus to form part of the a multiplex assay. The IAC was optimised for NoV GII as a one-step *rt RT-PCR* assay (Chapter 2), however, for the use in a two-step *rt* RT-PCR assay the concentration of the IAC was optimised. The IAC posed little competition to the detection of HAV (Figure 3.6) and NoV GII (Figure 3.7) despite the concentration of NoV GII used. These results were in contrast to the one-step *rt* RT-PCR results (Chapter 2) where the smaller PCR fragment size of NoV GII outperformed the amplification/detection of the larger IAC resulting in the detection of the IAC in very low concentrations or in the absence of NoV GII.

The triplex assay for HAV with the IAC and mengovirus, either individually or simultaneously, posed no significant deviation to the reaction when compared to the singleplex assay of HAV (Figure 3.5). What was interesting to note was the detection of HAV at the dilution H0 in duplex assays, a feature that was not possible when HAV was detected alone or in a triplex assay with these controls. The duplex assay of NoV GII and mengovirus were unsuccessful due to the cross reactivity of the mengovirus probe to that of the NoV GII PCR product. Despite the attempts made in optimising this *rt* RT-PCR assay, NoV GII, as also noted for NoV GII (based on sequence analysis) remained elusive at the lower concentrations (Nl = 3 x 10^4 and N0 = 3 x 10^3 copies/mℓ), with more than a $\log_{10}$-factor deviation at the dilution N2 = 3 x 10^5 copies/mℓ (data not shown). With the inability to simultaneously detect NoV GII and mengovirus in a single reaction, the duplex assays for NoV GII and the IAC followed by the triplex assay with HAV, NoV GII and the IAC was further optimised. The results of this triplex assay correlates well with the results of the individual singleplex assays for HAV

(Figure 3.6) and NoV GII (Figure 3.7), despite the presence of the individual concentrations of each of the viruses present. The detection of HAV at the dilution H0 = 3 x 10^3 copies/mℓ was only achieved in 1/3 of the replicates for the triplex assay. This indicated that the multiplex assay, as with the duplex assays for HAV with the individual controls, was more sensitive than that of the singleplex assay.

Norovirus GI, associated with shellfish linked gastroenteritis outbreaks (Atmar, 2010), and HRV, a leading cause of severe diarrhoea in young children under the age of 5 years (Parashar *et al*., 2006; Estes and Kapikian, 2007) were grouped into a triplex assay with SaV. In determining the sensitivity of the duplex and triplex assays a combination of the two and three viral targets at different concentrations/dilutions, respectively, were evaluated. The most notable deviations were noticed at the lower concentrations, in particularly for RV-SA11 and SaV when the other target(s) were present in the same or higher concentrations (Figure 3.8). Sapovirus showed little overall deviations in comparison to the singleplex reactions except in one combination (S0h3n3) where more than a $\log_{10}$-factor deviation was noted. Simian RV was not detectable in two combinations (h0S0 and h0S3n3), however, detection at the concentration h0 = 8.5 x 10^3 copies/mℓ varied. Little variation existed between the two assays and a similar level of sensitivity was noted between each of the assays.

Human AstVs, less known for foodborne outbreaks but most often associated with diarrhoeal outbreaks of gastroenteritis in children younger than 2 years of age (Kirkwood *et al*., 2005; Moser and Schultz-Cherry, 2005; Scallan *et al*., 2011a), was added to a third triplex assay along with the IAC, optimised for NoV GII but used as a non-competitive AC functioning as an ever present third target in the assay, and mengovirus. The mengovirus primers and probe sequence were, prior to the optimisation of the assay, aligned with the HAstV amplicon and a 46% (12/26) sequence similarity was seen of which 19% (5/26) could anneal to the HAstV forward primer region. Detection of HAstV in the triplex assay for the first two dilutions (A3 = 1.7 x 10^7 and A2 = 1.7 x 10^6 copies/mℓ) coordinated well with that of the singleplex assay (Figure 3.9), however a $\log_{10}$-factor deviation was seen in the A1 = 1.7 x 10^5 copies/mℓ dilution and the total absence of HAstV in the A0 = 1.7 x 10^4 copies/mℓ dilution was noticed. The decrease in the mengovirus primer concentration per reaction allowed the detection of HAstV at the A0 = 1.7 x 10^4 copies/mℓ lowest dilution (Figure

3.10), however, a $\log_{10}$-factor deviation at the dilution A1 = 1.7 x 10^5 copies/mℓ remained. The triplex assay would most likely perform well in the detection of HAstV in clinical samples where a high viral load is expected, but for environmental samples the risk exists that HAstV might not be accurately or at all detected. A singleplex assay was therefore suggested for HAstV if environmental samples were to be tested, with mengovirus and the IAC forming a duplex assay that serves well in identifying false negative test results prior to analysis with the above viral assays.

The triplex assays, besides being validated against their respective singleplex assays, were compared to commercially obtained singleplex one-step *rt* RT-PCR assays for NoV GI, NoV GII, HAV and mengovirus to compare the detection efficiency and sensitivity of the respective assays. The optimised multiplex *rt* RT-PCR assays had a clear advantage in the detection of NoVs (Table 3.6), making them ideal for the use in gastroenteritis outbreaks where these and other selected enteric viruses are suspected. The NoV GII/HAV/IAC and HAstV/IAC/mengovirus triplex assays, however, was not as sensitive in the detection of HAV and mengovirus, respectively, as that of the one-step *rt* RT-PCR assay. Possible reasons could be the different concentrations of nucleic acid used in each of the two assays and/or the use of random as compared to specific primers used in each assay. Additional viruses was also detected with the optimised multiplex *rt* RT-PCR assays and was more cost effective than the one-step *rt* RT-PCR assay.

3.5 CONCLUSION

The primary objective of this study was to develop cost effective, time saving multiplex assays that was more than or equally as sensitive as the individual singleplex assays. A large reduction in cost and an increase in sensitivity were made with the implementation of the RevertAid Premium™ First Strand cDNA synthesis kit (Fermentas Life Sciences) and EXPRESS qPCR Super Mix Universal kit (Invitrogen) for the optimisation of the individual multiplex assays. The implementation of an IAC allowed the identification of false negative test results, with mengovirus used as a process control for the viral recovery methods. Two triplex assays; i) NoV GII, HAV and the IAC, and ii) NoV GI, HRV and SaV were developed that are equally as sensitive and could be implemented for the detection of the selected enteric viruses in environmental samples. A third

triplex assay, HAstV, the IAC and mengovirus was optimised for the use in environmental samples, however, the assay would possibly perform better in the detection of HAstV in clinical samples than environmental samples. Additionally, a duplex assay containing both the IAC and mengovirus was shown to perform well in the pre-screening of samples for inhibitory compounds prior to the use of each of the above triplex assays. As a result in the lack of sensitivity of HAstV at lower concentrations, a sensitive singleplex assay was optimised for the use in environmental samples. Overall, the triplex assays developed and optimised in this study showed to have a higher or an equal amount of sensitivity to their individual singleplex assays or commercially obtained one-step *rt* RT-PCR assays. The triplex assays allowed for fast, accurate and sensitive results, with the exception to the triplex assay HAstV/IAC/mengovirus.

CHAPTER 4

OPTIMISATION OF METHODS FOR THE RECOVERY OF SELECTED ENTERIC VIRUSES FROM STRAWBERRIES

4.1 INTRODUCTION

The burden of human illness caused by foodborne pathogens remains largely unknown and is of growing public health concern worldwide (Newell *et al*., 2010; WHO, 2012). A large number of microbial, chemical and physical foodborne contaminants exist, although microbial contaminations, particularly in the industrialised world have been on the increase over the past two decades (Acheson, 1999; Newell *et al*., 2010). Enteric pathogens and other contaminants can be introduced via faecal discharge, soil, irrigation water, sewage, human handling and during the processing of food sources (Newell *et al*., 2010). Fresh produce, in particularly berries, have been increasingly associated with outbreaks of foodborne illness, such as gastroenteritis and hepatitis in several countries worldwide (Niu *et al*., 1992; Hutin *et al*., 1999; Friedman, 2005; Lynch *et al*., 2009; Maunula *et al*., 2009; Berger *et al*., 2010; Brassard *et al*., 2012).

Prevention of outbreaks relies on the optimisation of adequate methods for the recovery and detection of enteric pathogens (Kim *et al*., 2008). Various methods for the recovery of enteric viruses, namely NoVs, HAV, PV, aichi virus and FCV (used as a process control), from fresh produce and strawberries have been developed and optimised (Bidawid *et al*., 2000; Boxman *et al*., 2007; Butot *et al*., 2007; Croci *et al*., 2008; Fino and Kniel, 2008). However, a single universal validated and accepted method for the detection of enteric viruses from fresh produce may not be possible due to the differences encountered in the morphology, tissue composition, processing conditions as well as the hydrophobic interactions of different fruit and vegetables with the different enteric viruses (Croci *et al*., 2008).

The detection of viruses in berries is specifically problematic due to the presence of inhibitory factors and low pH. Viral recovery from berries has been overall poor and is believed to be attributed to a drop in pH due to the acidity of the berries (Butot *et al*., 2007). A possible solution to counter such pH changes is the addition of TRIS which is

used to maintain the pH of the elution buffer within a narrow range. Kim *et al*. (2008) compared the efficiency of five elution buffers for the recovery of NoV GII.4 from strawberries and other selected fresh produce. The study evaluated various factors in the recovery process and concluded that the molecular weight of PEG was not a contributing factor, that a buffer with a higher rather than a lower protein content was more efficient and that an elution period period of 4 h was more efficient. Based on the results of Butot *et al*. (2007) and Kim *et al*. (2008), five elution buffers were assessed for the recovery of selected enteric viruses from strawberries and the recovered virus suspension was screened by the optimised multiplex *rt* RT-PCR assays (Chapter 3). The elution buffers were evaluated based on the following:

i) protein concentration (3% beef extract vs 0.5% bovine serum albumin [BSA]),
ii) elution buffer pH (pH 7.2 vs pH 9.5),
iii) pH stability (elution buffers with or without TRIS), and
iv) an elution period of 20 min vs 5 h.

4.2 MATERIALS AND METHODS

4.2.1 Viral stock

The viral stocks used in this study were identical to those used in Chapter 3 (section 3.2.1). All seven viruses, namely, HAV, HAstV, RV-SA11, NoV GI, NoV GII, SaVs and mengovirus (process control) were used in this study. For the purpose of the seeding experiment, a 1 mℓ viral stock suspension was made where all seven viruses were pooled together. The individual viral concentrations per mℓ and per seeding volume (20 µℓ) are summarised in Table 4.1.

4.2.2 Primers and probes

Published sets of highly specific and sensitive primers and probes (Table 3.1), used in the optimisation of three multiplex *rt* RT-PCR assays (Chapter 3), were used in this study.

Table 4.1: Pooled viral stock concentration and working concentration.

Virus	Stock concentration: (copies per mℓ)	Working concentration: (copies per 20 µℓ)
HAV	1.0×10^7	2.0×10^5
HAstV	6.7×10^8	1.3×10^7
NoV GI	4.9×10^8	9.8×10^6
NoV GII	2.9×10^8	5.7×10^6
SaV	2.8×10^9	5.6×10^7
RV-SA11	1.9×10^8	3.9×10^6
Mengovirus	2.0×10^6	4.0×10^4

4.2.3 Optimisation of nucleic acid extraction method

In this study three nucleic acid extraction methods, namely,

i) the QIAmp® UltraSens® Virus kit (Qiagen),

ii) the semi-automated NucliSens® easyMAG™ system (Biomérieux), and

iii) the fully automated MagNA Pure LC instrument (Roche Diagnostics) using the MagNa Pure LC Total Nucleic Acid Isolation Kit (large volume) (Roche Diagnostics) were used according to the manufacturer's recommendations, respectively. The extracted nucleic acid was eluted into 100 µℓ elution buffer, aliquoted and stored at -70°C.

Strawberries (3) and blackberries (6) were divided equally into three groups A, B and C (each group containing a single strawberry for the strawberry groups or two blackberries for the blackberry groups). The berries were immersed in 30 ml of the TGBE-9.5 elution buffer for 30 min in a shaking incubator (100 rpm) and the pH of the suspension adjusted to pH 7 using 1 M HCℓ (Merck) and/or 1 M NaOH (Merck) as required. Each of the suspensions, containing berry fruit juices and other foreign particles, were seeded with NoV GII (1.1×10^4 copies/mℓ), HAV (1×10^4 copies/mℓ) and mengovirus (1.3×10^4 copies/mℓ). The viruses were further recovered from the suspensions using the optimised recovery method mentioned in section 4.2.7, with the exception that the pellet was re-suspended in 3 mℓ of PBS (Sigma-Aldrich Co.). A 1 mℓ of the PBS suspension was used for nucleic acid extraction for each of the three extraction method evaluated. In-house singleplex assays for the detection and quantification of NoV GII, HAV and mengovirus were used. The cDNA synthesis and PCR reaction preparation along with the cycling conditions used were identical to that described in Chapter 3 (section 3.2.9.1). An amendment for the HAstV/IAC/mengovirus *rt* RT-PCR assay was made

by decreasing the HAstV probe concentration from 4 µM to 3 µM as this resulted no change observed in the detection of HAstV.

4.2.4 Evaluation of the optimal viral recovery method for viruses from strawberries

4.2.4.1 Reagents and elution buffers

The elution buffers used in this study were as follows:

i) *Glycine 3% beef extract (GBE) buffer (pH 7.2 (GBE-7.2) or pH 9.5 (GBE-9.5))*: 3.8 g/ℓ glycine (Merck), 30 g/ℓ beef extract (BBL™ Becton Dickinson and Co.).

ii) *TRIS-glycine 3% beef extract (TGBE) buffer (pH 7.2 (TGBE-7.2) or pH 9.5 (TGBE-9.5))*: 12.1 g/ℓ TRIS base (Roche Diagnostics), glycine and beef extract as described above.

iii) *PBS/0.5% (PBS/BSA)buffer*: 5 g/ℓ BSA in 1 ℓ PBS. The buffer was filter sterilised using a Millex®-GP PES Express 0.22 µm syringe driven filter unit (Millipore Ireland Ltd, Cork, Ireland).

The GBE and TGBE elution buffers were sterilised by autoclaving and all elution buffers were stored at 4°C.

The following reagent was prepared;

i) *5 X PEG/NaCl solution (50% [w/v] PEG_{8000}, 1.5 M NaCl)*: 500 g PEG (AMRESCO LLC, Solon, OH), 87 g NaCl (Merck), as recommended by the European Committee of Standardisation (CEN TC275/WG6/TAG4 Technical Committee). The solution was made up to 1 ℓ using molecular grade water, sterilised by autoclaving and stored at room temperature (25°C).

4.2.4.2 Preparation and seeding of strawberries

Fresh strawberries were purchased from a commercial outlet. In the evaluation of each buffer, three samples consisting of five berries per sample were selected for each buffer and protocol evaluated, respectively. The leaves from the crown of the strawberry were removed and the strawberries were weighed. Three of the strawberries (1,2,3) were seeded with 20 µℓ of the prepared mixed viral suspension (Table 4.1) where as a fourth strawberry (process control) was artificially seeded with 20 µℓ of mengovirus (2.0×10^6 copies/mℓ), and the fifth strawberry with 20 µℓ of nuclease-free water (Promega Corp.). The seeded strawberries were left to dry for approximately 1-2 h in a dead box at room

temperature (25°C). To evaluate any effect the elution buffers might have on the recovery of the individual viruses, elution buffer controls were prepared by spiking 30 mℓ of each elution buffer with 20 µℓ of the mixed viral suspension.

4.2.4.3 Viral recovery protocol(s)

Using sterilised forceps individual seeded strawberries were placed into 50 mℓ centrifuge tubes (BD Biosciences, Franklin Lakes, NJ) to which 30 mℓ of the respective elution buffer had been added. The immersed strawberries were gently shaken at 120 revolutions per minute (rpm) at room temperature for either 20 min (protocol A) or 5 h (protocol B). After the respective elution periods the strawberries were removed from the centrifuge tubes and discarded. The pH of each suspension was adjusted to pH 7 using 1 M HCℓ (Merck) and/or 1 M NaOH (Merck) as required. Pectinase (5 µℓ/mℓ) (Sigma-Aldrich Corp.), used by Butot *et al.* (2007) in the reduction of PCR inhibitors, was added to each suspension and incubated for 30 min with gentle shaking at 60 rpm at room temperature. Secondary concentration was achieved by the addition of 0.25 w/v PEG_{8000}/NaCl. The suspension was allowed to incubate for 1 h with gentle shaking at 100 rpm at 4°C and concentrated into a pellet by centrifugation (Sorvall® Super T21 centrifuge, Du Pont Company, Wilmington, DE) at 10 000 x gravity for 30 min at 4°C. The supernatant was discarded and centrifuged (Sorvall® Super T21 centrifuge) at 10 000 x gravity for 10 min. The remaining supernatant was removed by aspiration and the pellet was resuspended into 2 mℓ PBS (Sigma-Aldrich Co.), aliquoted and stored at -70°C.

4.2.4.4 Multiplex *rt* RT-PCR assays

The optimised *rt* RT-PCR assays (Chapter 3) were used to determine the efficiency of each of the elution buffers for the recovery of the viruses from the berries. The *rt* RT-PCR assays used were as follows:

i) *NoV GII, HAV and IAC rt RT-PCR assay*: used to detect NoV GII and HAV with an IAC to discriminate between false negative PCR test results.
ii) *NoV GI, HRV and SaV*: used in the detection of NoV GI, HRV (RV-SA11 used as surrogate) and SaV.
iii) *HAstV, IAC and mengovirus*: used to detect HAstV and indicate the presence of inhibitory substances and the efficiency of the viral recovery method.

4.2.5 Statistical analysis

The data obtained, in the form of Ct-values, for both the nucleic acid extraction optimisation and the elution buffer assessment were analysed using the software STATA 12 version 12.1 (Statacorp. 2011 Stata Release12. Statistical Software. College Station TX: StataCorp LP.). Difference in the Ct-values of three cycles was considered analytically significant in this study.

4.3 RESULTS

4.3.1 Evaluation of nucleic acid extraction methods

The overall mean and standard deviations of the Ct-values for the three groups of berries: A, B and C for NoV GII, HAV and mengovirus and each extraction method are presented in Figure 4.1 and Table 4.2.

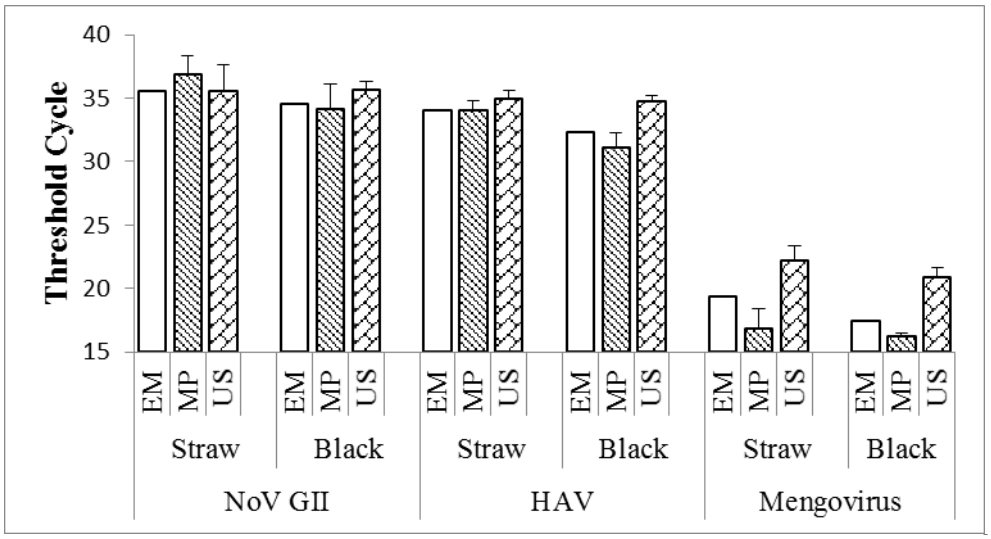

Figure 4.1: Comparative evaluation of the three different nucleic acid extraction methods namely, the NucliSens® easyMAG™ system (EM), the MagNA Pure LC instrument (MP), and the QIAmp® UltraSens® Virus kit (US) for the detection of NoV GII, HAV and mengovirus from blackberries (Black) and strawberries (Straw).

Table 4.2: Summary of the comparative evaluation of the three different nucleic acid extraction methods namely, the MagNA Pure LC instrument (Roche Diagnostics), the NucliSens® easyMAG™ system (Biomérieux SA) and the QIAmp® UltraSens® Virus kit (Qiagen) for the effective extraction of inhibitor free nucleic acid from blackberries (Black) and strawberries (Straw). The Ct-values are given as a mean of three replicates. In some instances, only two (*) or one (#) replicate could be detected of the three replicates done.

Virus	Berry	EasyMag			Overall Mean	Std. Dev§.	MagNa Pure			Overall Mean	Std. Dev.	UltraSens			Overall Mean	Std. Dev.
		A	B	C			A	B	C			A	B	C		
NoV GII	Straw	34.02	35.82	36.80*	35.55	1.41	34.50	37.76	38.46	36.91	2.11	37.00#	36.53#	33.13#	35.55	2.11
	Black	32.17	35.50	35.85*	34.51	2.03	33.37	34.43	34.56	34.12	0.65	35.63	0	0	35.63	-
HAV	Straw	33.89	33.32	34.94*	34.05	0.82	34.41	33.59	0	34.00	0.58	37.04#	31.97	35.99#	35.00	2.67
	Black	30.91	33.03	32.98*	32.3	1.21	31.38	30.60	31.31	31.10	0.43	34.05	35.15	34.98	34.73	0.59
Mengovirus	Straw	18.71	18.26	21.18	19.38	1.57	17.00	15.67	17.99	16.89	1.16	22.88	20.36	23.47	22.23	1.65
	Black	17.19	17.66	17.49*	17.45	0.24	16.97	15.46	16.42	16.28	0.76	20.18	21.19	21.17	20.85	0.58

§ = standard deviation

Analysing the mean detection Ct-value data for NoV GII from both strawberries and blackberries it was evident that the detection rates were of the same order (strawberries Ct 35.55 - 36.91; blackberries Ct 34.12 - 35.63) for all three nucleic acid extraction methods, indicating similar extraction efficiencies for all three kits. No analytical significant differences (>3 Ct-value differences) were noted between the overall Ct-value means recorded for either the blackberries or the strawberries. However, it was noted that for the NucliSens® easyMAG™ system (Biomérieux) and QIAmp® UltraSens® Virus kit (Qiagen) all three replicates for each assay were not extracted with the same efficiency as only one or two replicates from the strawberries could be detected while for blackberries no NoV GII was detected in groups B and C for the QIAmp® UltraSens® Virus kit (Qiagen). More intra-assay variation was noted when analysing the standard deviation data with the easyMAG™ system (Biomérieux) for the blackberries, whereas intra-assay variations were noted for the strawberries with the MagNA Pure LC instrument (Roche Diagnostics) and QIAmp® UltraSens® Virus kit (Qiagen). These variations were however not significant as the standard deviation was <3 deviation between the Ct-value means.

When the extraction efficiencies for individual viruses were analysed it was evident that the mean detection Ct-value data for HAV that the detection rates for the strawberries was of the same order (Ct 34.00 - 35.00) and similar extraction efficiencies were noted. However, the mean detection Ct-value data for HAV for the blackberries was not of the same order (Ct 31.10 - 34.73) with different extraction efficiencies recorded for the three nucleic acid extraction methods observed. Analytical significant differences were noted between the mean detection Ct-value data for the recovery of HAV from blackberries with the QIAmp® UltraSens® Virus kit (Qiagen) where a difference of >3 cycles were observed when compared to the MagNA Pure LC instrument (Roche Diagnostics). It was noted that the extraction efficiencies for all three replicates varied, with HAV only detected in one of the replicates in group A and C with the QIAmp® UltraSens® Virus kit (Qiagen) and in two of the replicates in group C for the NucliSens® easyMAG™ system (Biomérieux). Intra-assay variation of almost a $\log_{10}$ was noted in the recovery of HAV from the strawberries with the QIAmp® UltraSens® Virus kit (Qiagen) and almost half a $\log_{10}$ difference noted for the blackberries with the NucliSens® easyMAG™ system (Biomérieux).

As noted for HAV, the mean detection Ct-value data for mengovirus was not of the same order for both the strawberries (Ct 16.89 - 22.23) and blackberries (Ct 16.28 - 20.85) with differences of >3 Ct cycles noted in the extraction efficiencies. Analytic significant differences were mostly noted between the overall mean Ct-values of the MagNA Pure LC instrument (Roche Diagnostics) and the QIAmp® UltraSens® Virus kit (Qiagen) for both the strawberries and blackberries. Overall, similar intra-assays variations were noted among the groups for the three nucleic acid extraction methods in the recovery of mengovirus from the strawberries and blackberries. Mengovirus could efficiently be detected in all the extraction replicates for the strawberries but for the blackberries it could only be detected twice in group C when extracted with the NucliSens® easyMAG^{TM} platform (Biomérieux).

In summary, analytical significant differences were clearly noted for mengovirus. An almost $\log_{10}$ intra-assay variations was noted for HAV with the QIAmp® UltraSens® Virus kit (Qiagen) and for NoV GII with the MagNA Pure LC instrument (Roche Diagnostics) and QIAmp® UltraSens® Virus kit (Qiagen). Hepatitis A virus and mengovirus were more efficiently detected when the nucleic acid was extracted with the MagNA Pure LC instrument (Roche Diagnostics) (p = 0.0456).

4.3.2 Evaluation of recovery method with the individual elution buffers

Analysing the mean detection Ct-value data for HAV from the elution buffers evaluated it was evident that the elution efficiency of the majority of the buffers was of the same order (Ct 33.36 - 34.87) with the exception of the PBS/BSA elution buffer (Ct 36.90) (Figure 4.2A). A single analytical difference between the positive control (Ct 33.90) and the PBS/BSA Con A (Ct 36.90) were noted. The PBS/BSA B mean Ct-value (Ct 35.75) differed almost a $\log_{10}$ form the positive control but was not considered analytical significant. No analytical significant differences between the 3% beef extract buffers were noted. Based on the 95% confidence interval data a trend could be seen to which most of the data falls into, however, the PBS/BSA Con A data (Ct 36.90) did not fall into the same range as the majority of the data (Figure 4.2B).

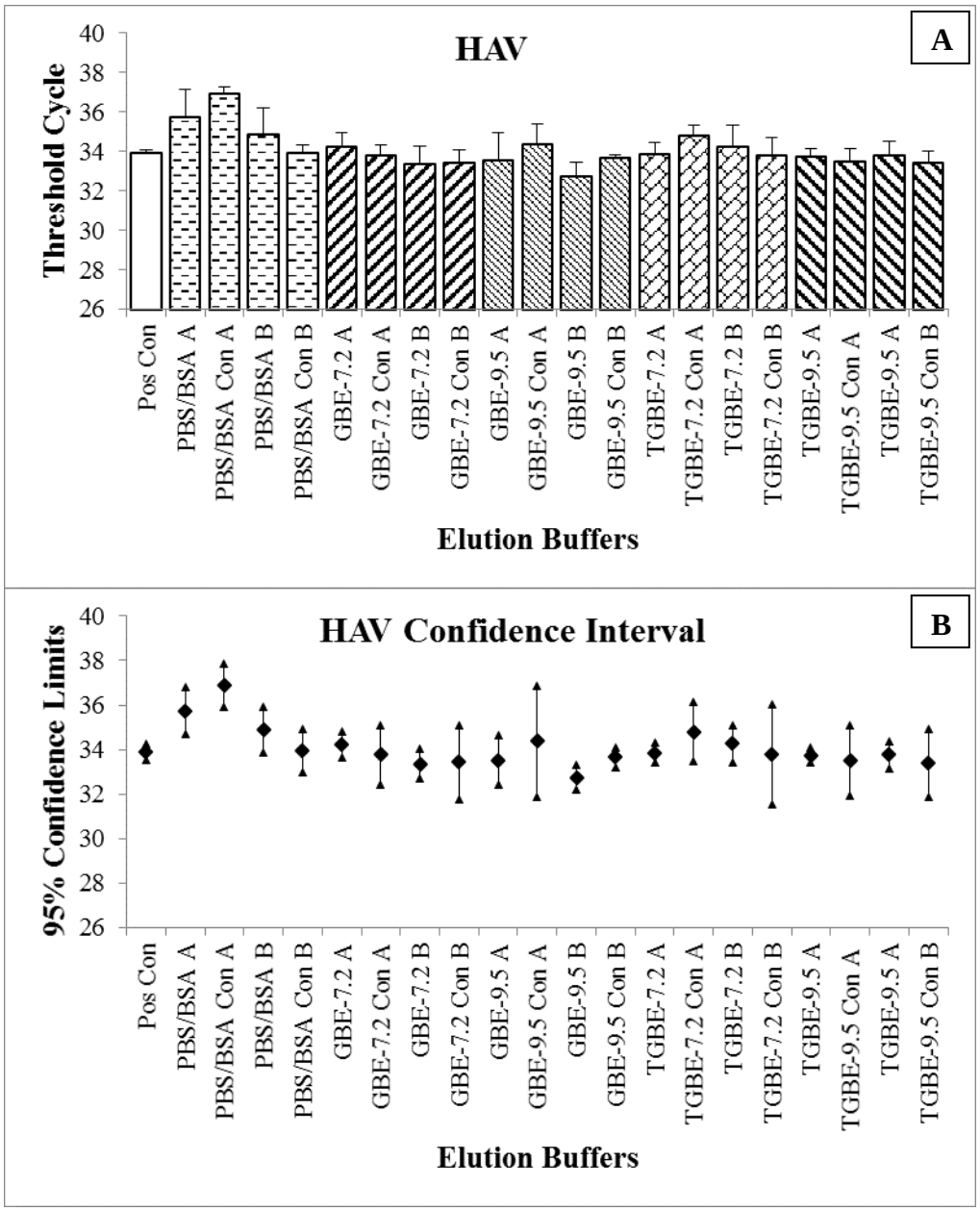

Figure 4.2: Summary of the analytical (A) and statistical (B) significant differences noted for HAV recovered from strawberries between the different elution buffers (PBS/BSA, GBE and TGBE), pH intervals (pH 7.2 and pH 9.5) and recovery protocols (A = 20 min and B = 5 h).

It was evident from the mean detection Ct-value data of the elution buffers evaluated for NoV GII that two different Ct-value ranges were noted (Figure 4.3A). The first range (Ct 33.4 - 34.33) was for the elution buffers in which the strawberries were immersed and the second range (Ct 30.39 - 32.11) for the elution buffer controls. No analytical significant differences existed between the elution buffer controls and the positive control (Ct 30.05). Analytical significant differences were noted between the two different Ct-value ranges and between the first range (elution buffers with the

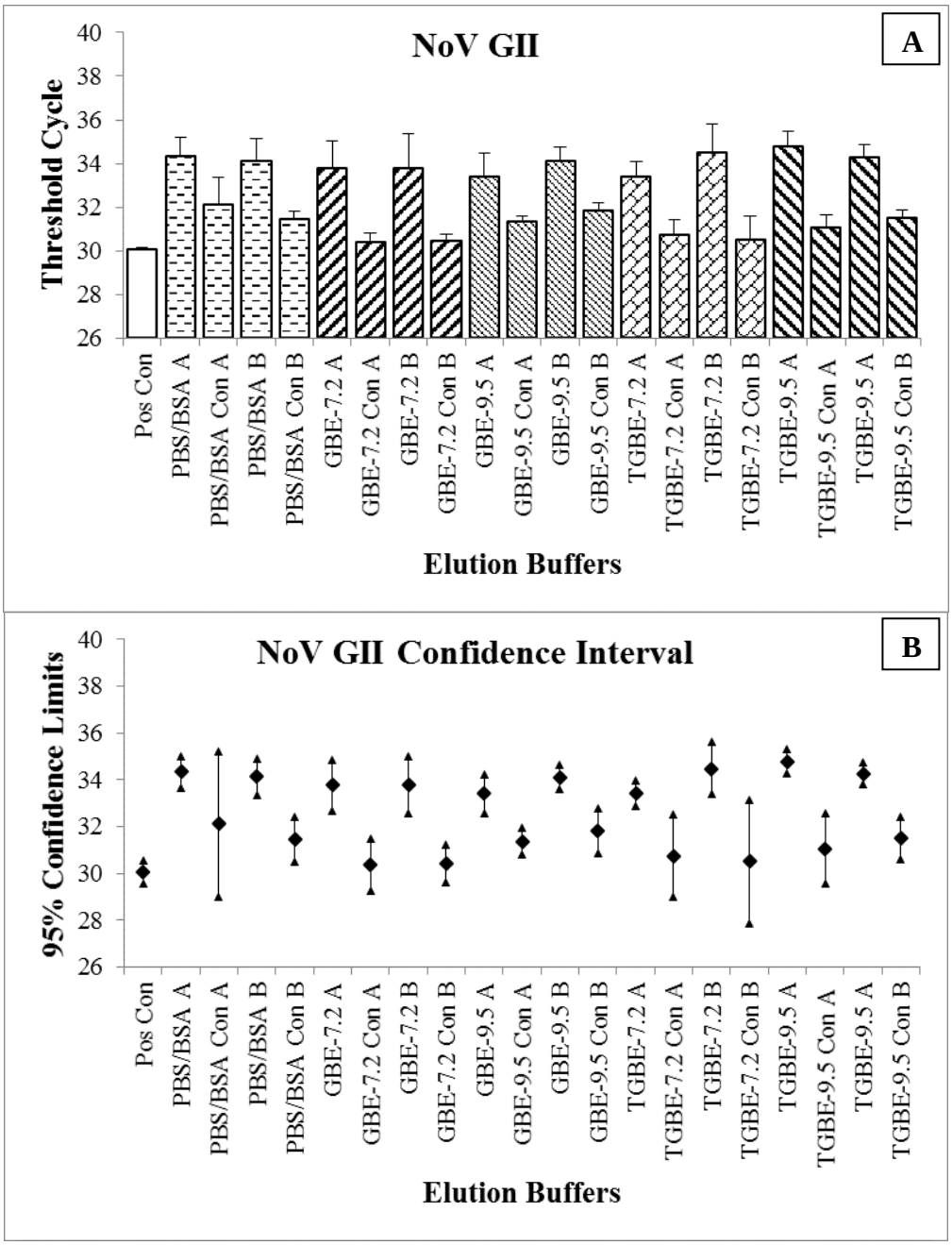

Figure 4.3: Summary of the analytical (A) and statistical (B) significant differences noted for NoV GII recovered from strawberries between the different elution buffers (PBS/BSA, GBE and TGBE), pH intervals (pH 7.2 and pH 9.5) and recovery protocols (A = 20 min and B = 5 h).

strawberries present) and the positive control (Ct 30.05). Analysis of the statistical data (Figure 4.3B) indicated the two Ct value ranges and analytical significant differences noted in the data.

In the analysis of the mean detection Ct-value data for NoV GI with the individual elution buffers it was evident that the detection rates were not within the same range and

different extraction efficiencies were noted, reflected by the mean Ct-value data for the elution buffers with the strawberries present (Ct 28.92 - 31.44) and for the elution buffer controls (Ct 26.40 - 29.79) (Figure 4.4A). Analytical significant differences, where the positive control mean Ct-value (Ct 26.33) differed >3 Ct cycles to that of recovered virus mean Ct-values, were noted for PBS/BSA A (Ct 31.31) and B (Ct 29.99), GBE-7.2 A (Ct 29.90), GBE-9.5 (Ct 29.73 - 31.04), TGBE-7.2 A (Ct 29.86) and B (Ct 30.01) and TGBE-9.5 (Ct 29.43 - 31.44). The differences noted between GBE-7.2 B (Ct 28.92) and GBE-7.2 Con B (Ct 26.40) was not considered analytically significant. Analysis of the confidence interval data indicated that the GBE-7.2/TGBE-7.2 and GBE-9.5/TGBE-9.5 grouped separately for the respective elution buffers with and without the presence of the strawberries (Figure 4.4B).

It was evident from the mean detection Ct-value data for SaV recovered from the respective elution buffers that the detection rates were not of the same Ct-value range yet most of the mean Ct-value data grouped within the range of Ct 28.60 – 31.13 with the exception of GBE-7.2 Con A (Ct 27.49), TGBE-7.2 Con A (Ct 27.54) and TGBE-9.5 A (Ct 31.71) (Figure 4.5A). In the comparison of the data to the positive control mean Ct-value (Ct 25.55), analytical significant differences were noted for all the elution buffers except for GBE-7.2 Con A and TGBE-7.2 Con A. In the evaluation of the confidence interval ranges, the trend noted in the detection ranges became evident with the exceptions that were mentioned above (Figure 4.5B).

In the analysis of the RV-SA11 mean detection Ct-value data it was evident that the majority of the data grouped into the same mean Ct-value range (Ct 33.79 - 36.54) with the exception of TGBE-9.5 A (Ct 36.98) (Figure 4.6A). Analytical significant differences were noted for the majority of the elution buffers when compared to the positive control mean Ct-value (31.67) with the exceptions of GBE-7.2 Con B (Ct 33.93), GBE-9.5 Con B (Ct 34.20) and TGBE-7.2 Con B (Ct 33.79). Analysis of the RV-SA11 confidence intervals it was evident that the majority of the elution buffers formed a basic trend with a couple of exceptions, as indicated above for Figure 4.6A, noted (Figure 4.6B).

Analysing the mean detection Ct-value data for the recovery of HAstV from the respective elution buffers, it became evident that the data was not of the same Ct-value

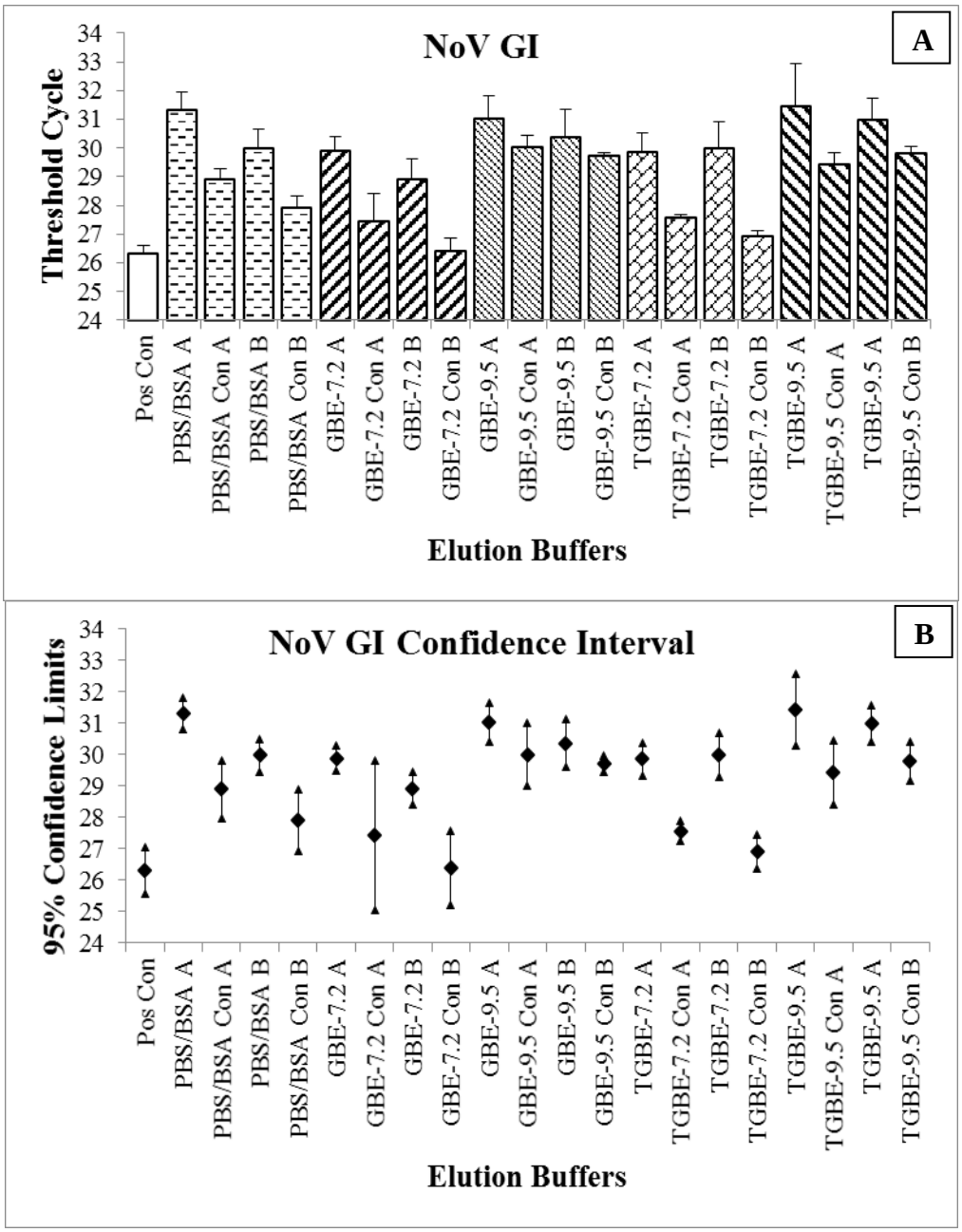

Figure 4.4: Summary of the analytical (A) and statistical (B) significant differences noted for NoV GI recovered from strawberries between the different elution buffers (PBS/BSA, GBE and TGBE), pH intervals (pH 7.2 and pH 9.5) and recovery protocols (A = 20 min and B = 5 h).

range, however, two different Ct-value ranges, the first for the elution buffer controls (Ct 31.63 - 33.59), with the exception of GBE-9.5 Con B and TGBE-9.5 Con B which grouped into the second range, and the second for the elution buffers with the strawberries present (Ct 33.90 - 36.42) were noted into which the data grouped together (Figure 4.7A). In the comparison of the two ranges to the positive control mean Ct-value (Ct 30.75), all of the data of the second range had mean Ct-values of >3 Ct cycle

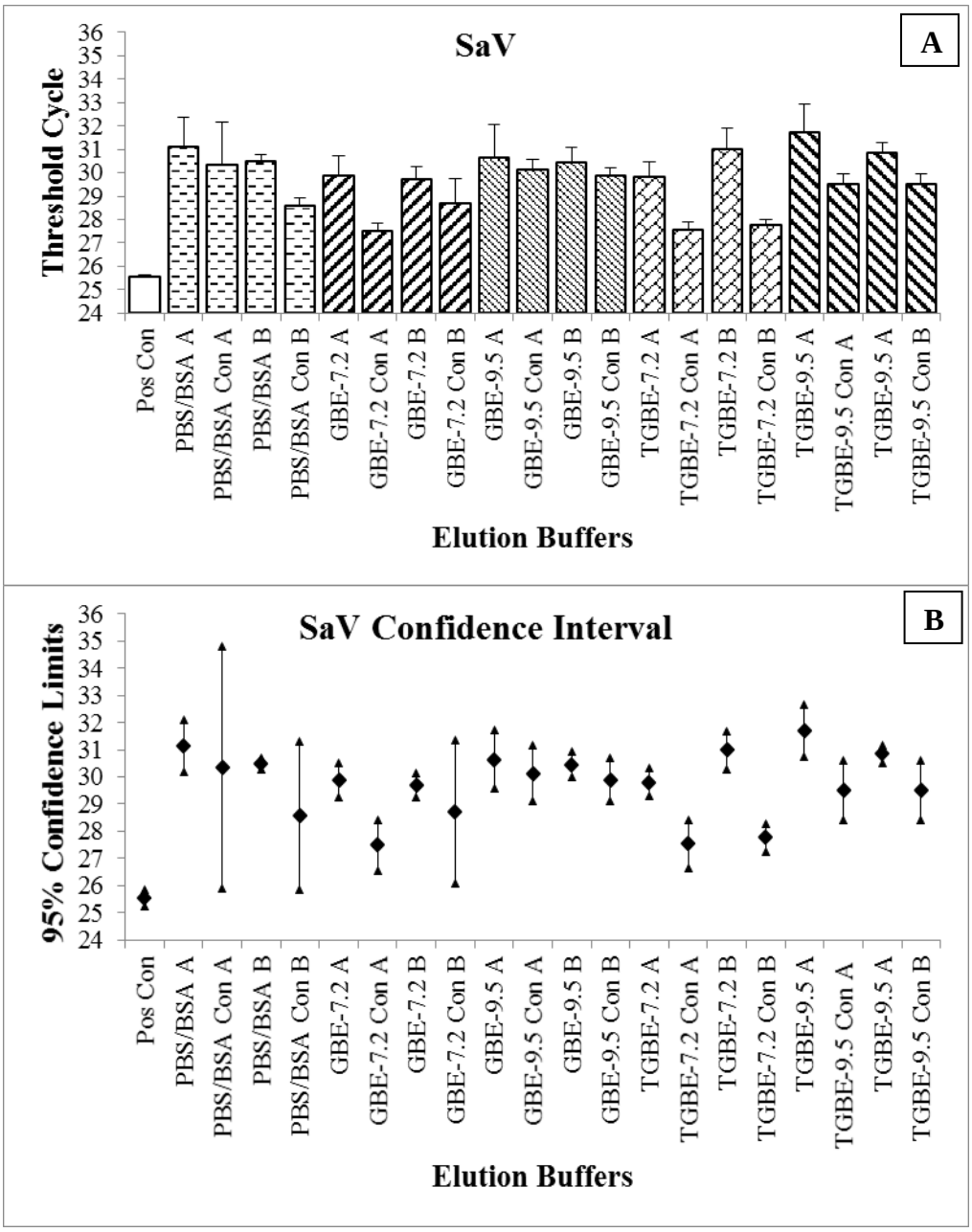

Figure 4.5: Summary of the analytical (A) and statistical (B) significant differences noted for SaV recovered from strawberries between the different elution buffers (PBS/BSA, GBE and TGBE), pH intervals (pH 7.2 and pH 9.5) and recovery protocols (A = 20 min and B = 5 h).

differences and was considered analytical significant. The two Ct-value ranges also resulted in the formation of two different confidence interval clusters (Figure 4.7B).

Separate Ct-value ranges (Ct 31.14 - 33.94 and Ct 34.84 - 37.12) were noted in the recovery of mengovirus from the respective elution buffers (Figure 4.8A). Analysis of the two Ct-value ranges showed that the elution buffer controls grouped separate from that of the elution buffers in which the strawberries were present. Furthermore, analytic

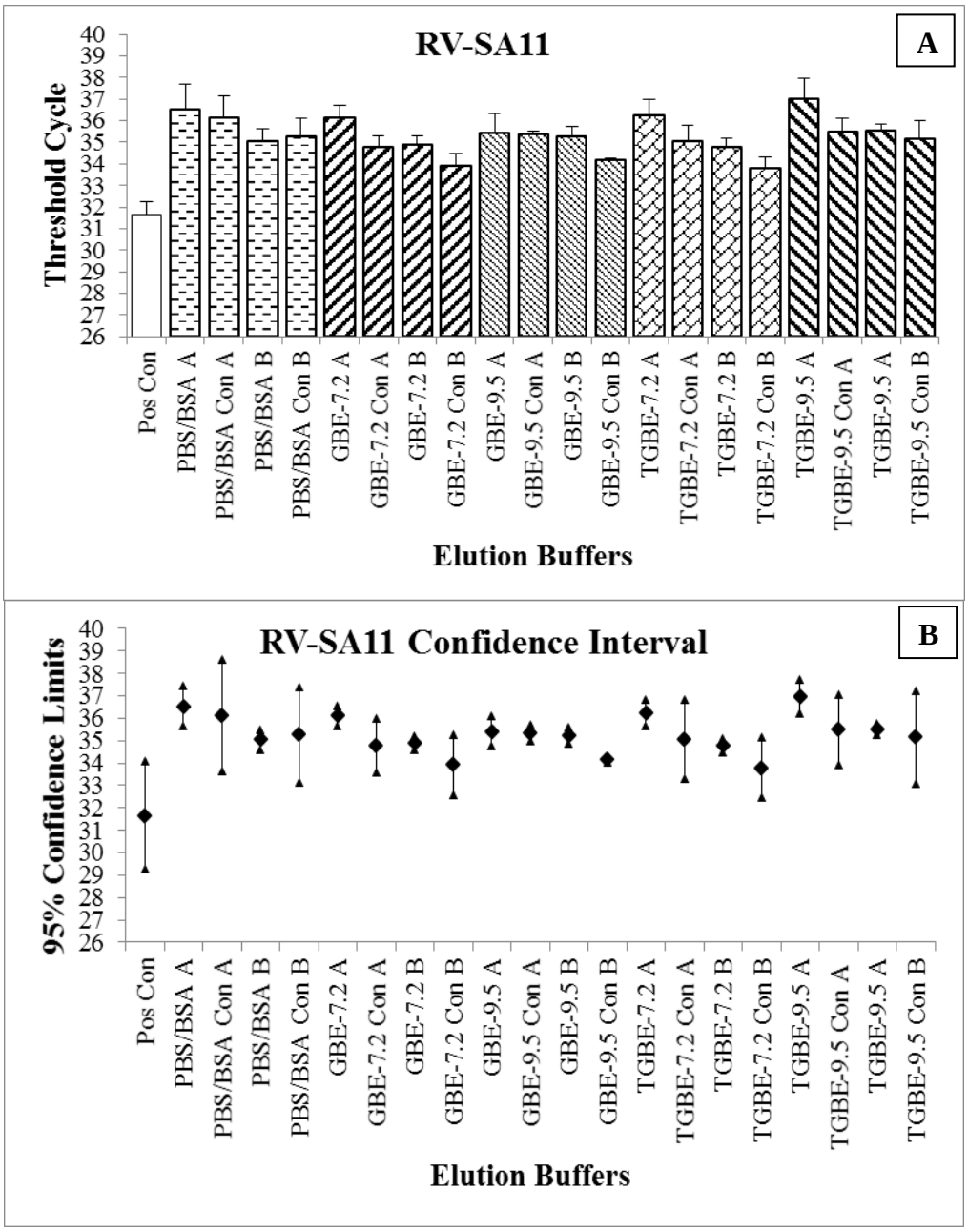

Figure 4.6: Summary of the analytical (A) and statistical (B) significant differences noted for RV-SA11 recovered from strawberries between the different elution buffers (PBS/BSA, GBE and TGBE), pH intervals (pH 7.2 and pH 9.5) and recovery protocols (A = 20 min and B = 5 h).

significant differences were noted for the second range when the data was compared to the positive control mean Ct-value (Ct 31.13). The two Ct-value ranges also formed two distinct confidence interval clusters (Figure 4.8B).

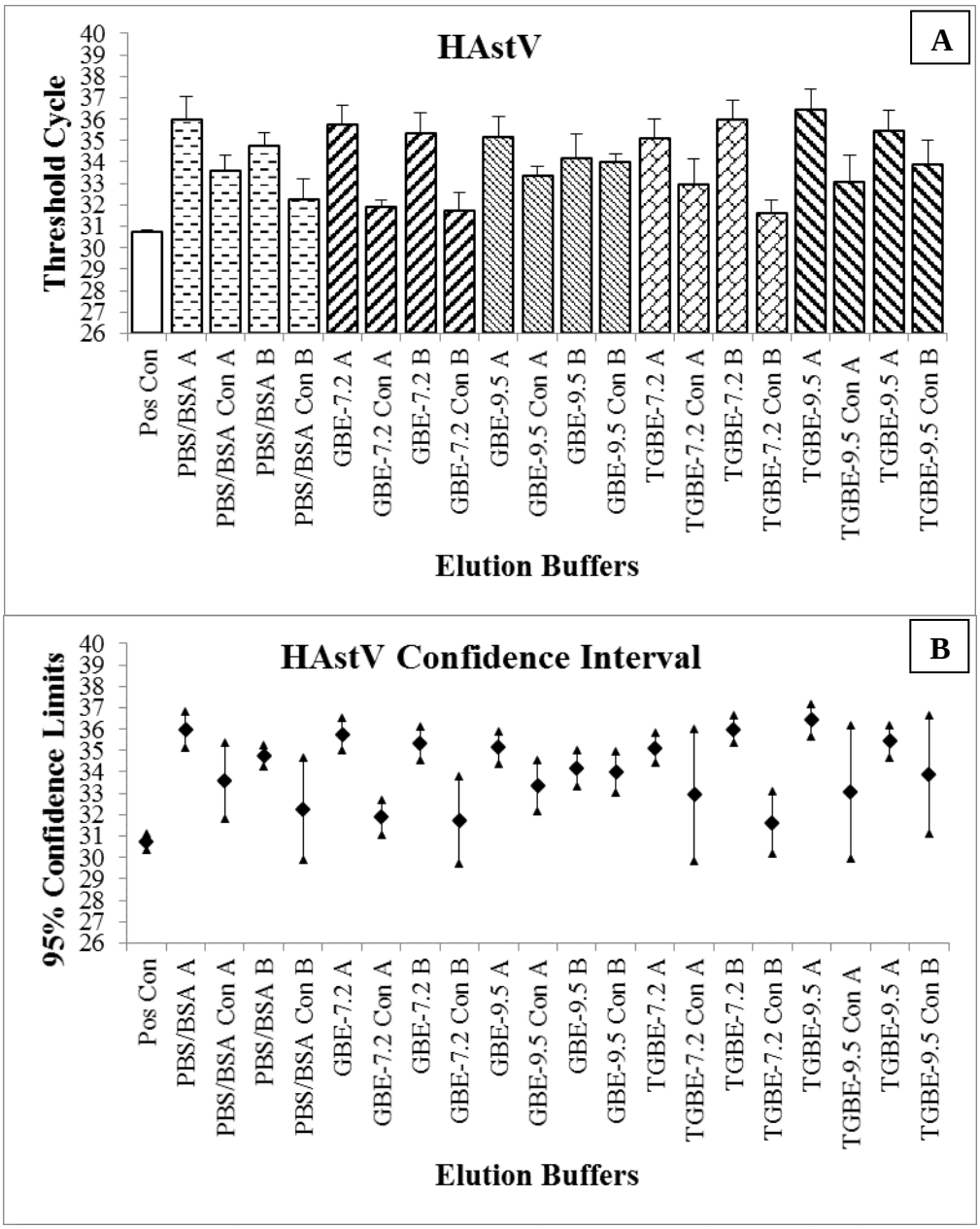

Figure 4.7: Summary of the analytical (A) and statistical (B) significant differences noted for HAstV recovered from strawberries between the different elution buffers (PBS/BSA, GBE and TGBE), pH intervals (pH 7.2 and pH 9.5) and recovery protocols (A = 20 min and B = 5 h).

4.4 DISCUSSION

The nucleic acid extraction techniques used for the isolation of DNA/RNA is a critical part of current molecular based assays for the detection of pathogenic microorganisms (Loens *et al.*, 2007; Verheyen *et al.*, 2012). Several evaluations and comparisons of different extraction methods from a variety of specimen types and target organisms have

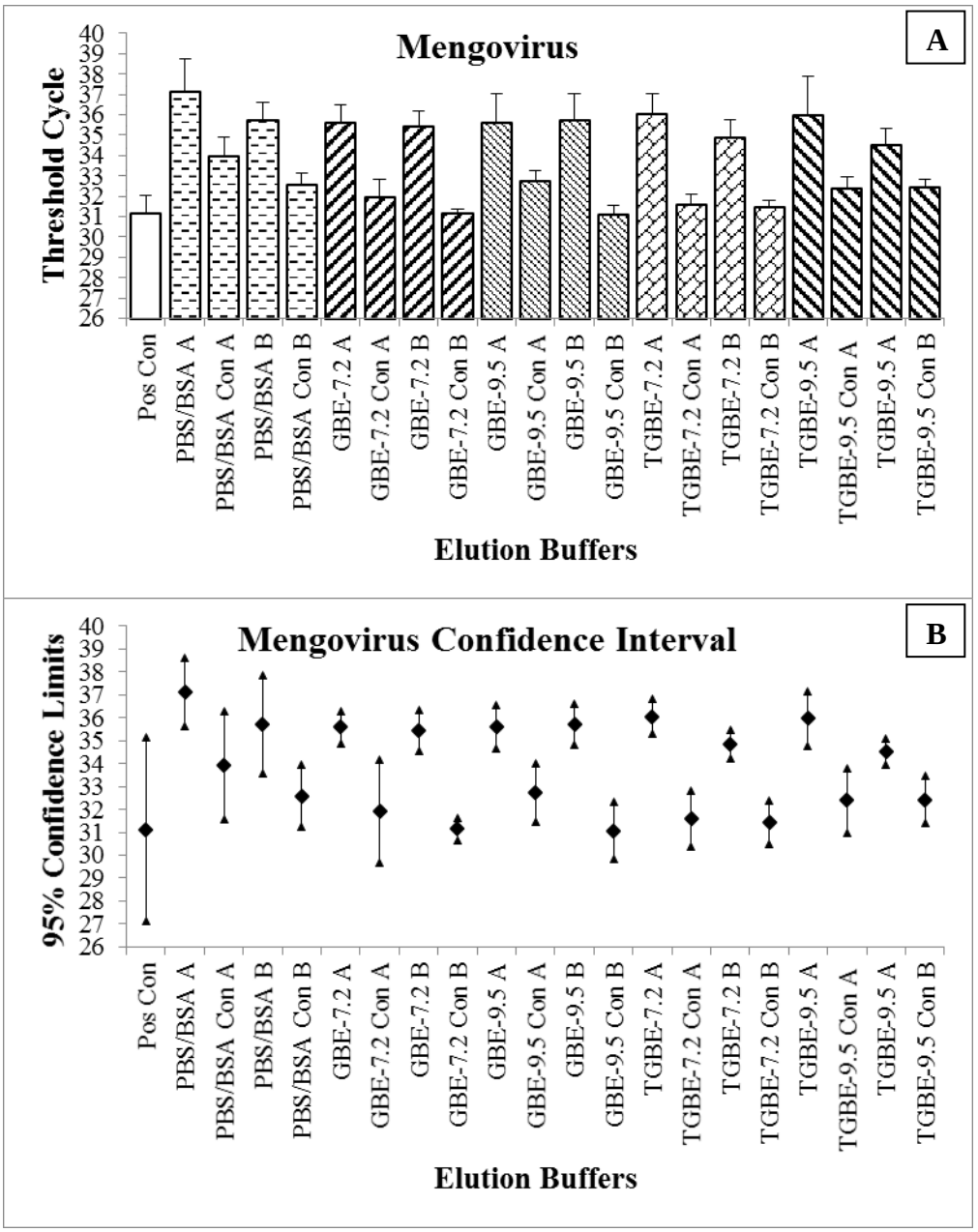

Figure 4.8: Summary of the analytical (A) and statistical (B) significant differences noted for mengovirus recovered from strawberries between the different elution buffers (PBS/BSA, GBE and TGBE), pH intervals (pH 7.2 and pH 9.5) and recovery protocols (A = 20 min and B = 5 h).

been performed (Knepp *et al*., 2003; Beuselinck *et al*., 2005; Petrich *et al*., 2006; Yang *et al*., 2011). However, the need to conduct such studies remains important in order to determine the effectiveness of obtaining pure concentrated nucleic acid extraction free of enzymatic inhibitors, since this has a direct influence on the amplification assay applied and the results obtained (Loens *et al*., 2007). The use of automated extraction platforms in preference to manual nucleic acid extraction methods, besides being less labour intensive and allowing two to three times the number of samples to be processed

in a given time period, had little differences in detection (Knepp *et al*., 2003; Witlox *et al*., 2008).

In this study the extraction efficiencies of the QIAmp® UltraSens® Virus kit (Qiagen) along with two automated nucleic acid methods, the NucliSens® easyMAG™ system (bioMérieux SA) and the MagNA Pure LC instrument (Roche Diagnostics) were evaluated for NoV GII, HAV and mengovirus. In the overall evaluation the QIAmp® UltraSens® Virus kit (Qiagen) proved to be less efficient for the recovery and purification of the nucleic acid (Table 4.2). It would seem that due to this lack in efficiency, NoV GII and HAV could not be detected in all the triplicate reactions and mengovirus experienced a $\log_{10}$ mean Ct-value difference in detection when compared to the mean Ct-values of the automated platforms. The nucleic acid extraction of NoV GII, HAV and mengovirus were less efficient from the strawberries than for the blackberries. The MagNA Pure LC instrument (Roche Diagnostics) allowed for better detection of the respective viruses and showed to have the least amount of intra-assay in comparison to the NucliSens® easyMAG™ system (Biomérieux) and the QIAmp® UltraSens® Virus kit (Qiagen). In this study, and in other similar studies done (Caliendo *et al*., 2007; Verheyen *et al*., 2012), equivalent results were obtained between the two automated platforms used, with the only difference being the maximum number of samples that can be extracted (24 samples with the bioMérieux platform and 32 samples with the Roche platform, respectively). Despite the lack in detection of HAV after nucleic acid extraction with the MagNA Pure LC platform (Roche Diagnostics) (Table 4.2), the method was applied to use in this study due to the larger number of samples that could be extracted with little hands on time required as opposed to the semi-automated NucliSens® easyMAG™ system (bioMérieux SA) in which the magnetic silica beads are dispensed manually.

A pilot study (results not given) indicated a real problem with PCR inhibitors present after the nucleic acid has been extracted resulting in the inability to detect some of the viruses. A serial ten-fold dilution for the nucleic acid allowed for the detection of the majority of the viruses detected in the study. This resulted in the serial ten-fold dilution of all extracted nucleic acid obtained in this study prior to cDNA synthesis. This allowed for the detection of the IAC in each of the reactions with differences remaining in their Ct-values, possibly due to the residual inhibitory substances (results not shown).

The presence or absence of the IAC in the *rt* RT-PCR assays was used for the indication of false negative results.

The detection of viruses in food remains challenging owing to the low virus titres and the components released from the food matrices that inhibit PCR-based assays (Wilson, 1997; Summa *et al*., 2012). This raises the need for optimised recovery methods and elution buffers for the detection of these viruses on different food matrices. The addition of a protein source, such as beef extract, has proven effective in the recovery of enteric viruses from a range of food matrices with various differences based on protein concentration, incubation periods and pH (Butot *et al*., 2007; Kim *et al*., 2008; Summa *et al*., 2012). The data obtained in this study was not reported as percentages, as done in other studies (Kim *et al*., 2008; Park *et al*., 2010), however, the Ct-values obtained from the individual multiplex *rt* RT-PCR assays were rather used for statistical analysis.

Analysis of the results indicated no analytical significant differences for a single buffer for any of the viruses when compared to the other buffers, however, small, less than three cycle differences in the mean Ct-values were noted but not found significant. For the viruses in the *Caliciviridae* family, the elution buffers GBE-7.2 and TGBE-7.2 allowed for more efficient elution and detection of the viruses when looking at the respective elution buffer controls (NoV GII: Figure 4.3, NoV GI: Figure 4.4, and SaV: Figure 4.5). Furthermore, the results showed no conclusive differences between the two protocols (A and B) evaluated for these viruses, with the small differences noted ascribed to pipetting errors. It was further evident, however, that differences in protein concentration had little effect in the recovery of NoV GII, NoV GI and SaV from the strawberries as opposed to the pH which allowed for better recovery. In the recovery of NoV GII though, all of the elution buffers evaluated faired equally well whereas for NoV GI and SaV the elution buffer GBE-7.2 B were slightly more efficient than the other elution buffers.

Hepatitis A virus could efficiently be detected with each of the elution buffers evaluated with the exception of PBS/BSA where an analytical significant difference was noted for PBS/BSA Con A. The reason for this was not well understood and could have been ascribed to the short interaction period with the elution buffer since the problem subsided when the 5 h protocol was tested (PBS/BSA Con B) (Figure 4.2A). It was

evident, when analysing the 3% beef extract buffers (GBE pH 7.2 and pH 9.5 and TGEB pH 7.2 and pH9.5) that none of the parameters evaluated (protein content, pH, presence or absence of TRIS and the two protocols) had any effect in the recovery of HAV from the respective elution buffers. It was further noted that the recovered HAV from the elution buffers had in some incidences a lower Ct-value than that of the positive control used. The reason for this was not fully understood and could have been the result of human error since the differences were <1 cycle.

In the recovery of RV-SA11, the 20 min protocol (A) for each of the elution buffers, except GBE-9.5 A, gave the highest mean Ct-value data (PBS/BSA A: Ct 36.54; GBE-7.2 A: Ct 36.11; TGBE-7.2 A: Ct 36.25 and TGBE-9.5 A: Ct 36.98) as opposed to the 5 h protocol for each of the respective elution buffers (PBS/BSA Con B: Ct 35.04; GBE-7.2 B: Ct 35.25; TGBE-7.2 B: Ct 34.79 and TGBE-9.5 B: Ct 35.52) (Figure 4.6A). This was further evident when the elution buffer controls were analysed for the 5 h protocol and compared to the 20 min protocol. The differences noted between the two protocols were not analytical significant and, as with HAV, it was evident that the other parameters evaluated contributed little to the detection of RV-SA11.

The recovery of HAstV from the strawberries was poor for all the elution buffers as noted for the majority of the other viruses tested except for HAV and RV-SA11. The elution buffer GBE-7.2 allowed for the consistent elution and detection of HAstV at both elution periods as opposed to the other elution buffers and was considered the appropriate elution buffer for HAstV. In the case of mengovirus, the use of the elution buffer GBE-7.2 allowed for more consistent elution and detection of the virus at both elution periods. However, the elution buffers TGBE-7.2 B and TGBE-9.5 B were considered more appropriate of all the elution buffers with the lowest mean Ct-values recorded. Aside from pH and protein concentration, protocol B with TRIS in the buffer gave the best results for mengovirus.

In the overall analysis of the results for each of the buffers evaluated, inconsistencies in the detection of each of the viruses except HAV and RV-SA11 were mostly seen between the elution buffers with the strawberries present and those that served as controls. This could in part be due to the presence of possible PCR inhibitors from the berries that remained after the single ten-fold dilution that was made prior to the

detection of each of the viruses. A further possibility could be due to the inability of the respective elution buffers to break the attachments made between the virus and strawberries. Another, more plausible explanation was that made by Verhaelen and colleagues (2012), who noted a lower decay rate of viruses on blackberries than strawberries whereas. Butot and colleagues (2007), however, found that a drop in pH a due to the acidity of the strawberries could account for the lowered recovery of the viruses from the strawberries. Despite these limitations, each of the viruses could be recovered from each of the strawberries with the elution buffer GBE-7.2 considered the more appropriate elution buffer of all the elution buffers evaluated.

4.5 CONCLUSION

In conclusion, nucleic acid extraction methods remain an important factor in the detection of viruses recovered from food matrixes. This was made evident in this study where the MagNA Pure LC platform (Roche Diagnostics) was found to provide the most consistent results and was thus considered to be the best method. In addition the elution buffer and elution period is also critical for the recovery of viruses from food matrices. In this study the buffer pH was found to be the most important factor in the recovery of the respective viruses from the strawberries where a neutral pH (pH 7.2) was more efficient than an alkaline pH (pH 9.5). This could in part be due to the difference that exists in the charges on the respective viral capsids at the different pH values. Furthermore, with the advent of the IAC, false negative test results were easily identified and could the necessary steps be taken to improve the detection of the viral nucleic acid. Mengovirus was equally as sensitive to inhibition as the majority of the viruses and along with the IAC could efficiently be implemented as a process control and AC, respectively.

CHAPTER 5

INVESTIGATION OF NOROVIRUSES AND HEPATITIS A VIRUS IN PAIRED IRRIGATION WATER AND STRAWBERRIES CULTIVATED IN SOUTH AFRICA

This Chapter of the dissertation formed part of larger Water Research Commission solicited research projects co-funded by Department of Agriculture, Forestry and Fisheries

i) Quantitative investigation into the link between irrigation water quality and food safety (WRC Project no K5/1773/4, Water Research Commission, 2006)

ii) An investigation into the link between water quality and microbiological safety of fruit and vegetables from the farming to the processing stages of production and marketing (WRC Project no K5/1875/4, Water Research Commission, 2009)

5.1 INTRODUCTION

Viral infections due to the consumption of contaminated food products have been described for more than five decades. Enteric viruses have been linked to foodborne illness either epidemiologically or by direct pathogen detection (Table 5.1) (Atmar, 2006).

Table 5.1: Enteric viruses associated with foodborne diseases (adapted Atmar, 2006).

Virus	Family	Disease	Foodborne Transmission
Human adenovirus	*Adenoviridae*	Gastroenteritis	No
Human calicivirus			
Norovirus	*Caliciviridae*	Gastroenteritis	Yes
Sapovirus		Gastroenteritis	Yes
Hepatitis A virus	*Picornaviridae*	Hepatitis	Yes
Hepatitis E virus	*Hepeviridae*	Hepatitis	Yes
Rotavirus	*Reoviridae*	Gastroenteritis	Yes
Human astrovirus	*Astroviridae*	Gastroenteritis	Yes

Noroviruses and HAV are recognized as important human foodborne viruses due to the number of outbreaks, the severity of disease caused by HAV infection, and the number of persons affected by NoVs (Hedberg, 2006). Raw produce are particularly vulnerable to contamination during pre-harvest (polluted soil and irrigation water) and post-harvest (washing water and food handlers) processes (Greening 2006; Hedberg, 2006; Papafragkou *et al*., 2006). Berry fruits have been associated with large outbreaks of hepatitis A (Niu et al., 1992; Le Guyader et al., 2004; Papafragkou et al., 2006; Food Safety news, 2013) and international outbreaks of NoV-linked gastroenteritis (Greening, 2006; Butot et al., 2008; Lysén et al., 2009; Sarvikivi et al., 2012). Strawberries are more prone to contamination by irrigation water, soil and farmworkers during harvesting and post-harvest handling than raspberries. However, an increased decay of viral particles on the surface of strawberries could in part explain the greater viral persistence noticed for raspberries than strawberries (Verhaelen et al., 2012). Furthermore, with the global production of strawberries exceeding that of raspberries (Food and Agricultural Organization of the United Nations, 2009), strawberry-linked gastroenteritis and hepatitis may possibly be far greater, yet less reported or documented than raspberry-linked infections.

Reports of foodborne NoV associated gastroenteritis outbreaks in SA have been previously reported (Taylor *et al*., 1993; Van Zyl *et al*., 2006), however, no published data on the prevalence of enteric viruses on the surface of strawberries and irrigation water used exists. The objective of this study was; i) to assess the quality of surface water used for irrigation of strawberries, ii) to assess the sensitivity of two extraction procedures, iii) to determine the presence of selected enteric viruses (NoVs and HAV) on strawberries and in irrigation water(s) used and, iv) to characterise and determine the phylogenetic relationship of these viruses detected.

5.2 MATERIALS AND METHODS

5.2.1 Viral stock

Mengovirus (1 x 10^6 copies/mℓ) (Table 3.1) served as a process control to monitor the viral recovery and nucleic acid extraction from the strawberries and irrigation water. From March 2011 to August 2011, 1 mℓ and 10 µℓ aliquots of mengovirus were added

to the irrigation water and inoculated onto the strawberry surface prior to viral recovery, respectively.

5.2.2 Primers and probes

Published sets of highly specific and sensitive primers and probes (Table 3.1) were used in this study. Furthermore, published sets of primers used for the genotyping of NoV GI and NoV GII was summarised in Table 5.2.

Table 5.2: Primer sequences used for genotyping of NoV GI and NoV GII.

Virus	Primer Set	Primer		Reference
		Forward	Reverse	
Norovirus GI	1	QNIF4	G1SKR	Da Silva *et al*. (2007) and Kojima *et al*. (2002)
	2	G1SKF	G1SKR	Kojima *et al*. (2002)
Norovirus GII	3	QNIF2	G2SKR	Loisy et al. (2005) and Kojima *et al*. (2002)
	4	G2SKF	G2SKR	Kojima *et al*. (2002)

5.2.3 Nucleic acid extraction

Viral nucleic acid was extracted from a 1 mℓ recovered viral suspensions of the water and strawberry samples using both of the following extraction platforms:

i) the fully automated MagNA Pure LC Total Nucleic Acid Isolation kit (large volume) (Roche Diagnostics) in a MagNA Pure LC instrument (Roche Diagnostics), and

ii) the Nuclisens® easyMAG™ semi-automated magnetic extraction platform (Boom method) (bioMérieux SA) according to the manufacturer's recommendations. For each batch of extractions, a 1 mℓ aliquot of nuclease-free water (Promega Corp.) served as a negative control. The extracted nucleic acid was eluted in 100 µℓ and 10 µℓ aliquots and stored at -20°C.

Nucleic acid from 11 irrigation water samples collected, excluding 2011/01/21 due to insufficient material, were re-extracted using the MagNA Pure LC Total Nucleic Acid

Isolation kit (large volume) (Roche Diagnostics), aliquot in in 100 µℓ and 10 µℓ and stored at -20°C.

5.2.4 Real-time RT-PCR assays

Individual *rt* RT-PCR assays were performed for the screening analysis of NoV GI, NoV GII and HAV in the water and strawberry samples. A 10 µℓ aliquot of the extracted nucleic acid was used for cDNA synthesis using the RevertAid Premium™ First Strand cDNA synthesis kit (Fermentas Life Sciences) following the manufacturer's recommendations. A 60 min incubation period was used as recommended by the manufacturer. The EXPRESS qPCR Super Mix Universal kit (Invitrogen) with 4 µM and 8 µM TaqMan primer and probe concentrations, respectively, was used (Table 3.1). Amplification was performed on a LightCycler v2.0 real-time platform (Roche Diagnostics) using the cycling conditions described in section 3.2.4. From December 2010 till August 2011 a 2 µℓ of the IAC (1 x 10^5 copies/ml) (Chapter 2) was added to each NoV GII *rt* RT-PCR reaction.

Eleven irrigation water samples was reassessed using optimised multiplex *rt* RT-PCR assays (Chapter 3). The cDNA synthesis, *rt* RT-PCR master mix and cycling conditions used was identical to that outlined in Chapter 3. A negative *rt* RT-PCR control using nuclease free water (Promega Corp.), an extraction negative control and a *rt* RT-PCR positive control, which consisted of the target viruses, were included in each batch of *rt* RT-PCR reactions.

For the characterisation of NoVs a highly conserved region at the 5'end of the NoV capsid gene was amplified and sequenced. Random primed cDNA synthesis was prepared as described in section 3.2.8.2. A two-step conventional PCR amplification was performed with published sets of primers. The KAPA Taq Hot Start PCR kit (KAPA Biosystems, Cape Town, SA) with 5 mM dNTP's (Fermentas Life Sciences), 37.5 mM magnesium chloride (KAPA Biosystems), and 5 µℓ of cDNA was used for the first round of amplification. For NoV GI, primer set 1 (Table 5.2) and for NoV GII primer set 3 (Table 5.2) with 25 µM and 50 µM forward and reverse primer concentration, respectively, was used. The reaction mix was prepared to a final volume of 20 µℓ. Amplification was performed on a Little Genius thermal cycler (BIOER

Technology, Hangzhou, China) using the following cycling conditions; pre-denaturation at 94°C for 10 min, followed by 45 cycles of 94°C for 30 sec, 50°C (NoV GI) or 55°C (NoV GII) for 45 sec, 72°C for 1 min, followed by a final extension step at 72°C for 5 min. A semi-nested PCR protocol was used for the second round of amplification. AmpliTaq® Gold DNA polymerase (0.025 U/µℓ) (Applied Biosystems) with 5 mM dNTPs (Fermentas Life Sciences), and 1 µℓ of the first round PCR product was used. For NoV GI, primer set 2 (Table 5.2) and for NoV GII primer set 4 (Table 5.2) with 5 µM and 25 µM forward and reverse primer concentrations, respectively, was used. Amplification was identical to that of the first round. The PCR products were analysed by 2% agarose gel electrophoresis (SeaKem® LE Agarose, Lonza, Rockland, NY) and visualised by EtBr staining and UV illumination using a 100 bp marker (O'GeneRuler, Fermentas Life Sciences, Burlington, Ontario, Canada) to determine the band size(s).

5.2.5 Site and sample selection

This study formed part of two Water Research Commission collaborative solicited research projects (Projects no K5/1773/4 and K5/1875/4). The samples were selected based on the recommended criteria that took the type of fruit or vegetable grown and corresponding irrigation water into account. A small scale strawberry farmer to the north of Pretoria, Gauteng was approached for the collection of strawberries, when in season or available, and irrigation water from the canal used for the irrigation of the strawberries. It was noted that the farmer used overhead irrigation at the start of the study but changed to drip irrigation soon thereafter when he became aware of preliminary test results.

5.2.6 Irrigation water and strawberry samples

5.2.6.1 Water samples

From September 2010 to August 2011 irrigation water samples were collected on a monthly basis from an irrigation canal flowing from the Bon Accord dam, Pretoria. Water samples were collected in sterile containers (10 ℓ with an additional 1 ℓ) and transported in a cooler bag to the laboratory and stored at 4°C until processed.

5.2.6.2 Strawberry samples

To obtain a representative sample size three samples, each comprising of three strawberries, were sampled from different areas of the field and analysed individually. Each strawberry was collected in a separate zip lock bag and gloves were changed between the picking of each strawberry to prevent possible cross contamination. The samples were placed in a cooler bag and transported to the laboratory and stored at 4°C until processed. The leaves from the base of the strawberries were removed before proceeding with viral recovery.

5.2.7 Viral Recovery

5.2.7.1 Irrigation water

A glass wool absorption-elution method, based on the method described by Vilaginès *et al*. (1997) and modified by Venter (2004) was used. The irrigation water was passed through the positively charged column using negative pressure at a rate of 10 ℓ/h. The viruses were eluted from the column to a final volume of 100 mℓ using a glycine-beef extract buffer (pH 9.5) (3.754 g/ℓ glycine (Merck) and 5 g/ℓ beef extract powder (Difco™ Becton, Dickinson and Company, MD). The solution was brought to a neutral pH (pH 7) using 1 M HCl (Merck) or 1 M NaOH (Merck). Secondary concentration of the viral suspension was achieved by a PEG_{6000} (Merck)/ NaCl (Merck) precipitation method based on the methods described by Vilaginès et al. (1997) and Minor (1985). The final pellet was resuspended in PBS (pH 7) (Sigma-Aldrich Co.) and stored (-20°C) prior to further analysis.

5.2.7.2 Strawberries

A 3% beef extra elution buffer (Kim *et al*., 2008) was used in the recovery of viruses from the surface of strawberries. The method described by Netshikweta. (2012) for the recovery of HAV and NoVs from fresh produce was used with the following amendments: i) 50 ml of the extraction buffer was used instead of 30 ml due to the larger strawberries, ii) a 30 min strawberry/buffer elution period was used, iii) the secondary precipitation period was extended to 4 h, and iv) the second centrifugation step of 6000 rpm for 10 min was not applied. The amendments were made prior to the optimisation of this method in Chapter 4 for strawberries. The recovered viral pellet was resuspended in 10 mℓ PBS (Sigma-Aldrich Co.) and stored at -20°C till further use.

5.2.8 Analysis for indicator organism

5.2.8.1 Thermotolerant (faecal) coliforms

Thermotolerant (faecal) coliforms were enumerated from a 100 mℓ aliquot of sampled irrigation water (1 ℓ) using a membrane filtration method (0.45 µm Satorius Stedim filters). Turbid water samples that could possibly restrain or inhibit the flow of the water through the filter were diluted ten-fold using Modified Scholtens' Broth (MSB). Faecal coliform selective agar plates (m-FC agar plate (55 mm diameter) (Selecta Media, Quantum Biotechnologies [Pty] Ltd, Ferndale, SA) were used onto which the filters were placed and incubated overnight at 44.5°C. Individual blue colonies on the surface of the filter(s) were used to calculate the number of colony forming units (cfu)/100 mℓ present for each water sampled. In cases where individual colonies could not be counted due to the higher number present/over growth, serial ten-fold dilutions of the water were made with PBS (Sigma-Aldrich Co.) and the enumeration process repeated.

5.2.8.2 *Escherichia coli*

The process for the enumeration of *E. coli* from the irrigation water samples was identical to that for the thermotolerant (faecal) coliforms with the following differences; i) a 0.45 µm filter from Merck Millipore (Merck) was used, ii) m-ColiBlue24 *E. coli* specific broth (Merck) was used and iii), the plates were incubated overnight at 35°C. The cfu/100 mℓ was calculated by counting the individual blue colonies present. Serial ten-fold dilutions, similar to that made in section 5.2.4.1, of the water were made in cases where individual colonies could not be enumerated.

5.2.9 Characterisation of NoVs

5.2.9.1 Cloning of PCR products

Amplified PCR fragments of approximately 300 - 400 bp were recovered from the gel and concentrated to 10 µℓ using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research Corp., Irvine, CA). The purified PCR fragments were cloned into a plasmid vector using the CloneJET™ PCR cloning kit (Fermentas Life Sciences) according to the manufacturer's recommendations. Four microliter of the ligation reaction was used to transform the *E. coli* competent cells (10 µℓ) (Lucigen Corp., Middleton, WI). The transformed *E. coli* cells were incubated at 37°C for 1 h in 900 µℓ recovery media

(Lucigen Corp.) under vigorous shaking. A 100 µℓ aliquot of the suspension was plated on LB enriched agar containing 100 µg/mℓ ampicillin (Sigma-Aldrich Co.) and incubated overnight at 37°C. Ten individual colonies were randomly isolated from the plates and screened for the specific insert by PCR amplification. GoTaq® Flexi DNA Polymerase (5u/µℓ) (Promega Corp.) with 2.5 mM dNTP's (Fermentas Life Sciences) and 4 µM pJET 1.2/blunt specific primers according to the manufacturer's recommendations was used. Amplification was performed on a Little Genius thermal cycler (BIOER Technology) using the following cycling conditions; pre-denaturation at 95°C for 3 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min with a final extension step at 72°C for 10 min. The PCR fragments were analysed by electrophoresis in a 2% agarose gel (SeaKem® LE Agarose) and visualised under UV illumination after staining with EtBr. A 100 bp marker (O'GeneRuler) was used to determine the band size(s).

An additional ten clones were sequenced in those samples where NoV could be typed. The amplification process were repeated for those samples in which an appropriate fragment size could not be obtained with changes made to the nucleic acid concentration (serial tenfold dilution series), the Taq polymerase used for the first and second round amplification steps (KAPA Taq vs. AmpliTaq Gold), the volume of PCR product used for second round amplification (1 vs. 2 µℓ) and the cycling conditions of the PCR assays were adjusted.

5.2.9.2 Sequencing and sequence analysis of PCR products

Amplified PCR products were purified using the DNA Clean & Concentrator™-25 Kit (Zymo Research Corp.) and eluted in 10 µℓ. The fragments were sequenced based on the Sanger sequencing method using the ABI Prism BigDye® Terminator v3.1 Cycle sequencing Kit (Applied Biosystems) and pJET 1.2/blunt specific primers (3.2 pmol) according to the manufacturer's recommendations. A 2 µℓ aliquot of the purified PCR fragments were used in each PCR reaction. Amplification was performed on a Little Genius thermal cycler (BIOER Technology) under the following cycling conditions; pre-denaturation at 94°C for 3 min followed by 25 cycles of 94°C for 30 sec, 50°C for 10 sec and 60°C for 4 min. Precipitation of the PCR products prior to sequence analysis was as outlined in section 2.2.6. The ABI 3130 automated analyser (Applied Biosystems) was used to analyse the amplified PCR products. Sequencher™ v4.10.1

and MEGA version 5.1 (Tamura *et al*., 2011) was used for the analysis of the sequences Norovirus reference were strains obtained from (http://0-blast.ncbi.nlm.nih.gov.innopac.up.ac.za/) (Table 5.3). MAFFT version 6 (http://0-mafft.cbrc.jp.innopac.up.ac.za /alignment/server/index.ht) was used to align the sequences. After manual adjustments of the alignment, phylogenetic analysis was performed with MEGA version 5.1 using the neighbour-joining method. Genotypes were assigned based on the clustering of the individual samples in the phylogenetic tree with a bootstrap support of >70%.

Table 5.3: Norovirus reference strains used in phylogenetic analysis of field strains.

Genotype	GenBank Accession no.	Strain	Year	Country of Origin
GII.1	HCU07611	Hawaii	1971	United States
GII.2	X81879.1	Melksham	1994	United Kingdom
GII.3	GU980585.1	CBNU1	2006	Korea
GII.3	HM635200.1	Seoul	2009	Korea
GII.4	X76716.1	Bristol	1993	United Kingdom
GII.5	AJ277607.1	Hillingdon	1990	United Kingdom
GII.6	AJ277620.1	Seacroft	1990	United Kingdom
GII.7	AJ277608.1	Leeds	1990	United Kingdom
GII.8	AF195848.1	98-18	1998	Amsterdam
GII.9	AY038599.2	VA97207	1997	United States
GII.10	AF427118.1	Erfurt	2000	Germany
GII.11	AB074893.1	Sw918	1997	Japan
GII.12	AJ277618.1	Wortley	1990	United Kingdom
GII.13	AY113106.1	Fayetteville	1998	United States
GII.14	AY130761.1	M7	1999	United States
GII.15	AY130762.1	J23	1999	United States
GII.16	AY502010.1	Tiffin	1999	United States
GII.17	AY502009.1	CS-E1	2002	United States
GII.18	AY823304.1	OH-QW101	2003	United States
GII.19	AY823306.1	OH-QW170	2003	United States
GII/Unknown	HM560937.1	205	2010	Taiwan

5.3 RESULTS

5.3.1 Analysis of indicator organisms

The thermotolerant and *E. coli* count ranged from 3 100 to 48 000 and 30 to 2 200 cfu/mℓ, respectively, with no seasonal pattern noticed (Table 5.4).

Table 5.4: Summary of a 12 month survey of the Bon Accord irrigation canal for the presence of indicator organisms of faecal contamination.

Sample	**Collection Date**	**Thermotolerant coliforms (cfu/100 mℓ)***	***Escherichia coli* (cfu/100 mℓ)***
Irrigation water	2010/09/22	9900	250
Irrigation water	2010/10/25	11000	200
Irrigation water	2010/11/22	11400	250
Irrigation water	2010/12/15	48400	1600
Irrigation water	2011/01/21	10000	1000
Irrigation water	2011/02/22	3100	470
Irrigation water	2011/03/22	9600	2200
Irrigation water	2011/04/21	5000	900
Irrigation water	2011/05/21	16000	1100
Irrigation water	2011/06/21	6000	600
Irrigation water	2011/07/26	5000	1500
Irrigation water	2011/08/24	7000	30

* cfu/100 mℓ = colony forming units (cfu)/100 mℓ

5.3.2 Viral detection from paired irrigation water and strawberry samples

From September 2010 to August 2011 a total of 24 samples comprising of 72 strawberries and 12 irrigation water samples were collected and analysed for the presence of NoV GI, NoV GII and HAV (Table 5.5). Possible PCR inhibitors were removed by diluting the nucleic acid ten-fold prior to amplification. Norovirus GI, NoV GII and HAV were detected in 8.3% (1/12), 41.7% (5/12) and 0% (0/12), respectively, of the irrigation water samples. Similar results were noted in the strawberry samples with an overall detection percentage of 4.2% (1/24), 20.8% (5/24) and 4.2% (1/24) recorded for NoV GI, NoV GII and HAV, respectively.

Table 5.5: Summary of results from the comparison of two nucleic acid extraction isolation methods.

Sample	Sampling date	Real-time RT-PCR analysis						
		NoV GI		NoV GII		HAV		Mengo-virus
		easy MAG	Mag NA Pure	easy MAG	Mag NA Pure	easy MAG	Mag NA Pure	
Irrigation water	10.09.22	+	-	-	-	-	-	N/D
Strawberry (a)		-	-	-	+	-	-	N/D
Strawberry (b)		-	-	-	-	-	-	N/D
Strawberry (c)		-	-	-	+	-	-	N/D
Irrigation water	10.10.25	-	-	+	+	-	-	N/D
Strawberry (a)		-	-	-	-	-	+	N/D
Strawberry (b)		-	-	-	-	-	-	N/D
Strawberry (c)		-	-	-	-	-	-	N/D
Irrigation water	10.11.22	-	-	+	-	-	-	N/D
Strawberry (a)		-	-	-	-	-	-	N/D
Strawberry (b)		-	-	-	-	-	-	N/D
Strawberry (c)		-	-	-	-	-	-	N/D
Irrigation water	10.12.15	-	-	-	-	-	-	N/D
Strawberry (a)		-	-	-	-	-	-	N/D
Strawberry (b)		-	-	-	-	-	-	N/D
Strawberry (c)		-	-	-	-	-	-	N/D
Irrigation water	11.01.21	-	-	+	+	-	-	N/D
Irrigation water	11.02.22	-	-	-	-	-	-	N/D
Strawberry (a)		-	-	-	-	-	-	N/D
Strawberry (b)		-	-	-	-	-	-	N/D
Strawberry (c)		-	-	-	-	-	-	N/D
Irrigation water	11.03.22	-	-	-	-	-	-	+
Irrigation water	11.04.21	-	-	-	-	-	-	+
Irrigation water	11.05.21	-	-	-	-	-	-	+

ND = not done

Table 5.5: (continued) Summary of results from the comparison of two nucleic acid extraction isolation methods.

Irrigation water	11.06.21	-	-	-	-	-	-	+
Strawberry (a)		-	-	-	-	-	-	+
Strawberry (b)		-	-	+	-	-	-	+
Strawberry (c)		-	-	+	+	-	-	+
Irrigation water	11.07.26	-	-	-	+	-	-	+
Strawberry (a)		-	-	-	-	-	-	+
Strawberry (b)		-	-	-	-	-	-	+
Strawberry (c)		-	-	-	-	-	-	+
Irrigation water	11.08.24	-	-	-	+	-	-	+
Strawberry (a)		-	+	+	-	-	-	+
Strawberry (b)		-	-	-	-	-	-	+
Strawberry (c)		-	-	-	-	-	-	+

The sensitivity and possible viral preference for one of the two extraction methods were assessed. With the use of the MagNA Pure extraction method a total of 2.8% (1/36), 19.4% (7/36) and 2.8% (1/36) of NoV GI, NoV GII and HAV, respectively, could be detected. Similar results were noted for the Nuclisens® easyMAG™ semi-automated magnetic extraction platform (Boom method) (bioMérieux SA) for NoV GI (2.8% (1/36)), NoV GII (16.7% (6/36)) and HAV 0% (0/36). The extraction methods assessed showed similar sensitivity with no viral preference detected.

The usefulness of mengovirus as a process control for the recovery of viruses from paired irrigation water and strawberry samples were evaluated. Mengovirus could be detected in all the irrigation water and strawberry samples tested with little inhibition noticed during amplification. The introduction of an IAC for NoV GII enabled the detection of false negative test results, however, the IAC was not yet optimised for the use in a two-step PCR assay and so amplification and detection was problematic at best (results not shown).

The use of optimised multiplex *rt* RT-PCR assays allowed for the detection of the above and additional viral species (Table 5.6). Norovirus GI, NoV GII and HAV could be detected in 9.1% (1/11), 36.4% (4/11) and 0% (0/11) in irrigation water samples with the singleplex *rt* RT-PCR assays, respectively. However, with the multiplex *rt* RT-PCR

Table 5.6 Summary of the results from the comparison of singleplex and multiplex *rt* RT-PCR assays for the detection of selected enteric viruses.

Sample		Singleplex *rt* RT-PCR* assay				Multiplex *rt* RT-PCR* assays							Total viruses detected
	Sample date	HAV	NoV GI	NoV GII	PC	HAV	NoV GI	NoV GII	PC	HAstV	HRV	SaV	
Irrigation water	2010.09.22	-	+	-	-	-	-	-	-	-	-	-	1
	2010.10.25	-	-	+	-	-	+	+	-	-	-	-	3
	2010.11.22	-	-	+	-	-	-	-	-	+	-	-	2
	2010.12.15	-	-	-	-	-	+	-	-	+	+	-	3
	2011.02.22	-	-	-	-	-	-	-	-	+	-	-	1
	2011.03.22	-	-	-	+	-	-	-	+	-	-	+	2
	2011.04.21	-	-	-	+	-	-	-	+	-	-	-	-
	2011.05.21	-	-	-	+	-	-	-	+	+	+	-	2
	2011.06.21	-	-	-	+	-	-	-	+	-	-	-	-
	2011.07.26	-	-	+	+	-	+	+	+	-	-	-	3
	2011.08.24	-	-	+	+	-	-	+	+	-	-	-	2

**rt* RT-PCR = real-time reverse transcriptase polymerase chain reaction

assays NoV GI, NoV GII and HAV was detected in 27.3% (3/11), 27.3% (3/11) and 0% (0/11) of the irrigation water samples, respectively. An additional three NoV GI positive irrigation water samples were detected by the multiplex *rt* RT-PCR assay. Human AstV, HRV and SaV were detected in 36.4% (4/11), 54.5% (6/11) and 9.1% (1/11) of the irrigation waters collected. Mengovirus was consistently detected by both assays.

5.3.3 Phylogenetic analysis

The 5' end of the NoV GII capsid gene (273 bp) was used in the phylogenetic analysis. The NoV GII strains could be genotyped from three of the 10 positive samples (Table 5.7), however, no strains from the five NoV GI positive samples could be genotyped. No further NoVs strains could be detected from the additional clones sequenced. A 100% sequence similarity between the clones in an individual sample existed, resulting in the use of a single sequence for further phylogenetic analysis (Figure 5.1).

A neighbour-joining tree using the amino acid sequence alignments of the reference NoV GII strains (Table 5.3) and unknown NoV strains from this study (Table 5.7) was drawn (Figure 5.1). Phylogenetic analysis could closely match the NoV strain in the irrigation water as swine NoV GII.18 and that of the strawberry as human NoV GII.7. BLAST results of the irrigation water strains found an unidentified NoV GII strain (Table 5.3) recovered from an oyster in Taiwan as the closest match (99% nucleotide sequence identity) (Figure 5.1) (Table 5.8). Analysis of this sequence strain and that of the individual irrigation water sequences closely grouped together with swine NoV GII.18. A pair wise alignment of the amino acid and nucleotide sequences of the Jul/Aug Bon Accord Irrigation Water (BIW) clones, the swine NoV GII.18 and the NoV strain from the oyster (Table 5.6) revealed an overall 81.8% amino acid and 76.3% nucleotide similarity of the clones to the swine NoV GII.18 strain (Table 5.8). An overall 99.5% amino acid and a 93.47% nucleotide sequence identity existed between the Jul/Aug BIW clones and that of the oyster NoV GII strain. In the majority of the Jul/Aug BIW clones, insignificant changes were noticed between the NoV GII strain collected in July and that in August 2011 (Figure 5.1) (Table 5.8). Separate groupings of two clones (Gauteng Province (GP) July BIW clone 4 and GP July BIW clone 4)

Table 5.7: Summary of genotyping results of NoV GI and NoV GII strains detected in the irrigation water or on the strawberries.

Sample	Date	Norovirus genogroup	Genotyping
Irrigation waters	2010.09.22	GI	Untypable
	2010.10.25	GII	Untypable
	2010.10.25	GI	Untypable
	2010.11.22	GII	Untypable
	2010.12.15	GI	Untypable
	2011.01.21	GII	Untypable
	2011.07.26	GII	GII.18
	2011.07.26	GI	Untypable
	2011.08.24	GII	GII.18
Strawberries	2010.09.22	GII	Untypable
	2010.09.22	GII	Untypable
	2011.06.21	GII	Untypable
	2011.06.21	GII	Untypable
	2011.08.24	GII	GII.7
	2011.08.24	GI	Untypable

were noticed but these difference were not supported by bootstrap analysis. Similarly, Bon Accord Strawberries (BST) clones 3 and 7 (Figure 5.1) grouped together but separate from the other clones, yet the differences noticed were not supported by bootstrap analysis.

5.4 DISCUSSION

Viruses transmitted by the faecal-oral route can result in disease if contaminated water or food is consumed. To date, it remains a difficult task to determine the presence of all the possible viruses in food and water (Farrah, 2006). *Escherichia coli* and thermotolerant coliforms are used as indicator organisms for the presence of enteric viruses in food and water samples because of the cost effectiveness and effortlessness in their detection (Farrah, 2006; Jofre, 2007). These organisms were used to assess the quality of irrigation water and to correlate their presence with that of selected enteric

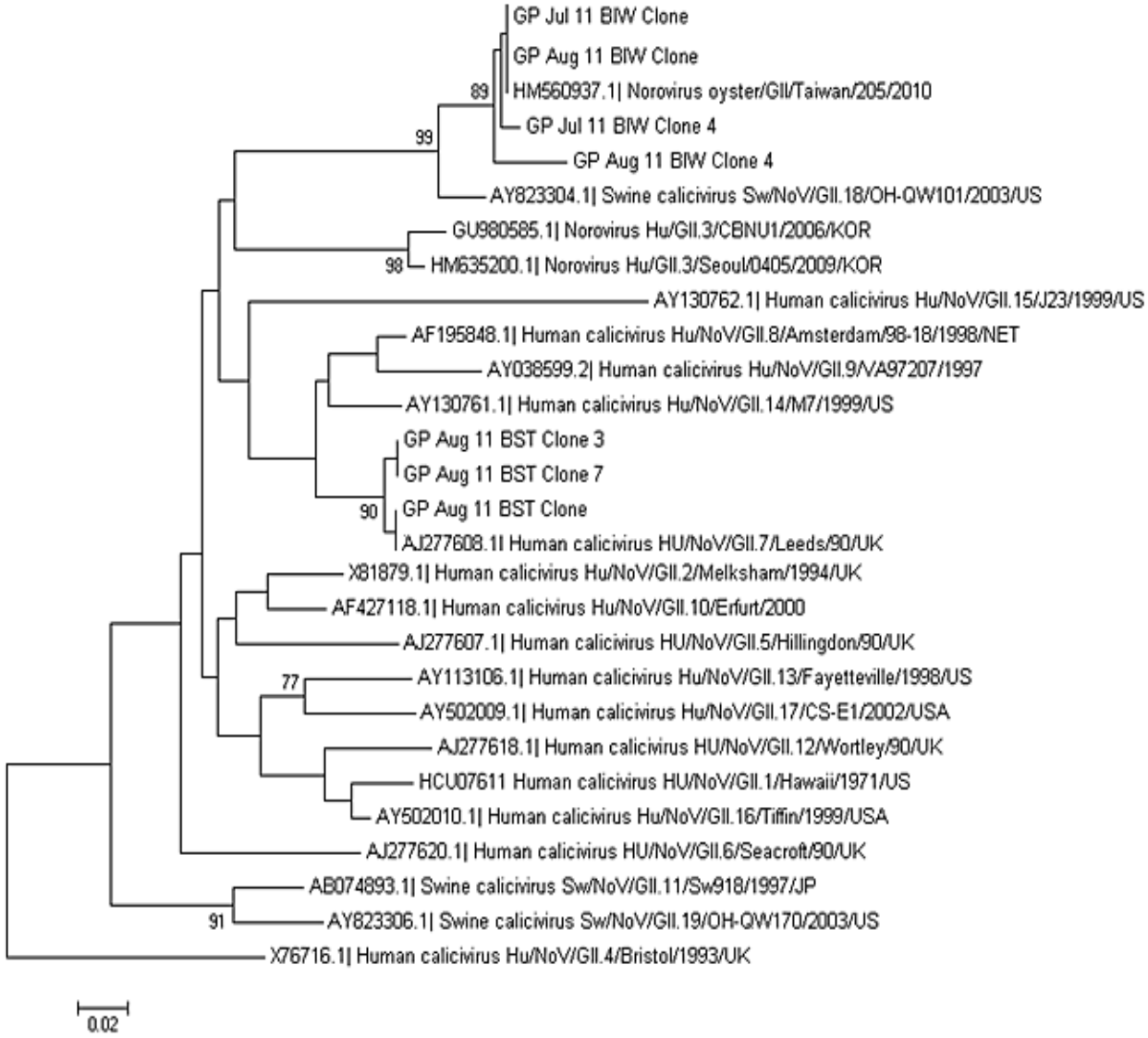

Figure 5.1: Neighbour-joining phylogenetic tree based on the amino acid sequence of the NoV capsid gene region. The NoV strains from irrigation water and strawberry samples along with reference NoV GII strains were used to draw the tree. Norovirus GII.4 was used to root the tree. Bootstrap percentages are indicated with the bar representing the nucleotide changes. BIW (Bon Accord Irrigation Water) and BST (Bon Accord Strawberries)

viruses. The findings indicated that the quality of the water used for irrigation does not meet the standards (thermotolerant coliforms: <1000 colony forming units per 100 mℓ and *E.coli* counts: ≤1 counts/100ml) set by the Department of Water Affairs and Forestry of SA in 1996 (Table 5.4). The use of bacterial indicator organisms is considered by some as imperfect for the indication of the presence of enteric viruses (Farrah, 2006; Joffre, 2007; Sinclair *et al*., 2009). However, in this study an almost

Table 5.8 Pairwise analysis of the amino acid (the upper section of the table) and nucleotide (the lower section of the table) sequences of four swine NoV GII.18 strains, with two additional clones of each of the samples respectively, were used to determine the relatedness of the strains to each other and to the two swine NoV GII.18 reference strains (AY823304 and HM560937). A 306 bp region of the NoV GII capsid protein sequence was aligned and used for analysis.

		AY823304	HM560937	Jul 11 BIW				Aug 11 BIW				
		Swine	Oyster	Clone 1	Clone 2	Clone 3	Clone 4	Clone 1	Clone 2	Clone 3	Clone 4	Clone 5
AY823304 Swine			82.18	82.18	82.18	83.17	81.12	82.18	82.18	82.18	79.21	82.18
HM560937 Oyster		77.23		100	100	99	99	100	100	100	97.03	100
	Clone 1	76.24	93.34		100	99	99	100	100	100	97.03	100
Jul	Clone 2	76.57	93.73	99.34		99	99	100	100	100	97.03	100
2011	Clone 3	76.57	93.4	98.68	99		98.02	99	99	99	96.04	99
BIW	Clone 4	75.91	93.1	99.97	99	98.35		99	99	99	96.04	99
	Clone 1	76.57	93.73	99.67	99.67	99	99.39		100	100	97.03	100
Aug	Clone 2	76.57	93.4	98.69	98.35	98.02	98.35	98.35		100	97.03	100
2011	Clone 3	76.57	93.73	99.67	99.67	99	99.34	100	98.35		97.03	100
BIW	Clone 4	75.58	93.1	98.35	98.68	98.35	98.02	98.68	97.69	98.68		97.03
	Clone 5	76.24	93.73	99.67	99.34	99	99.34	99.34	99	99.34	98.68	

perfect correlation between the presence of indicator organisms (Table 5.4) and the presence of selected enteric viruses detected (Table 5.6) could be made. Several methods have been tried and tested for the recovery of enteric viruses from the surface of strawberries (Butot et al., 2007; Kim et al., 2008). The recovery method used in this study deviated from the optimised method in Chapter 4, however, both methods made use of a 3% beef extract buffer. Kim et al. (2008) concluded that the concentration of the beef extract used could explain the difference noted in the recovery of NoV.

Insignificant differences existed between the two extraction methods assessed. Hepatitis A virus was only detectable with the MagNA Pure extraction, however, no conclusion could be made about the viral preference for HAV for a particular extraction method based on these results. This also held true for the recovery and detection of NoV GI and GII (Table 5.5). A 40% (4/10) and 30% (3/10) loss in detection of NoV GII positive samples extracted by the MagNA Pure and Nuclisens® easyMAG™ semi-automated magnetic extraction platform (bioMérieux SA), respectively, would have been undetectable if only one of the two extraction methods were used (Table 5.5). The further use of the MagNa Pure system (Roche Diagnostics) was based on financial and time convenience as well as the number of samples that can be extracted by each system (32 samples with the MagNa Pure system (Roche Diagnostics) compared to 24 by the Nuclisens® easyMAG™ semi-automated magnetic extraction platform (bioMérieux SA).

A wide range of NoVs have been documented to circulate in SA with NoV GII.4 being the most prevalent strain both in the surface waters and in paediatric patients in SA (Mans et al., 2010, 2013). Real-time RT-PCR assays in the form of multi-and singleplex assays were used to determine the presence of NoV GI, GII and HAV in paired irrigation water and strawberry samples. Noroviruses, in particularly NoV GII, were most frequently detected in the irrigation water and on strawberries (Table 5.5). The multiplex *rt* RT-PCR assays applied in this study could detect three additional NoV GI positive samples, however, this could partially be due to the re-extraction of nucleic acid. Additional enteric viruses (HAstV, HRV and SaV) were detected in the irrigation waters used for the strawberries. Human RV was frequently detected in the irrigation water samples (54.54%) (Table 5.6). Further studies will be required to determine the possible carryover effect of these viruses from the water onto the strawberries.

The use of mengovirus as a process control effectively aided in the evaluation of the viral recovery method. Mengovirus were detected in each of the samples, however, it was also detected in samples where the amplification of other viruses, such as NoV GII, were impeded. An IAC was used to detect the presence of these false negative results (results not shown). These results supported the use of a process control and an IAC for the recovery and PCR-based detection of possible human pathogenic viruses from strawberry fruits and associated irrigation water samples.

Environmental sources could potentially be seen as reservoirs for animal pathogens, most specifically RNA viruses, which could potentially amount to cross-species transmission (Heeney, 2006). The genus *Norovirus* comprises of viruses capable of infecting humans, pigs, cattle and mice species (Patel *et al*., 2009). A risk of zoonotic transmission between human and animal NoV strains could possibly exist. In such an event a novel virus with a new host tropism and increased or decreased pathogenicity may arise (Almanza *et al*., 2008). Swine NoVs are genetically and antigenically related to the most prevalent human NoVs strain (GII) (Van der Poel *et al*., 2000; Wang *et al*., 2005; Mattison *et al*., 2007). Mattison *et al*. (2007) demonstrated the presence of NoV GII.4 human-like strains in pigs whereas Wang *et al*. (2005) identified a potential human-swine recombinant NoV strain that could infect gnotobiotic pigs. The possibility for pigs as potential reservoirs for the emergence of new human NoVs strains therefore exists (Van der Poel., 2000; Wang *et al*., 2005; Mattison *et al*., 2007; Almantza *et al*., 2008).

During the period Sept 2010 to Aug 2011, 12 water samples were collected of which 42% (5/12) tested positive for NoV GII. Swine NoV GII.18 was genotyped from two irrigation water samples and human NoV GII.7 from a single strawberry sample. Mans *et al*. (2013) had similar success rates in the genotyping of NoVs from surface waters. To our knowledge, swine NoV has not been detected in SA (Mans *et al*., 2010, 2013) and thus makes this the first reported incidence of swine NoV GII.18 in SA. BLAST analysis of this strain indicated a 94% sequence identity with a NoV strain detected in 2010 in Taiwan from an oyster (Figure 5.1). Two clones; GP Jul BIW 11 clone 4 and Aug BIW 11 clone 4 grouped separately from the other strains but these differences were not supported by boodstrap values (Figure 5.1). Despite these differences, the

closest representatives remained swine NoV GII.18 and human NoV GII.7 detected in the water and on the strawberries, respectively.

5.5 CONCLUSION

The use of thermotorelant coliforms and *E. coli* as indicators for the identification of viral pathogens in water samples remains an important, inexpensive tool. Strawberries cultivated in SA and irrigated with below standard irrigation water could pose a health threat. The underreporting and/or misdiagnosis of strawberry-linked gastroenteritis and hepatitis mask the problem at hand. The combined use of an effective elution buffer and PCR-based assay could increase the chances of identifying such incidences. This study focussed on the assessment of fast, reliable and cost effective techniques for the surveillance and detection of human pathogenic viruses in water and strawberry samples. The use of a 3% beef extract buffer (Chapter 4) in conjunction with three optimised multiplex *rt* RT-PCR assays (Chapter 3) could effectively identify the possible risked posed by the consumption of contained irrigation water and strawberries. In addition to this, the use of appropriate controls, such as the use of a process control and IAC, for the recovery and detection of selected enteric viruses is strongly recommended.

This was the first report on the presence of swine NoV GII.18 in SA. Although a definite link between the viruses detected in the irrigation water and those on the strawberries could not be established at this point of time, a more concerning problem of the possible human-swine NoV variants was brought to light. Further studies will be needed to establish such a link along with increased surveillance for the possible occurrence of possible zoonotic variants among the SA population.

CHAPTER 6

VALIDATION OF AN OPTIMISED VIRUS RECOVERY METHOD AND MULTIPLEX REAL-TIME RT-PCR ASSAYS FOR THE DETECTION OF SELECTED ENTERIC VIRUSES ON "MOCK IRRIGATED" STRAWBERRY SAMPLES

6.1 INTRODUCTION

Noroviruses, HAV and to a lesser extent HRV are thought to be the leading cause of foodborne illness in industrialised countries (Butot et al., 2007). Other enteric viruses such as SaV, HAstV and HEV have also been linked to cause food and to a lesser extent waterborne gastroenteritis and hepatitis, respectively (Greening, 2006). Enteric viruses can remain infectious for prolonged periods of time in various transmission vehicles such as sewage, soil, water, food, air or surfaces. Environmental contamination occurs through the discharge of faecal matter from an infected person which can be transmitted back to a susceptible host through the consumption of contaminated food products, drinking and/or recreation waters (Le Guyader et al., 2004; Bosch *et al*., 2006; Goyal, 2006; Butot et al., 2008). Billions of gastrointestinal cases occurs annually worldwide largely due to faecal contamination of the environment, whereas hepatitis caused by HAV and HEV are more linked to contaminated shellfish, water, and crops (Bosch et al., 2006).

Simple, rapid and sensitive methods for the detection of viruses in water and food products can assist to determine the cause of an outbreak. The main limitations that these methods must overcome is: i) the low presence of these viruses on or in food products, ii) the variety and complexity of food products, iii) the distribution of the viruses in the food product thus complicating sampling, and iv) the presence of inhibitory substance (Goyal, 2006). As the detection of viruses from fresh produce is a multistage process the recovery of the viruses from the food matrix is critical for sensitive detection. Viruses can be recovered from the produce by rinsing in an elution buffer followed by a secondary concentration step using a PEG_{6000} or PEG_{8000} precipitation method (Kim *et al*., 2008). Detection generally involves the extraction of

the viral nucleic acid and used in molecular based assays such as multiplex *rt* RT-PCR assays. The objective of this section of the investigation was to determine the presence of viruses on strawberries artificially contaminated with polluted irrigation water using an optimised viral recovery method (Chapter 4) and multiplex *rt* RT-PCR assays (Chapter 3).

6.2 MATERIALS AND METHOD

6.2.1 Viral stock

The individual viral stock suspensions used for this study were identical to those described in section 3.2.1. A 100 µℓ aliquot of the stock suspensions of each virus (section 3.2.1) was pooled and made up to a final volume of 1 mℓ with nuclease-free water (Promega Corp.). The concentrations of the respective viruses in the 1 mℓ viral suspensions and 20 µℓ working stock, as determined using *rt* RT-PCR quantification, are presented in Table 4.1.

6.2.2 Primers and probes

Optimised *rt* RT-PCR assays (Chapter 3) using published sets of highly specific and sensitive primers and probes (Table 3.1) were used in this study.

6.2.3 Nucleic acid extraction

Nucleic acid was extracted from the recovered virus concentrates using two different technologies. Genomic viral nucleic acid was extracted from 1 mℓ of the recovered virus concentrate using the Nuclisens® easyMAG™ semi-automated magnetic extraction platform (Boom method) (bioMérieux SA) following the manufacturer's instructions and from a second 1 mℓ aliquot using the MagNA Pure LC Total Nucleic Acid Isolation Kit (large volume) (Roche Diagnostics) in a MagNA Pure LC Robotic instrument (Roche Diagnostics), following the manufacturer's instructions. Nucleic acid was eluted in 100 µℓ, aliquoted and stored at -20°C until use.

6.2.4 Multiplex *rt* RT-PCR assays

Qualitative multiplex *rt* RT-PCR assays (Chapter 3), based on TaqMan technology and published primers and probes (Table 3.1), were applied for the detection of the selected enteric viruses. Briefly RevertAid™ Premium Reverse Transcriptase (Fermentas Life Sciences) was used for cDNA synthesis. The EXPRESS qPCR Universal Mix (Invitrogen) with primers (4 µM), probe (2 µM) and 5 µℓ cDNA in a final volume of 20 µℓwas used in the setup of the reaction mastermix. The reactions were carried out in a 7300 Real Time PCR System (Applied Biosystem). Included in each assay was an extraction negative control (nuclease-free water; Promega Corp.), an *rt* RT-PCR negative control (nuclease free water; Promega Corp.) and a *rt* RT-PCR positive control containing nucleic acid of the targeted viruses. The samples were initially screened with Reaction C (HAstV, IAC and mengovirus) to control for efficient recovery and to validate the recovery and nucleic acid extraction through the amplification of the process control and IAC. Ten-fold dilutions of the nucleic acid were prepared in cases where no amplication was detected and then re-tested. Thereafter samples were screened with Reactions A and B (Chapter 3) at the appropriate nucleic acid concentration.

6.2.5 Strawberry samples

Strawberries were purchased from a commercial outlet and three strawberries were randomly selected for each experiment, with an additional three strawberries used for the negative and positive controls, respectively. Prior to seeding and viral recovery the stem and leaves were carefully removed from the base of the strawberry.

6.2.6 “Mock irrigation” water

Surface water samples from the Rietspruit River (RV), Gauteng, referred for testing over a 4 mo period containing one or more of the following enteric viruses; HAV, HAstV, NoV GI, NoV GII, HRV and SaV by commercial singleplex *rt* RT-PCR assays were used as “mock irrigation” water samples. A 100 mℓ aliquot of the water samples were dispensed into a container for use. A 100 mℓ aliquot of distilled water was used as a negative water control.

6.2.7 "Mock irrigation" of strawberries with river water

Individual strawberries were impaled, at their base, onto a disposable inoculation loop (Thermo Fisher Scientific, Rochester, NY). Three strawberries were immersed ten times into a "mock irrigation" river water sample. The step was repeated for the negative control. For the positive control, three strawberries were seeded with 20 µℓ of the working virus stock suspension. The inoculation loops were firmly placed into a polystyrene base with the "irrigated" strawberries in the air. To prevent cross contamination the negative and positive control strawberries were placed onto a separate polystyrene base to the experimental strawberries (Figure 6.1).

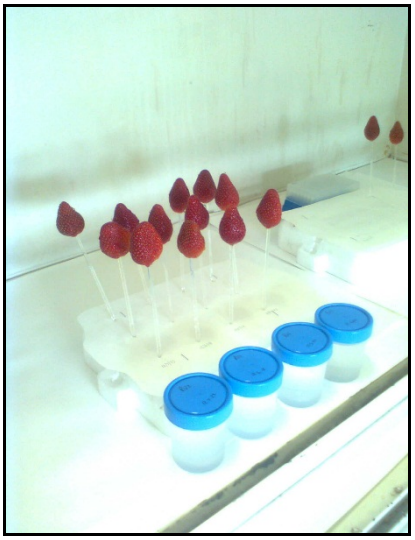

Figure 6.1: Impaled "mock irrigated" strawberries drying in a safety cabinet.

The experimental and positive control strawberries were seeded with 10 µℓ of mengovirus (process control) and dried at room temperature (~25˚C) in a safety cabinet until all the water drops had evaporated (~ 1-2 h)

6.2.8 Viral recovery and concentration

An optimised recovery method (Chapter 4) was used in the recovery of viruses from the surface of the strawberries. Briefly, the individual berries were immersed in 30 mℓ GBE-7.2 elution buffer in a 50 mℓ centrifuge tube (BD Biosciences) and shaken gently at room temperature (+/- 25˚C) for 20 min. The berries were discarder afterwards and

the buffer pH adjusted to pH 7 using 1 M HCl (Merck) or 1 M NaOH (Merck) as required. Pectinase (Pectinex® Ultra SPL, Sigma, Buchs Switzerland) was added to a final concentration of 5 µℓ/mℓ. The buffer suspensions were gently shaken at 60 rpm (Labnet 211DS Shaker Incubator: Labnet International. Inc,) for 30 min at room temperature (25˚C). The eluted viruses were concentrated to a final volume of 5 mℓ in PBS (pH 7.2) (Sigma-Aldrich Co.) using a PEG_{8000} (Amresco, Solon, OH)/NaCl (Merck) precipitation method recommended by the European Committee of Standardisation (CEN) Technical Committee (2010).

6.3 RESULTS AND DISCUSSION

Initial results (not shown) indicated that the process control and IAC were not detected by the triplex *rt* RT-PCR assay (HAstV, IAC and mengovirus) when nucleic acid had been extracted using the Nuclisens® easyMAG™ automated magnetic extraction platform (bioMérieux SA). The reason for this observation remains unclear and will require further investigation. Results are therefore only reported for nucleic acid extracted using the MagNA Pure LC Robotic instrument (Roche Diagnostics) (Table 6.1). The detection of mengovirus from all the strawberries, except Berry B irrigated with water dated 2012-04-23, demonstrated that the viral recovery and nucleic acid extraction processes were optimal and successful. The detection of the IAC and process control in reactions where no other viruses were detected indicates that the amplification reaction was not inhibited and the detection results for the enteric viruses are valid. As no viruses were detected in the negative control no cross contamination occurred during the analytical process. The results using water samples 2012-05-21 and 2012-07-23 for "mock irrigation" clearly indicate that enteric viruses such as group A RV and NoV GI (Table 6.1) present in irrigation water can be transferred to and detected on soft fruits. It is also evident from the data obtained from strawberries "mock irrigated" with the water sample dated 2012-06-18 that enteric viruses may be present at undetectable levels in the water sample but could still result in contamination of irrigated soft fruits.

From these experiments, where optimised virus recovery methods and multiplex *rt* RT-PCR assays were applied, it became evident that faecal contaminated surface waters used for irrigation purposes retains the possibility to contaminate soft fruit. The

Table 6.1: Summary of the virus detection results from the "mock irrigated" strawberries and the river water samples used to "mock irrigate" the strawberries using the optimised multiplex real-time reverse transcription-polymerase chain reaction assays.

Water Sample	Berry	Mengo	IAC**	HAstV	HAV	Group A RV	NoV GI	NoV GII	SaV
RV	A	+	+	-	-	-	-	-	-
2012-04-23	B	-	+	-	-	-	-	-	-
	C	+	+	-	-	-	-	-	-
Control		nd*	+	+	-	-	-	-	-
RV	A	+	+	-	-	-	-	-	-
2012-05-21	B	+	+	-	-	-	-	-	-
	C	+	+	-	-	-	+	-	-
Control		nd	+	+	-	-	+	-	-
RV	A	+	+	-	-	-	-	-	-
2012-06-18	B	+	+	-	-	-	-	-	-
	C	+	+	+	-	+	+	-	-
Control		nd	+	-	-	-	-	-	-
RV	A	+	+	-	-	+	+	-	-
2012-07-23	B	+	+	-	-	+	+	-	-
	C	+	+	-	-	+	+	-	-
Control		nd	+	+	-	+	+	-	-
Positive control	A/B/C	+	+	+	+	+	+	+	+
Negative control	A/B/C	nd	+	-	-	-	-	-	-

* nd = No process control added

** IAC = Internal amplification control

detection of NoV GI more frequently that NoV GII was relevant as NoV GI is more frequently associated with food- and waterborne outbreaks (Mattison et al, 2010). Human AstV infections have mostly been limited to shellfish and water with the exception of the 1991 Japan foodborne gastroenteritis outbreak involving thousands of children (Greening, 2006). The lack of detection of HAstV on the surface of strawberries could be; i) that HAstV could not effectively be retained on the strawberry surface, ii) that HAstV was neutralised by the strawberry juices, and iii) that HAstV was present in too a low concentration to be detected. However, HAstV remains a contributor to gastrointestinal disease among SA children (Taylor *et* al., 1997; Mans et al., 2010). The methods applied to demonstrate the viral contamination of strawberries in this study used appropriate quality control procedures and were therefore validated.

CHAPTER 7

GENERAL DISCUSSION

Food has become a well-known vehicle for the transmission of infectious diseases and has since become a public health problem for millions of people worldwide. More than 250 diseases are known to be transmitted by food and food products with an annual health cost of approximately $5-6 bil (Arora *et al*., 2011). Despite the advancements made in preventative health, enteric virus transmission by food, food products and water remains a well-recognised, largely underestimated widespread public health problem (Koopmans *et al*., 2002; Papafragkou *et al*., 2006; Cliver, 2008; O'Brein, 2008). Gastroenteritis and hepatitis, frequently caused by NoVs and HAV, but not excluding other enteric viruses such as HAstV, HRV and SaV, remains the leading cause of foodborne outbreaks (Smith De Waal *et al*., 2008; Scallan *et al*., 2011b). It is therefore imperative for the development of rapid, competent and reliable methods for the detection and recovery of these pathogens in and from food and environmental samples (Bosch *et al*., 2011; Arora *et al*., 2012; Rodríguez-Lázaro *et al*., 2012).

Molecular techniques have now been used and implemented on a routine basis in virology laboratories with real-time quantitative PCR being the method of choice for the detection of enteric viruses (Rodríguez-Lázaro *et al*., 2012). In one of the objectives of this study was to develop and optimise sensitive multiplex *rt* RT-PCR assays for the detection of selected enteric viruses, namely, NoV GI, NoV GII, SaV, HAV and HRV. In order to validate these assays, appropriate quality controls were implemented to distinguish between false negative test results by using an AC and a process control (mengovirus) was used.

An AC is used both in the presence or absence of the target and serves as an indicator for the adequacy of the amplification conditions (Bosch *et al*., 2011; Rodríguez-Lázaro *et al*., 2012). With the majority of nonbacterial gastroenteritis outbreaks over the past decade caused by NoV GII.4 strains (Siebenga *et al*., 2009) and with the frequent detection of NoV GII in SA surface waters (Mans *et al*., 2013), a competitive RNA AC was suggested and developed specifically for the NoV GII *rt* RT-PCR assay. The

internal region of the MBA gene of *U. parvum* was used as the non-target DNA for the construction of the AC fragment. Oligonucleotide primers were designed to contain both the *U. parvum* and NoV GII forward and reverse primers, respectively, along with an SP6 RNA polymerase promoter sequence to allow the amplified PCR fragments to be *in vitro* transcribed to RNA. A one-step *rt* RT-PCR assay for the detection of the AC in a duplex reaction as an IAC or in a separate single reaction as an EAC to NoV GII was designed and evaluated. The duplex one-step *rt* RT-PCR assay using the AC as an IAC could more effectively distinguish between false negative test results and was overall more cost effective and less time consuming. The competition for primers and nucleotides was minimised due to the size difference of ~174 bp between the IAC (262 bp) and NoV GII (88 bp) amplicons, similar to what was done in the study by Oikonomou *et al*. (2008). A shared sensitivity to inhibition by the IAC and NoV GII was noticed and was thought to be due to the primer sequences shared by both, since no other characteristics were shared by their respective amplicons.

Three multiplex *rt* RT-PCR assays for the detection of NoV GI, NoV GII, SaV, HAV, HAstV, HRV and mengovirus were optimised with the IAC implemented both as a competitive and non-competitive AC. A total of four multiplex *rt* RT-PCR amplification kits with two cDNA synthesis kits were evaluated and it was evident from the results that two-step EXPRESS qPCR Super Mix Universal kit (Invitrogen) with the RevertAid Premium™ First Strand cDNA synthesis kit (Fermentas Life Sciences) was the method of choice. In the assessment viral stocks of SaV, NoV GI, RV-SA11 and HAstV were unquantified. Furthermore, insufficient clinically-derived HAstV stock was available and along with NoV GI, RNA transcripts were made from DNA PCR fragments. In a study done by Bowers and Dhar. (2011), DNA standard curves were shown to be as effective as RNA standard curves when used to quantify nucleic acid. Based on these findings, DNA standard curves for each of these viruses using quantified PCR products were made and were used to quantify the RNA fragments generated of each of these viruses. Once quantified, RNA standard curves were constructed for each of the viral stocks to be used. Despite the inability to simultaneously amplify NoV GII with NoV GI and NoV GI and NoV GII with mengovirus, three multiplex *rt* RT-PCR assays were proposed:

i) NoV GII, HAV and the IAC,
ii) NoV GI, SaV and HRV, and

iii) HAstV, mengovirus and the IAC.

The multiplex *rt* RT-PCR assays were optimised by comparing the Ct-value means of each of the respective singleplex *rt* RT-PCR assays to that of the multiplex *rt* RT-PCR assays generated from the amplification of each of the respective RNA standard curve nucleic acid dilutions. In the majority of the results less than a $\log_{10}$ deviation between the singleplex and multiplex *rt* RT-PCR assays existed with the exception noted for HAstV. This was ascribed to a 46% sequence overlap between the HAstV amplicon and the mengovirus forward primer which resulted in a log10 decrease in the detection of HAstV when it was present at low concentrations (1.7×10^5 - 1.7×10^4 copies/mℓ).

In the second objective of this study, the optimised multiplex *rt* RT-PCR assays was to be compared to monoplex or singleplex assays currently in use. Due to financial constraints NoV GI, NoV GII, HAV and mengovirus were the viruses decided upon to be used in the comparison and to fulfil this objective. A total of 19 water samples collected over a 5 mo period were subjected to viral recovery from water, primary and secondary viral concentration and nucleic acid extraction. The extracted nucleic acid was used in the detection of these viruses with the individual environmental one-step *rt* RT-PCR ceeramTools™ Detection Kits (Ceeram s.a.s) and the results compared to that of the optimised multiplex *rt* RT-PCR assays. The objective was to determine if the same virus could be detected by both assays. It was evident from the results that the one-step *rt* RT-PCR ceeramTools™ Detection Kits (Ceeram s.a.s) could more effectively detect HAV and mengovirus from the water samples and could possibly be attributed to the difference in the nucleic acid concentration used per PCR reaction. However, the NoV GII/HAV/IAC multiplex assay could in one instance detect HAV in a single water sample in which the one-step *rt* RT-PCR assay could not. Furthermore, an increased sensitivity in the detection of NoV GI and NoV GII from the water samples were noted in the respective multiplex *rt* RT-PCR assays when compared to the one-step *rt* RT-PCR assays. The major advantages of the multiplex *rt* RT-PCR assays over that of the singleplex *rt* RT-PCR assays as was seen in this comparison was the ability to improve the turnaround time, to reduce the cost needed and with the cost and time needed for the detection of a single virus additional viruses (HRV, SaV and HAstV not tested for) could be detected at the same time. As to determining which *rt* RT-PCR assay to implement in a specific laboratory setting, the choice will ultimately be based

on cost, availability of equipment and the discretion of that setting due to a lack in standardised techniques (Evander *et al*., 1992; De Paula *et al*., 2004; Wacker and Godard, 2005; Wong and Medrano, 2005; Rodríguez-Lázaro *et al*., 2012).

The successful application of molecular-based techniques largely depends on the effective recovery of viruses from food and water matrixes (Hurt *et al*., 2001). Enteric viruses can attach to the surface of food matrices by either ionic and/or hydrophobic interactions, van der Waals forces, ligands/receptors or the uptake into plants (Le Guyader and Atmar, 2008). Many studies have been directed towards the recovery of enteric viruses from food, food products and water matrices (Boxman *et al*., 2007; Fino and Kniel, 2008; Kim *et al*., 2008; Ikner *et al*., 2012; Liu *et al*., 2012; Prata *et al*., 2012; Summa *et al*., 2012). In order to break these attachments, an elution step is generally require to free the viruses from the food surfaces as naturally contaminated food matrices can carry virus particles on the surface (Butot *et al*., 2007). The third objective of this study was to optimise methods for the recovery of selected enteric viruses from the surface of food matrices, with the focus on berry fruits as they have been implicated and considered a potential future cause of outbreaks (Niu *et al*., 1992; Hutin *et al*., 1999; Brassard *et al*., 2012). Five washing/elution buffers were evaluated based on their effectiveness in the recovery of seeded enteric viruses on strawberries. The elution protocols were based upon the following parameters:

i) protein content (0.5% vs. 3%),
ii) pH (pH 7.2 vs. pH 9.5),
iii) elution period (20 min vs. 5 h), and
iv) the addition of TRIS to the buffers.

In the analysis of the results it was evident that no single buffer was more effective and efficient in the recovery of all the viruses tested. The elution buffers GBE- and TGBE-7.2 showed no analytical significance in the recovery of the selected enteric virus from the strawberries compared to the other elution buffers evaluated. The pH more so than the protein concentration was found to be the biggest contributor in the recovery of these viruses, however, analytical significant difference was noted for NoV GI (GBE-7.2 Con A; TGBE-7.2 Con A and B) when compared to the other elution buffer controls. In the recovery of each of the viruses from the strawberries, no analytical significant differences was noted for the two 3% beef extract elution buffers when either of the parameters were considered. Kim *et al*. (2008) demonstrated that the 3% beef

extract buffer with a pH of 9.5 was more effective and could have been attributed to the higher protein concentration, however, the 3% beef extract buffers evaluated in this study proved to be more effective at a pH 7.2 but this was not supported by analytical significant differences.

In achieving the fourth objective, which was to apply the optimised methods to analyse irrigation and processing water and berry fruits for the presence of enteric viruses, irrigation water and strawberries were collected from the period September 2010 to August 2011. For the water samples a glass wool absorption-elution method was used to recover the viruses and a PEG_{6000}/NaCl precipitation method was used to further concentrate the viruses. It was evident that no analytical significant differences existed between the 3% beef extract elution buffers for the recovery of HAV (Figure 4.2), NoV GII (Figure 4.3), NoV GI (Figure 4.4) and mengovirus (Figure 4.8) from the surface of strawberries and so the 3% TGBE-9.5, as shown by Kim *et al*., (2008) was the most effective elution buffer evaluated and was used in the recovery of these viruses from the surface of the collected strawberries. Since little differences existed between the efficiencies of the nucleic acid extraction methods (Table 4.2), both the semi-automated NucliSens® easyMAG™ system (Biomérieux) and the fully automated MagNA Pure LC instrument (Roche Diagnostics) using the MagNa Pure LC Total Nucleic Acid Isolation Kit (large volume) (Roche Diagnostics) were used. Both the methods showed similar sensitivity with no viral preference detected for either of the two methods assessed.

From February 2011 the farmer implemented drip irrigation as appose to overhead irrigation. Overhead irrigation with polluted water substantially increases the likelihood of leaving residual contaminants on fresh produce (Cheong *et al*., 2009; Berger *et al*., 2010). The water and strawberry samples were tested for NoV GI, NoV GII. HAV and mengovirus with optimised singleplex assays used in the development of the individual multiplex assays. It was evident from the results that NoV GII was more frequently detected than NoV GI and HAV in both the water samples and strawberry samples during this period. An increase in the detection of enteric viruses from June 2011 to August 2011 on the strawberries was noted, even though drip irrigation was still being used with the absence of a sprinkler system noted. Post-harvest contamination of fresh produce can be attributed to unhygienic practises by food handlers (Baert *et al*., 2009)

whereas pre-harvest contamination are considered to be due to contaminated irrigation water or organic-based fertilisers (Carter, 2005). Fertiliser was considered a possible reason for the contamination noted on the strawberries during June 2011 to August 2011 when the field was prepared for the new crops planted in March 2011.

For the final objective, 11 irrigation water and six strawberry samples collected during the period September 2010 to August 2011 were tested again using the optimised multiplex assays (Chapter 3). The detected viruses were characterised, but only three of the NoVs could be successfully genotyped (Table 5.7). Human NoV GII.7 were identified on the surface of the strawberries and a NoV GII.18 in the irrigation water. It was interesting to note, and for the first time in SA, the presence of swine NoV GII.18 in sampled irrigation water. This begs the question as to the possibility of zoonotic NoV infection. To establish such a link, known water samples, obtained from a sewage polluted river over a 4 mo period, were used in mimicking the contamination of strawberries by irrigation water. The strawberries were dunked into the water and left to dry. This was followed by viral recovery and nucleic acid extraction of the possible viruses on the surface of the strawberries and in the water. From the analysis of the results it was evident that such a link exists with the viruses detected on the surface of the strawberries being also present in the water samples (Table 6.1). Furthermore, the water sampled on 2012-07-23 contributed to a higher degree of contamination as opposed to the water sampled in April - June (Table 5.5). Sewage polluted irrigation water could be a definite cause of contamination on the strawberries and other fresh produce. Due to insufficient typing data link between polluted irrigation water and strawberries could not be confirmed.

In conclusion, the outcomes of this study includes:

i) a functional AC that can be applied in a duplex and multiplex *rt* RT-PCR assay,
ii) sensitive and specific multiplex *rt* RT-PCR assays that can be used for surveillance and in outbreak situations for selected enteric viruses, and
iii) new data on the possible role of irrigation water as a source of contamination on berry fruits in SA.

CHAPTER 8

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APPENDIX A

A.1: **de Ridder GA,** Taylor MB, Cook N. Amplification controls for the real-time PCR analysis of noroviruses in water and food: which is best [Poster]. 2nd Regional Conference of the Southern African Young Water Professionals 3-5 July 2011: CSIR International Convention Centre, Pretoria, South Africa.

Outbreaks of waterborne disease may result from exposure to faecally polluted water used for domestic purposes, recreation or irrigation. Noroviruses (NoVs) are one of the most common causes of waterborne disease. These viruses are shed in large numbers with a low infectious dose, ranging from 1 to 100 particles. Analysis for viruses in water and food is a complex and expensive process due to the viruses low titers and is often not done. As NoV cannot be isolated in cell culture, real-time reverse transcriptase-polymerase chain reaction (*rt*RT-PCR) is considered the gold standard for the detection of these viruses. As these assays are very sensitive to inhibitory compounds in environmental samples, therefore effective quality control procedures are required to exclude false positives and negative results. To ensure the integrity of the amplification reaction an amplification control (AC) can be used. An AC is an oligonucleotide that contains the same primer-complementary sequences as the target but is otherwise dissimilar to the target viral nucleic acid. The AC is included in each *rt*RT-PCR reaction to indicate the presence or absence of inhibitors. The objective of this study was to establish whether an external (EAC) or internal (IAC) AC was more effective for monitoring reaction failure in *rt*RT-PCR-based assays for NoV in water and food samples. An AC was constructed using *Ureaplasma parvum* as the non-target DNA, flanked with NoV GII primers and a SP6 RNA polymerase promoter. The AC *rt*RT-PCR was optimised and assessed to determine whether it out competed the target sequence in the *rt*RT-PCR reaction. Faecally polluted irrigation water, which previously tested NoV positive or negative, were retested using the AC in both IAC and EAC format to compare which AC performed the best. A statistically insignificant level of competition was noted between the target nucleic acid and AC. Norovirus GII positive field samples which re-tested positive for NoV GII showed no amplification of either of the ACs. Norovirus GII negative field samples which re-tested negative for NoV GII also showed no amplification of either of the ACs indicating the presence of inhibitors. The latter sample's nucleic acid was diluted and retested for NoV GII using both AC formats. The appearance of a signal in the diluted nucleic acid indicated the presence of inhibitory substances in the neat extract. These results indicate that inhibitory substances in water influence the amplification of the AC and NoV GII target sequences. False negative results were clearly identified by the lack of signal from the AC. Both ACs performed equally well but the IAC was found to be less laborious and more cost-effective as only one reaction is required compared to the two reactions required for the EAC.

A:2: **de Ridder GA,** Taylor MB, Cook N Amplification controls for the real-time PCR detection of noroviruses in water and food samples [Presentation]. Faculty Day, Faculty of Health Sciences, University of Pretoria 30-31 August 2011: HW Snyman Building North, Pretoria.

Noroviruses (NoVs) are one of the most common causes of water- and foodborne gastroenteritis. These viruses are shed in large numbers with a low infectious dose, ranging from 1 to 100 particles. The analysis of water and food for viruses is a complex and expensive process. Noroviruses cannot be isolated in cell culture, consequently real-time reverse transcriptase-polymerase chain reaction (*rt*RT-PCR) is considered the gold standard for the detection of these viruses. Effective quality control procedures are required to ensure the validity of the *rt*RT-PCR results as *rt*RT-PCR assays are sensitive to inhibitory substances. To ensure the integrity of the amplification reaction an amplification control (AC) can be used. An AC is included in each *rt*RT-PCR reaction to indicate the presence or absence of inhibitors. The objective of this study was to establish whether an external (EAC) or internal (IAC) AC was more effective for monitoring reaction failure in the NoV *rt*RT-PCR. An AC was constructed using *Ureaplasma parvum* as the non-target DNA, flanked with NoV GII primers and a SP6 RNA polymerase promoter. The *rt*RT-PCR assays for the AC and NoV GII were optimised and then assessed in a duplex reaction to determine whether the AC outcompeted the target sequence. A statistically insignificant level of competition was noted between the target nucleic acid and the AC. Ten faecally polluted irrigation water samples were tested using the AC in either the IAC or the EAC format to compare which AC was more suitable to be applied in field samples. Using the IAC format true positives were detected in 30% and true negatives in 50% of the samples whereas the EAC format indicated 40% true positives and 10% true negatives. These results suggest that inhibitory substances in water influence the amplification of the AC and NoV GII target sequences. The IAC format was found to be less laborious and more cost-effective as only one reaction was required compared to the two reactions required for the EAC.

A.3: de Ridder GA, Taylor MB. Strawberries: Healthy friend of foe [Poster]. Faculty Day, Faculty of Health Sciences, University of Pretoria 28-29 August 2012: HW Snyman Building North, Pretoria.

Introduction: Enteric viruses, mainly hepatitis A virus (HAV) and noroviruses (NoVs) have been identified as the leading cause of foodborne outbreaks of hepatitis and gastroenteritis, respectively. Imported and exported berry fruits have resulted in an increasing number of outbreaks of gastroenteritis and hepatitis over the past decade. These viruses have been detected previously on lettuce, parsley and tomatoes cultivated in South Africa (SA) and in surface waters to irrigate fresh produce. To date, no data on the virological quality of strawberries grown and harvested in SA exists.

Aim: To try and establish a link between viral contamination in irrigation water and that on irrigated strawberries cultivated in SA.

Methods: From September 2010 to August 2011, seasonal strawberries (3 samples comprising of 3 berries each) and irrigation water samples (10L) were collected monthly from a small scale commercial farmer and tested for the presence of HAV and NoVs. A 3% beef extract buffer and the glass wool absorption-elution method was used to recover the viruses from the strawberries and water samples, respectively. Secondary concentration was achieved using an optimised PEG_{8000}/NaCl precipitation method. An internal amplification control was used to determine the presence of real-time PCR inhibitors while a process control (mengovirus) were used to validate that the sample processing procedure. In samples where inhibition was detected the RNA was diluted 10-fold and retested.

Results: A total of 72 strawberries, representing 3 samples from 8 months, were collected of which, 1.4% (1/72), 8.3% (6/72) and 1.4% (1/72) were positive for NoV GI, GII and HAV, respectively. Dual contamination of strawberry and water samples with NoV GI and/or NoV GII was recorded in the months of September 2010 and August 2011. Twelve (10L) irrigation water samples were collected of which NoV GI was detected in 16.7% (2/12) and NoV GII in 41.67% (5/12), however, HAV was not detected in any of the water samples.

Discussion/Conclusion: Potentially pathogenic enteric viruses were detected on strawberries cultivated in SA. As the strawberries were collected pre-harvest in the field the surface water from an irrigation canal used for irrigation was identified as the source of contamination.

APPENDIX B

Internal Amplification Controls in Real-time Polymerase Chain Reaction-based Methods for Pathogen Detection

4

Nigel Cook, Gabriel A. de Ridder, Martin D'Agostino and Maureen B. Taylor

Abstract

Assays based on nucleic acid amplification are highly efficient, but they can be affected by the presence of matrix-derived substances which can interfere or prevent the reaction from performing correctly. Careful sample treatment must be applied/used to remove these inhibitory substances. However no sample treatment can be relied on completely, thus an amplification control should be employed to be able to verify that the assay has performed correctly. An internal amplification control (IAC) is a non-target NA sequence present in the very same reaction as the sample or target nucleic acid extract. If it is successfully amplified to produce a signal, any non-production of a target signal in the reaction is considered to signify that the sample did not contain the target pathogen or organism. If, however, the reaction produces neither a signal from the target nor the IAC, it signifies that the reaction has failed. An IAC concentration optimisation protocol is described.

Introduction

The technological progression from the end-point detection of polymerase chain reaction (PCR) products to their detection in real-time has been widely taken up by the research community, and now nearly all studies which involve nucleic acid amplification-based pathogen detection utilize real-time PCR or quantitative PCR (qPCR). The inherent advantages of qPCR-based methods for pathogen detection over conventional PCR and/or culturing, e.g. shorter turnaround, improved detection limits, specificity and potential for automation. (Rutjes *et al.*, 2005; Bosch *et al.*, 2011) have fostered their widespread implementation in analytical laboratories, especially those dedicated to analysis of food and water (de Paula *et al.*, 2007; Le Guyader *et al.*, 2009; Nenonen *et al.*, 2009; Baque *et al.*, 2011). Currently PCR and RT-PCR is considered to be the 'gold standard' for virus detection (Rolfe *et al.*, 2007; Bosch *et al.*, 2008) and PCR was predicted to be established as a routine reference by 2010 (Hoorfar and Cook, 2003). This however has not happened for a variety of reasons (D'Agostino and Rodriguez-Lazaro 2009), including the fact that there are currently no standard procedures for the detection of viruses in environmental samples (Mattison and Bidawid, 2009; Verheyen *et al.*, 2009). Among the main issues that must be addressed for the effective adoption of PCR-based techniques in analytical laboratories is the concern that they can not always be used effectively for analysis of complex matrices such as foods because of substances inimical to the amplification reaction which may exist in these matrices (Abu Al Soud and Radstrom, 2000; Rossen *et al.*, 1992; Mattison and Bidawid, 2009; Plante *et al.*, 2011). This may cause a negative signal to be obtained when the sample did in fact contain the target microorganism. A false-negative result of analysis of a contaminated sample could result in the exposure of the public to a potential pathogen, with serious consequences.

qPCRs require that nucleic acids be extracted from a sample then delivered to the assay. As qPCR assays use small (microliter) volumes of nucleic acid as template, the microorganism and/or extracted nucleic acids must be concentrated during sample preparation to ensure that target nucleic acids are represented within the template. This process however can also concentrate inhibitory substances with the target nucleic acids. Among the substances which can cause inhibition of a qPCR are bile salts (Widjojoatmodjo *et al.* 1992), haem from blood and humic acids (Hata *et al.*, 2011), polysaccharides and polyphenols (Wei *et al.*, 2008; Plante *et al.*, 2011) which may be co-extracted with the nucleic acids. No process can be relied on to completely remove all of the inhibitory substances (Parshionikar *et al.*, 2004) and thus effective quality control procedures need to be implemented to ensure the validity of the qPCR result (Wong *et al.*, 2007; Pintó and Bosch, 2008; Bosch *et al.*, 2011). An important control for verification of the correct operation of the qPCR assay is an amplification control (AC) (Rolfe *et al.*, 2007; Hoffmann *et al.*, 2009; Lees, 2010; Bosch *et al.*, 2011). From the literature it is evident that different AC systems are being applied. One of the approaches is to use a house-keeping gene which is present in the test sample (Wei *et al.*, 2008) and this is referred to as an endogenous internal control (IC) (Hoffmann *et al.*, 2009). The other approach, which is more suitable for the analysis of water and food samples, is to use an exogenous AC which comprises a nucleic acid sequence that does not occur naturally in the sample under investigation and is unrelated to the target nucleic acid sequence (Hoorfar *et al.*, 2004; Hoffmann *et al.*, 2009; Rip and Gouws, 2009) or is a modification of the target sequence (mimic ICs)(Escobar-Herrera *et al.*, 2006; Hoffmann *et al.*, 2009). The exogenous AC can be constructed in various ways (Rodríguez-Lázaro *et al.*, 2004; Oikonomou *et al.*, 2008; Rip and Gouws, 2009; Deer *et al.*, 2010; Diez-Valcarce *et al.*, 2011; Gregory *et al.*, 2011) and applied in different formats, namely as a competitive or non-competitive internal amplification control (IAC) (Hoorfar *et al.*, 2004; Parshionikar *et al.*, 2004; Deer *et al.*, 2010; Hata *et al.*, 2011) or as a external amplification control (EAC) (Costafreda *et al.*, 2006; Lees, 2010). In the literature the IAC is also referred to as an IC (Rolfe *et al.*, 2007; Hoffmann *et al.*, 2009) or internal positive control (IPC) (Lauri and Mariani, 2009).

IAC rationale

An IAC is a non-target nucleic acid sequence present in the same reaction as the sample nucleic acid extract, and which can be amplified simultaneously with the target. An IAC signal should always be produced when there are no target sequences present in the qPCR. In a qPCR without an IAC, the absence of a target signal can be interpreted as signifying that the sample did not contain the target. However, it could have been that the qPCR had failed through the presence of inhibitory substances, or for other reasons such as thermocycler failure or incorrect mastermix preparation. In this case a false-negative interpretation of the result is possible. The use of an IAC will prevent false-negative interpretations.

To be most effective, the IAC should possess the same primer complementary sequences as the target, in other words, it should be amplifiable by the same primers as are used to amplify the target. However, it should be readily distinguishable from the target amplicon. In conventional PCR this is achieved by making the IAC amplicon a different size to the target, but in qPCR an IAC amplicon-specific probe is used containing a different fluorescent dye from that used to detect the target (Rodríguez-Lázaro *et al.*, 2005a,b). The use of the same primers for amplification of IAC and target will result in competition between the IAC and the target for the primers. This competition means that if there is a greatly higher concentration of target sequences within the qPCR then the IAC may not be amplified, or be amplified to a lesser extent than the target. In such a case, only the target signal will appear. It is not necessary in that case for an IAC signal to be obtained, for of course the presence of a target signal means that the qPCR has operated correctly.

Table 4.1 shows the pattern of results which can be obtained when using an IAC in an qPCR, and how to interpret them.

Table 4.1 Interpretation of pattern of signals which can be observed when an IAC is incorporated into a qPCR-based method for pathogen detection

Observed result	Meaning	Action
Target signal and IAC signal present	qPCR has operated correctly. Sample is target-positive	Record sample as contaminated
Only target signal present	qPCR has operated correctly. Sample is target positive with high amount of target	Record sample as contaminated
IAC signal present and target signal absent	qPCR has operated correctly. Sample is target-negative	Record sample as uncontaminated
IAC and target signal absent	qPCR has failed	Repeat using new mastermix and/or test a dilution of sample nucleic acid extract

IAC construction for qPCR

The basic steps in IAC construction are described below.

Construction of internal amplification control

1 Design primers containing target, non-target, and RNA polymerase sequences.
2 Get non-target DNA.
3 Amplify non-template DNA with hybrid primers.
4 Purify amplicon.
5 Transcribe amplicon (if RNA IAC required).
6 Purify IAC.
7 Quantify IAC.

General procedures for construction of IACs are given in Hoorfar *et al.* (2004). As mentioned, the signal from amplification of an IAC for qPCR needs to be distinguishable from the signal from the target, and this is most often achieved by using different probes. Therefore the IAC oligonucleotide must contain specific sequences to which its probe can bind, and these sequences must be totally different to those targeted for amplification within the pathogen's genome.

An IAC construction procedure that has been widely used involves utilizing probe–sequence combinations from qPCR assays for microorganisms other than the target. For example, when constructing an IAC for a qPCR for *Mycobacterium avium* subsp. *paratuberculosis*, Rodriguez-Lazaro *et al.* (2005a) incorporated sequences from *Listeria monocytogenes* which hybridized with a probe which incorporated the VIC dye. The amplicon could thus be distinguished from the target amplicon, which hybridized with a probe incorporating the FAM dye.

To construct the IAC oligonucleotide containing these non-target sequences along with sequences complementary to the target primers, it is necessary to first construct chimeric primers which link the target primer sequences to primers for the non-target sequences. These chimeric primers are then used to amplify the non-target sequences, and the resulting amplicon contains the full IAC sequence. The amplicon can then be purified and used in the intended qPCR, or cloned into a plasmid which is subsequently used as the IAC.

It is advisable to construct the IAC oligonucleotide such that it is ~100–200 bp larger than the target sequence. In this way, competition between the IAC and target is more favourable to the target.

Fig. 4.1 shows a schematic diagram of this procedure.

Optimization of IAC concentration

It is essential that a competitive IAC be incorporated in an qPCR in an amount which will not out-compete the amplification of low copies of target. To achieve this, it is necessary to optimize the quantity of IAC which is used in the qPCR. The protocol below will mediate optimization of the IAC concentration.

1 Dilute the quantified IAC solution to, for example, 10^{-10} using nuclease-free water with 0.1 mg /ml bovine serum albumin (BSA) added.

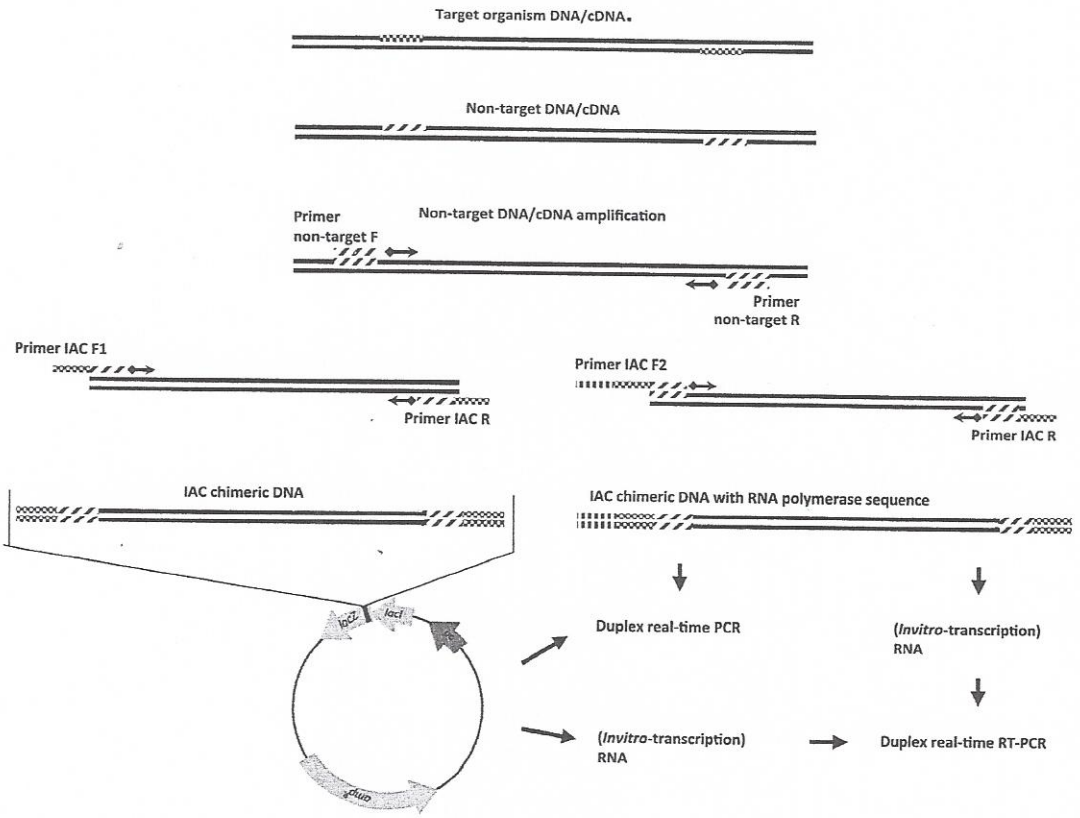

Figure 4.1 Representation of two methods to construct an IAC. (A) Primer IAC F1 comprises the target non-target forward primer sequences. (B) Primer IAC F2 comprises the target/non-target forward primer sequences as well as an RNA polymerase promoter sequence. Primer IAC R comprises the target/non-target reverse primer sequences.

2 Prepare a mastermix for 11× reactions. Do not add IAC to this mastermix. Replace the volume of target as quoted in the relevant protocol, with the equivalent volume of water.
3 Add *n** µl of each IAC dilution from step 1, to 10 reactions. To the 11th reaction, instead of the IAC, add the same amount of water (negative control). *The volume of IAC which is specified in the relevant protocol.
4 Perform PCR. If it is a nested PCR, perform both rounds.
5 Choose the highest dilution of IAC which produces a positive signal.
6 Prepare a mastermix for four reactions. Again, do not add IAC to this mastermix. Replace the volume of target as quoted in the relevant protocol, with the equivalent volume of water.
7 Add *n* µl of the dilution chosen in step 5 to the first reaction.
8 Add *n* µl of the next highest dilution (e.g. if the dilution chosen in step 5 is 10^{-6}, then use 10 µl this step) to the second reaction.
9 Add *n* µl of the next lowest dilution (e.g. if the dilution chosen in step 5 is 10^{-6}, then use 10^{-5} in this step) to the third reaction.
10 In the fourth reaction use water instead IAC dilution sample.
11 Perform PCR. If it is a nested PCR, perform both rounds.
12 Repeat steps 6–11 twice more.
13 Choose the highest dilution of IAC which consistently gives three positive signals from the three separate mastermixes. This is the working dilution of IAC which is to be used in all subsequent tests which include this particular batch of IAC.

14 Determine quantity of working IAC by extrapolating dilution from the quantity in the neat stock.

It will be found that the concentration of IAC set using this protocol will not out-compete low copies of the target.

Examples of IAC use in qPCR-based methods for pathogen detection

IACs are especially important when a culture-based detection of a bacterium can be very slow, as with mycobacteria, and therefore PCR must be used on nucleic acids extracted directly from a sample, which may contain inhibitors. Rodriguez-Lazaro *et al.* (2005a) developed a real-time PCR assay for quantitative detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) which amplified sequences from the IS*900* insertion element which is specific for this bacterium, and included an IAC. The IAC consisted of a 64-bp fragment of a listerolysin (*hly*) gene from *L. monocytogenes* (Mengaud *et al.*, 1988) flanked by primers for IS*900* amplification. It was constructed by amplifying *L. monocytogenes* sequences using hybrid primers where each IS900 sequence flanked 5′ a *hly* sequence. A second PCR reaction performed with the *IS900*-specific primers confirmed that the chimeric DNA produced in the first round PCR contains the complete sequence of the target primers. One hundred molecules of IAC were used per reaction in subsequent tests. The final assay consistently could detect 12 MAP cells with 99% probability, demonstrating its sensitivity and the lack of interference with target amplification by the IAC. Harnessing the assay to a sample pretreatment for milk based on protein and fat removal allowed the resulting method to be able to detect down to 100 MAP cells in 20 ml milk, with no failure of any reaction.

The most significant food-transmitted or environmentally transmitted viruses, such as norovirus, hepatitis A virus or hepatitis E virus, can not be grown, or can be grown only with difficulty, *in vitro* in cell culture, and therefore direct extraction of nucleic acids from a sample followed by PCR are essential for their detection. This being the case, an amplification control is an absolute necessity to ensure accurate interpretation of results. Diez-Valcarce *et al.* (2011) constructed IACs for existing qPCR or reverse transcription qPCR (RTqPCR) assays for a range of viruses which could be found in the environment or foods: bovine polyomavirus, hepatitis A virus, hepatitis E virus, human adenovirus, human norovirus genogroup I, human norovirus genogroup II, murine norovirus and porcine adenovirus. The IACs for the assays for the DNA viruses were plasmid-based, and those for the RNA virus assays were themselves RNA molecules which were transcribed from chimeric sequences cloned into a plasmid transcription vector, following the principles for design of RNA IACs devised by Rodriguez-Lazaro *et al.* (2004). Addition of optimized (following the procedure outlined above) amounts of IAC into the assays did not affect the limits of detection for each specific target virus. To demonstrate how the IACs would indicate reaction failure, a situation in which purification of nucleic acid from a food sample was poorly performed was simulated, by adding 50 µl of non-extracted strawberry puree to 50 µl of nucleic acid extract prior to nucleic acid amplification; no IAC signal was obtained, whilst robust signals were obtained from nucleic acid extract correctly obtained from strawberry samples (by the method of Dubois *et al.*, 2006).

The study of Radin and D'Souza (2011), in which IACs were developed for two broadly reactive qRTPCR assays for noroviruses, provided further demonstration that properly optimized IAC-containing reactions have the same limit of detection as the corresponding assays without IAC. This shows that concerns over competition between IAC and target can be overcome by careful optimization.

The other approach to the use of ACs is the EAC. This involves performing two separate reactions for each sample – one (the test) reaction contains only the sample nucleic acid, but the other (the control reaction) contains the sample nucleic acid plus the amplification control (Costafreda *et al.*, 2006). If it is successfully amplified to produce a signal, any non-production of a target signal in the test reaction is considered to signify that the sample did not contain the target.

If, however, no signal is produced in both the test and control reactions, it signifies that the nucleic acid extract contains inhibitory substances and the reaction has failed. The potential competition issue (see above) has led some workers to adopt the EAC approach. However, using EACs one can never be completely certain that the test reaction has not individually failed, for example through pipetting error or non-homogeneous contamination by inhibitory substances. For example, if an EAC signal is produced in the control reaction, but no target signal is produced in the test reaction, can one be completely certain that the test reaction has not failed? However, using an IAC eliminates this ambiguity, since it is present in the mastermix and a signal will always appear when the reaction has not failed or high levels of competing target are not present (if they are, a target signal will be produced anyway). The concern of the proponents of the EAC approach regards the possibility that a low level of target may be outcompeted by the IAC, leading to a false negative result. However, a thoroughly optimized assay should not present these problems (D'Agostino *et al.*, 2004). A recent study has moreover demonstrated, by a direct comparison between an IAC- and EAC-containing qPCR-based method for detection of NoV GII in foods, that an optimized IAC-containing PCR assay will mediate the same level of detection as an EAC-containing qPCR assay and that it is more cost-effective than an EAC-containing method (de Ridder *et al.*, unpublished data).

A recently proposed (Rossmanith *et al.*, 2011) approach is the use of internal sample process controls (ISPCs). ISPCs are added at the beginning of the sample treatment, like normal sample process controls (SPCs; D'Agostino *et al.*, 2011), then passaged through all subsequent analytical steps. An ISPC can be a recombinant organism containing a single-copy artificial DNA sequence for coamplification. This approach would appear convenient, in that it combines two controls (the SPC and the IAC) in one. However, in routine analysis it would not allow for troubleshooting, e.g. if no ISPC signal is obtained, it will not be clear whether it was the sample process or the amplification which has failed.

Concluding remarks

If qPCR-based monitoring of food supply chains for pathogens is to be effectively performed as part of a food safety programme or an epidemiological investigation, then it is vitally necessary that the reliability of the analytical results can be verified (Rodriguez Lazaro *et al.* 2007). Therefore, during routine monitoring of foodstuffs with a molecular screening method an appropriate suite of controls must be used to verify that the method has performed correctly (D'Agostino *et al.*, 2011), and the AC is an essential component of this suite.

Although an IAC in a qPCR offers an unambiguous control to conclusively show that the assay has performed correctly it must however be borne in mind that the IAC or EAC may be detected in the qPCR even though the nucleic extraction procedure has failed. It is therefore important to implement effective quality control procedures to control the entire analytical process from elution, concentration, nucleic acid extraction to detection (Mattison and Bidawid, 2009; Bosch *et al.*, 2011; D'Agostino *et al.*, 2011) to ensure the validity of results.

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APPENDIX C

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

22/04/2010

Number	:	**S45/2010**
Title	:	The development and application of multiplex real-time reverse transcriptase polymerase chain reaction assays for the detection of enteric viruses on berry fruits and in water samples
Investigator	:	Gabriel de Ridder, Department of Medical Virology, University of Pretoria (SUPERVISOR: Prof MB Taylor)
Sponsor	:	**None**
Study Degree:		MSc. Medical Virology

This Student Protocol was approved by the Faculty of Health Sciences Research Ethics Committee, University of Pretoria on 20/04/2010. The approval is valid for a period of 3 years.

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