Sveriges lantbruksuniversitet
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Faculty of Natural Resources and Agricultural Sciences

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Abstract

Food-poisoning is a major health problem and an estimated half a million Swedes are food-poisoned annually, with acute gastroenteritis as a consequence. One of the major causes of contaminated foods is related to food- and waterborne viruses. To be able to trace back the source of contaminant, the method of detecting viruses must be specific and sensitive. No standardized method for detecting foods for sapovirus exists today.

The aim of the work described in this bachelor thesis is to implement and optimize a real-time RT-PCR method for the detection of all genogroups of human sapovirus in foods. The optimization involves modifications of an already existing assay.

Three synthetic DNA templates, representing the different genogroups of human sapovirus, were amplified by real-time one-step RT-PCR. The modification of the RT-PCR assay involved testing TINA modified primers over a temperature gradient and primers used by Karolinska University Hospital. All genogroups of human sapovirus were detected, but there was no notable improvement using the TINA modified primers or the primers used by Karolinska University Hospital. The optimum temperature for the RT-PCR was determine to 60°C and was further used in the experiments.

A monoplex assay was set up to test the specificity of the multiplex RT-PCR.The monoplex assay revealed that only the primers and probe for DNA template representing genogroup V was specific against its target.

Fecal samples from sapovirus positive patients, a gift from Karolinska University Hospital, were extracted and screened for genogroup V. All fecal samples were positive for sapovirus, whereof one sample was positive for genogroup V.

Three synthetic single-stranded DNA (ssDNA) templates were used as positive controls, representing the target sequences for the different genogroups of human sapoviruses. Problems with dilution series of these led to an investigation where different diluents were tested, as well as heating the samples. The troubleshooting of the DNA templates was not solved.

The real-time RT-PCR assay developed in this work did not have the same sensitivity as for Oka *et al.* (2006). The sensitivity of the assay must improve to be used for foods, and hopefully will this project serve as a pilot study for further investigation and development of a RT-PCR assay for detection of human sapoviruses in foods.

Sammanfattning

Matförgiftningar är ett stort hälsoproblem och en halv miljon svenskar uppskattas råka ut för matförgiftningar årligen, med magsjuka som följd. En av de viktigaste orsakerna till kontaminerade livsmedel är relaterade till livsmedel- och vattenburna virus. För att kunna spåra smittan måste metoden för detektering av virus vara specifik och känslig. Det finns i dagsläget ingen standardmetod för detektering av sapovirus i livsmedel.

Syftet med kandidatarbetet som beskrivs i den här uppsatsen är att implementera och optimera en realtids RT-PCR metod för att samtidigt detektera alla genogrupper av humana sapovirus i livsmedel. Optimeringen innebär modifikationer av en redan existerande metod.

Tre syntetiska DNA templat vilka representerar de olika genogrupper av humana sapovirus amplifierades med ett-stegs realtids RT-PCR. Modifieringen av RT-PCR metoden inkluderade test av TINA modifierade primers under en temperaturgradient, och primers vanligen använda av Karolinska Universitetssjukhuset. Alla genogrupper kunde detekteras men igen märkbar förbättring observerades med TINA modifierade primers eller primers använda av Karolinska. Den optimala temperaturen för RT-PCR bestämdes till 60°C och användes vidare i experimenten.

En monoplex analys gjordes för att undersöka specificiteten hos multiplex analysen. Monoplex analysen visade att endast primers och prob för DNA templat som representerar genogroup V var specifika mot sitt target.

Fecesprover från sapovirus positiva patienter, en gåva från Karolinska Universitetssjukhuset, extraherades och analyserades för genogrupp V. Alla fecesprover var positiva för sapovirus, varav ett prov var positivt för genogroup V.

Som positiv kontroll användes tre syntetiska enkelsträngade DNA templat, som representerar målsekvenserna för de olika genogrupper av humana sapovirus. Problem med spädningsserier av dessa ledde till en utredning där olika spädningsvätskor testades, liksom upphettning av dem. De problem som uppkom gällande DNA templaten kunde ej lösas.

Känsligheten för RT-PCRen måste förbättras för att kunna användas på livsmedel. Förhoppningsvis kommer detta projekt att tjäna som en pilotstudie för vidare undersökning och utveckling av en RT-PCR metod för detektion av humana sapovirus i livsmedel.

Table of contents

List of Tables

List of Figures

Abbreviations

bp	Base pair
cDNA	Complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
kb	Kilobases
MGB	Minor groove binder
NoV	Norovirus
NTC	No template control
ORF	Open reading frame
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT	Reverse-transcriptase
SaV	Sapovirus
ssDNA	Single-stranded deoxyribonucleic acid
TINA	Twisted intercalating nucleic acid

1 Introduction

1.1 The purpose of the bachelor thesis

The purpose of the work described in this bachelor thesis is to implement and optimize a real-time RT-PCR method for foods, which will be able to detect human sapovirus. The method should detect all sequence variations in the four known genogroups of human sapovirus. The optimization involves modifications of an already existing PCR assay published by Oka *et al.* (2006).

In this work, synthetic ssDNA templates representing the different genogroups of human sapovirus will be amplified by multiplex real-time one-step RT-PCR using several different primers and probes targeting sapovirus specific sequences. The method will be validated by screening fecal samples from sapovirus positive patients.

This independent project is placedat the National Food Agency of Sweden, and covers ten weeks of work including writing this thesis.

1.2 Food- and waterborne viruses

An estimated half a million Swedes are food-poisoned annually, with acute gastroenteritis as a consequence (Toljander & Karnehed, 2010). Food-poisoning is a major health problem which leads to, besides personal suffering, economic losses for both the affected person and companies. One of the major causes of contaminated foods is related to food- and waterborne viruses (Fig 1.).

Viruses are sub-microscopic particles which are unable to replicate without a host. They exist in both aquatic and terrestrial environments, where they persist very well due to their ability to withstand moderate heat, acidity, drying and freezing. (Richards *et al.*, 2013)

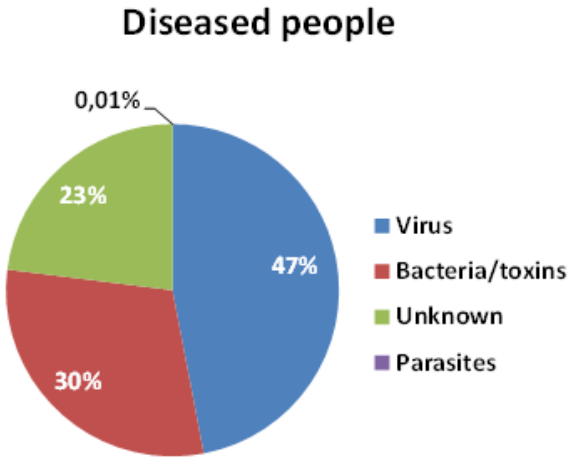

Figure 1. People in Sweden which suffered from reported food-poisoning outbreaks during 2003-2007 from different causative agents *(Lindblad et al., 2009)*

Viral pathogens associated to food- and waterborne outbreaks are the enteric virusesrotavirus, calicivirus, adenoviruses and astroviruses. Hepatitis A virus (HAV) and hepatitis E virus (HEV) are also food- and waterborne viruses and besides giving enteric symptoms, also cause hepatitis.The most common viruses causing non-bacterial human gastroenteritis belong to the family *Caliciviridae*, which consists of five genera whereof norovirus (NoV) and sapovirus (SaV) cause disease in humans. NoV differs in comparison to SaV by causing winter-vomiting disease and infects all age groups whilst sapovirus preferentially infects children. (Greening, 2006)

1.3 Food- and waterborne transmission of infectious gastroenteritis

Some foods frequently connected to viral foodborne outbreaks include raspberry, bivalves, ready-to-eat foods and bakery products.

The transmission of enteric food- and waterborne viruses is through the fecal-oral route. The infectious dose of calicivirus is small, meaning a dosage of 10 viral particles or less may result in illness. The virus will multiply in the ill person's gut, and then be secreted in large amounts through the feces for several weeks. Viruses in wastewater will persist although water is treated and filtered by a sewage treatment, reducing the amount of virus moderately, before being let out into the nature. The prevalence of intact viruses in aquatic systems increases the risk for contamination forirrigation of crops, such as raspberries. Bivalves on the other hand, filter loads of water and may naturally concentrate great amounts of viral

particles in them. (Richards *et al.*, 2013) Haramoto *et al.*(2007) showed that sporadic and possibly asymptomatic infections, apart from food-borne outbreaks, also occurred throughout the year due to SaV being abundant in wastewater and aquatic environments.

The occurrence of food-borne viruses on bakery products and ready-to-eat foods is mainly due to contamination by infected food handlers, good hygiene is essential to reduce this kind of risk (Richards *et al.*, 2013).

The causative agents of food-borne outbreaks are often difficult to determine thus the contaminated food has already been eaten or the onset of the disease occur rather late, making it less likely for the affected persons to remember what they consumed the time of infection (Lindblad *et al.*, 2009).

Although the majority of non-bacterial food-borne outbreaks and cases are caused by NoV (Lindblad *et al.*, 2009), SaV have frequently been found in young children (<5 years old) with gastroenteritis, and there are sporadic cases and outbreaks where adults have been diseased (Farkas *et al.*, 2004; Okada *et al.*, 2002).

1.4 Sapoviruses as a causative food-poisoning agent

The first SaV was found in an orphanage home in Sapporo, Japan, in 1977 by Chiba *et al*. (1979). The virus was detected by electron microscope (EM) and had the typical calicivirus configuration of a Star-of-David (Chiba *et al.*, 2000). The virus was called Sapporo-like virus until 2002, when it was changed to sapovirus (Mayo, 2002).

The non-enveloped SaV particle has a diameter of 27-40 nm, with a genome consisting of approximately 7.5 kb of positive-sense single-stranded RNA (ssRNA) with two or three open reading frames (ORF) (Nakanishi *et al.*, 2011).

SaV have been divided into five genogroups based on their capsid protein gene sequences. Genogroup I (GI), II (GII), IV (GIV) and V (GV) infect humans. Genogroup III (GIII), also known as porcine enteric calicivirus (PEC), has only been found in swine. Genogroups can further be divided into clusters, or genotypes. (Oka *et al.*, 2012)

Ever since the first isolation of SaV (Sapporo/82) in Sapporo, Japan, multiple new strains from all over of the world have been isolated.

Overall, there is a great genetic diversity between SaV. However, Nakanishi *et al.* (2011) describes the global variation of isolated strains and conclude that strains classified to the same cluster remained genetically conserved over years.

Table 1 shows an overview of the proposed classification of SaV according to Oka *et al*. (2012), prototype strain from each genogroup and cluster, as well as the country from where it was isolated.

Table 1. Overview of the classification of sapoviruses (Oka *et al.*, 2012)

Genogroup/cluster	Prototype strain	Country in which isolated	Accession no.
GI/1	Sapporo/82	Japan	HM002617
GI/2	Parkville/94	Unknown	U73124
GI/3	Mexico14917/00	Mexico	AF435813
GI/4	Chiba000496	Japan	AJ606693
GI/5	Yokote1	Japan	AB253740
GI/6	Chiba000764	Japan	AJ606694
GI/7	Ehime04-1680	Japan	AB258428
GII/1	Bristol/98	UK	AJ249939
GII/2	Mc10	Thailand	AY237420
GII/3	Syd53	Australia	DQ104360
GII/4	Kumamoto6	Japan	AB429084
GII/5	Cruise ship/00	USA	AY289804
GII/6	SaKaeo15	Thailand	AY646855
GII/7	20072248	Japan	AB630067
GIII	PEC/Cowden	USA	AF182760
GIV	Houston7-1181/90	USA	AF435811
GV	Argentina39	Argentina	AY289803

Sapovirus from GI and GII occurs more frequently than GIV and GV (Okada *et al.*, 2002), which also can be observed by looking at the clusters of every genogroup (Tab 1).

1.5 Molecular techniques

The detection method for screening potentially infected foods, and trace back the source of contamination, must be highly sensitive to be able to detect very low amounts of viral particles.Conventional methods such as electron microscope (EM), ELISA and end-point PCR have been used to detect calicivirus in stool specimens are too insensitive for foods. No standardized method for detecting foods for sapovirus exists today. (Richards *et al.*, 2013)

Polymerase chain reaction (PCR) is a molecular method based on the amplification of specific sequences within DNA or cDNA templates. The amplification requires thermal cycling with the presence of several reagents such as oligonucleotides, heat stable DNA polymerase and buffer. In end-point PCR, the amplicon will need a post-PCR analysis, e.g. gel electrophoresis and image analysis, for detection and quantification.

In real-time RT-PCR TaqMan assay, used in this work, the PCR product is measured at the end of each cycle using fluorescence reporter, probes. The probes are relatively short oligonucleotides labelled with a fluorescent dye (e.g. FAM and Texas Red) in the 5' end, and a fluorescence-suppressor, named quencher in the 3' end. The probe will first hybridize with the DNA template, then the DNA-polymerasecleaves the probe by 5' -> 3' exonuclease activity, and the fluorescent dye and quencher are physically separated. Excitation of the fluorescent dye will lead to light emission at a specific wavelength, and can thereby be detected by the PCR instrument. The amplification of PCR products will theoretically increase exponentially, meaning that the fluorescence signals will increase in proportion to the amplified products. The signals give rise to an amplification curve, which can be monitored in real-time. Standard curves for quantitative measurements of the initial amount of targets can be prepared by using target nucleic acid of known quantity as modified plasmids containing the target sequence or synthetic oligonucleotides of DNA or RNA.

The threshold cycle (C_t) is the cycle where the fluorescence signal crosses the threshold, a set value above the background noise, which is positioned at the exponential phase of the reaction. C_t is related to the initial amount of target, and used in the calculation of number of DNA copies. For example, if a reaction tube containing five DNA copies and another one twice as much, the C_t value should be situated one cycle apart as theoretically the product should be doubled every PCR-cycle. The amplification gives rise to a log-linear curve. The C_t value is equivalent to the slope of the log-linear curve, and assuming the PCR works with 100% efficiency, the value of the slope should be -3.32. Inhibition, experimental factors, non-optimal reagents etc. influence the efficiency.

The collected data from the PCR instrument are transferred to a software program which automatically calculates the C_t values, and other related analysis.

Real-time RT-PCR is commonly used to detect sapovirus and has several advantages over conventional methods such as electron microscope (EM) and end-point RT-PCR (Richards *et al.*, 2013; Logan *et al.*, 2007). Real-time RT-PCR is fast, has the ability to detect a small amount of virus, and quantitative measurements are possible (Richards *et al.*, 2013).

The viral RNA has to undergo a reverse-transcriptase step where it will be transcribed into complementary DNA (cDNA). The RT-step is either made separately from the PCR reaction (two-step RT-PCR) or in the same reaction tube (one-step RT-PCR). The two-step RT-PCR requires an additional step where the tube needs to opened and the risk of contamination increases. The one-step RT-PCR decreases the risk of contamination but may be compromised in reaction conditions as the reaction buffer has to be optimized for two polymerases, the reverse transcriptase

and the DNA polymerase (Richards *et al.*, 2013). One-step real-time RT-PCR is used in this work.

A multiplex real-time RT-PCR will detect more than one target in a single run by specific primer-pairs and probes in each reaction. To test that the primers and probes are specific against the targets, a monoplex assay can be set up (Richards *et al.*, 2013).

Strains within the different genogroups of sapovirus have sequence variations, even in the most conserved regions of their genome. The use of so called degenerated primers makes it possible to amplify these variable strains with greater specificity than regular primers. Degenerated primers are a mixture of similar primers specific to their target with a variation of bases in specific positions in their sequence, also known as wobbles (Richards *et al.*, 2013). Degenerated primers are used in this PCR assay to target sequence variations in the primer regions. For example, in this work, the reverse primer SaV124R has been designed with the wobble Y, which implies the primer mixture contain both primers with nucleotide C and T in one specific position in the primer sequence.

Both primers and probes may be modified with molecules to improve the PCR reaction. Twisted intercalating nucleic acid (TINA) is a molecule that binds to primers at the 5' end and has, according to the manufacturer (Eurofins Genomics), the ability of increase the melting temperature (T_m) up to 8°C and lower the optimum primer concentration by 30 to 50% and reduce the cycles of quantification. A minor groove binder (MGB) is a molecule bound to a probe and has, according to the manufacturer (Life Technologies), the properties of stabilizing shorter probes when hybridizing with DNA, and raising the T_m.

In regards of descriptions of molecular methods, the handbook of real-time PCR from Life Technologies has been used.

2 Materials and methods

2.1 Design of primers and probes

The most conserved regions of ten sequenced genomes of the four genogroups were according to Oka *et al.* (2006) found in the ORF 1, surrounding nucleotide 5100 which is located in the very 5' end of the coding region of the viral RNA dependent RNA polymerase gene. Four primers and two probes designed to fit within the conserved region was selected according to Oka *et al.* (2006). The set of primers were also ordered with TINA-modifications. (Tab 2)

Two additional forward primers, SaVF1 and SaVF2, used by Karolinska University Hospital (KS) correspond to primer SaV124F, but with less wobbles. SaVF1 has only one wobble in comparison to SaV124F's two, and SaVF2 contains of no wobbles at all. (Tab 2)

Primers and probes were purchased from Eurofins Genomics and Applied Biosystems.

Table 2. Overview of primers and probes for real-time RT-PCR used in this study

	Primer or probe	**Sequence (5'->3')[a]**	**Orientation[b]**	**Length**	**T_m**	**Reference**
Primer	SaV124F	GAY CAS GCT CTC GCY ACC TAC	+	21	63.7	Oka *et al.*, 2006
	SaV1F	TTG GCC CTC GCC ACC TAC	+	18	60.5	Oka *et al.*, 2006
	SaV5F	TTT GAA CAA GCT GTG GCA TGC TAC	+	24	61.0	Oka *et al.*, 2006
	SaV1245R	CCC TCC ATY TCA AAC ACT A	-	19	53.4	Oka *et al.*, 2006
	SaV124F TINA	GAY CAS GCT CTC GCY ACC TAC	+	21	63.7	Oka *et al.*, 2006
	SaV1F TINA	TTG GCC CTC GCC ACC TAC	+	18	60.5	Oka *et al.*, 2006
	SaV5F TINA	TTT GAA CAA GCT GTG GCA TGC TAC	+	24	61.0	Oka *et al.*, 2006
	SaV1245R TINA	CCC TCC ATY TCA AAC ACT A	-	19	53.4	Oka *et al.*, 2006
	SaVF1	GAC CAG GCT CTC GCY ACC TAC	+	21	64.7	KS, 2014
	SaVF2	GAT CAC GCT CTC GCC ACC TAC	+	21	63.7	KS, 2014
Probe	SaV124TP	FAM[c]-CCR CCT ATR AAC CA-MGB[d]	-	14	N.D.	Oka *et al.*, 2006
	SaV5TP	FAM[c]-TGC CAC CAA TGT ACC A-MGB[d]	-	16	N.D.	Oka *et al.*, 2006

[a]Degenerated primers and probes contain a mixture of bases: Y=C/T, S=C/G, R=A/G

[b]plus-sense, and anti-sense

[c]FAM, 6-carboxyfluorescein, fluorescence reporter dye

[d]MGB, minor groove binder, non-fluorescence quencher

Stock solutions of freeze-dried primers were dissolved in nuclease free water to a concentration of 100 µM according to the manufacturer's instruction. 100 µl of stock solutions were added into test tubes with 900 µl nuclease free water which resulted in 1 ml of 10 µM solution.Tenfold serial dilutions of stock solutionwere stored as aliquots at -20°C.

Stock solutions of freeze-dried probes were dissolved in nuclease free water resulting in 500 µl of 10 µM solution according to the manufacturer's description. Probe solutions were stored as aliquots at -20°C.

2.2 Design of synthetic DNA templates

In contrast to Oka *et al.* (2006) which uses modified plasmids for all genogroups, as standard curves and positive controls in this work were prepared using three synthetic single-stranded DNA templates, SaV GII, SaV GI.2 and SaV GV, representing the four genogroups of human sapovirus (Tab 3).

The genetic variation among the strains of genogroup I led to the decision of using an additional synthetic DNA template, SaV GI.2, which represents the strains within genogroup I, cluster 2 (Tab 3).

The synthetic DNA templates were purchased from Biomers.net.

Table 3. Overview of synthetic DNA templates

Synthetic DNA templates	Genogroups	Location[a]	Accession no.
SaVGI.2	I[b]	N.D.[c]	U73124
SaVGII	I, II and IV	5066-5192	AY237420.2
SaVGV	V	5103-5229	AY646856.2

[a]Nucleotide position

[b]Only strains in the same cluster as Parkville (U73124)

[c]No whole genome sequence was available

Stock solutions of freeze-dried synthetic DNA templates were dissolved in nuclease free water to a concentration of 100 µM according to the manufacturer's description. The templates were diluted into tenfold serial dilutions, and thereafter saved in aliquots to avoid freeze thawing. All templates were stored at -54°C.

Additional tenfold dilutions series of DNA templates were made with nuclease free water as well as with QuantiTect nucleic acid dilution buffer (Qiagen). Half of the dilution series diluted with water were made at 95°C, and a second heating was done before added to the master mix and analyzed by RT-PCR.

2.3 Fecal samples

Eleven fecal samples from sapovirus positive patients were a kind gift from Karolinska University Hospital (KS) (Tab 4). The eleven fecal samples were sampled over four years, from different time of the year and from different age groups. The variation of sampling time points and age group was intentional.

Table 4. Overview of fecal samples used in this study

Sample name	Ct value[b]	Age group	Sampling[a]
11504	20.95	> 65 year	February 2011
11510	22.89	25-60 year	June 2011
11520	19.11	< 5 year	November 2011
12507	17.41	> 65 year	March 2012
12511	17.29	< 5 year	June 2012
12512	23.67	25-60 year	July 2012
13507	19.91	< 5 year	April 2013
13519	23.97	> 65 year	November 2013
14500	13.63	< 5 year	January 2014
14502	18.72	> 65 year	February 2014
14504	22.94	25-60 year	March 2014

[a]Year and month
[b]C_t value according to KS diagnostic analysis

Nucleic acid was extracted from fecal samples using the extraction robot BioRobot EZ1 with EZ1 Virus Mini Kit v2.0 according to the manufacturer's description (Qiagen, 2011); The kit extracts both DNA and RNA and 100 µl of fecal samples were used for the extraction of viral RNA with an elution volume of 120 µl (Qiagen, 2011). Samples werediluted with nuclease free water 10^{-1} and 10^{-2} before stored into aliquots at -54°C.

2.4 Real-time RT-PCR

Synthetic DNA templates and RNA targets were amplified by real-time RT-PCR using QuantiTect Virus Kit (Qiagen).

Each RT-PCR reaction contained 400 nM of each primer, 200 nM of each FAM labelled MGB probe, 5 µl of DNA template, 5 µl of 5x QuantiTect Virus Master Mix, 0.25 µl 100x QuantiTect Virus RT Mix and 9.75 µl added RNase-free water for a total reaction volume of 25 µl.

The cycling conditions were used according tothe handbook's protocol 3, multiplex RT-PCR for LightCycler PCR instrument, of the QuantiTect Virus Kit (Qiagen) and included an RT-step for 20 minutes at 50°C, hot start thermostable DNA

polymerase activation and RT-enzyme inactivation for 5 minutes at 95°C followed by 50 cycles of a denaturation for 15 seconds at 95°C and annealing and elongation for 45 seconds at 60°C. The PCR amplifications were performed with LightCycler (Roche Diagnostics). Experiments for determination the optimum temperature for the PCR reaction were made with temperature gradients and performed with Bio-Rad (Bio-Rad Laboratories Inc., USA), otherwise the conditions were as for LightCycler.

Non-template controls (NTC) in form of water in duplicate wells were always included. Positive controls in form of synthetic DNA templates were added for the experiment with extracted RNA.

3 Results

3.1 Optimum temperature for annealing and elongation

Five hundred thousand DNA copies of all three DNA templates were run with primer sets used by Oka *et al*. (2006). The templates were amplified with, and without, TINA modified reverse-primer, on a temperature gradient from 50-60°C. Another PCR assay was made with TINA modification on all primers, on a temperature gradient from 55-65°C.

C_t values of the DNA templates at different temperatures can be observed in Table 5. There was no notable difference of C_t values between the templates in the PCR assay with TINA primers and without. The temperature at the lowest observed C_t value ranged from 56.3-63.3°C.

The optimum temperature was determine to 60°C and was used in further experiments.

Table 5. Experiment with, and without TINA-modified primers over a temperature gradient

	Without TINA (50-60°C)		With TINA reverse-primer (50-60°C)		With TINA-primers (55-65°C)	
DNA templates	**$C_t$value[a]**	**Temperature**	**$C_t$value[a]**	**Temperature**	**$C_t$value[a]**	**Temperature**
SaVGI.2	23.3	50-60°C[b]	22.85	56.3°C	22.99	59°C
SaVGII	27.3	60°C	27.16	60°C	26.49	61.4°C
SaVGV	22.73	59.4°C	22.85	59.4°C	22.53	63.3°C

[a]Lowest C_t value observed
[b]The C_t values were more or less constant over 50 to 60°C

3.2 KS primers vs. Oka primers

Standard curves with DNA templates were set up with primer sets used by Oka *et al*. (2006). An additional standard curve was made with the DNA templates using the forward-primers SaVF1 and SaVF2 from KS (2014) instead of SaV124F from Oka *et al*. (2006). DNA templates concentrations of 10^5 to 10^0 were used for both standard curves.

Both RT-PCR assays were able to detect approximately 50 copies of SaVGI.2 and SaVGV, and 500 copies of SaVGII. The difference in efficiency and sensitivity of the two analyses were marginal, with a slightly better slope value for KS primers although the standard deviation was larger. All values and log-linear curves are presented in Figure 2 and Figure 3.

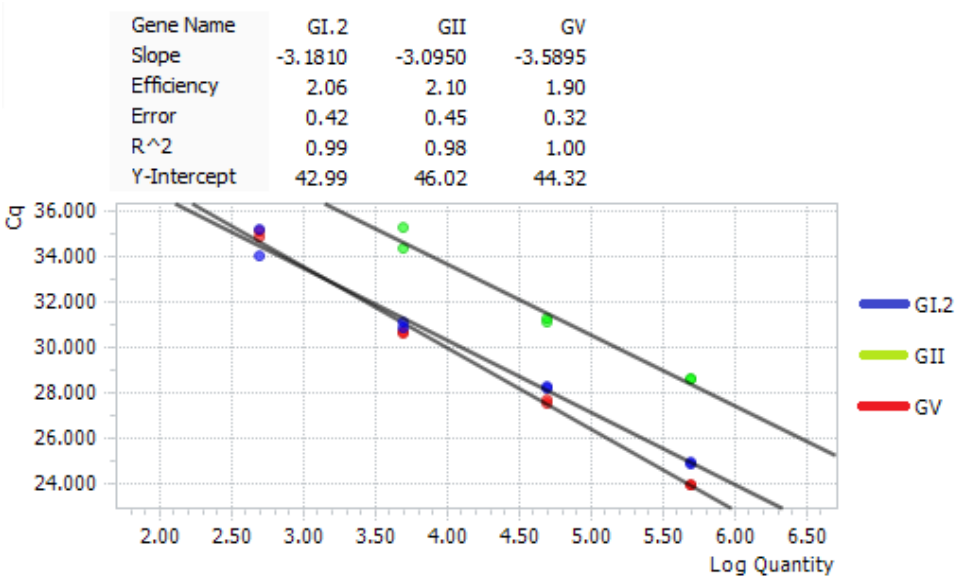

Figure 2. Log-linear curves and data for DNA templates amplified with Oka et al. (2006) primers.

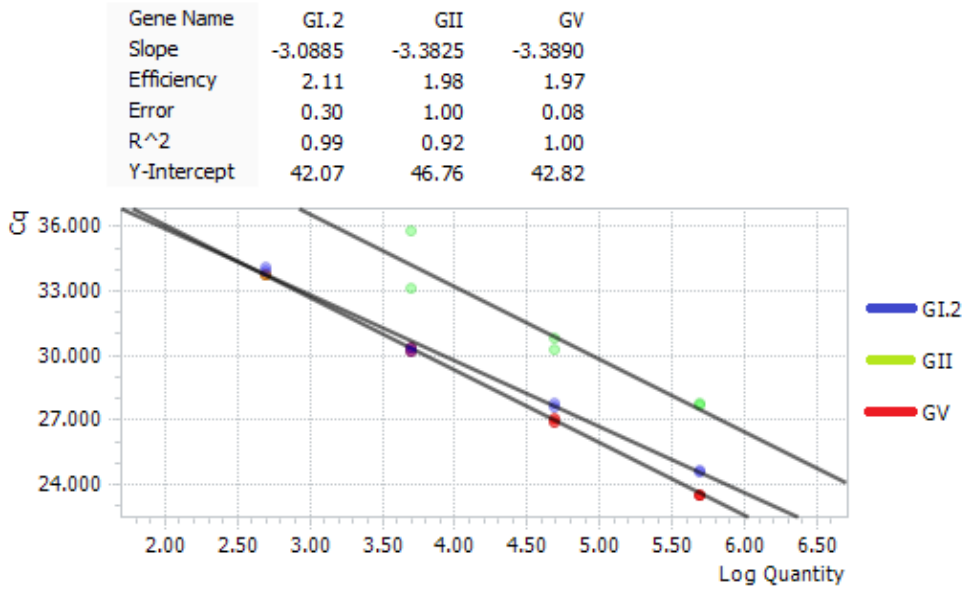

Figure 3. Log-linear curves and data for DNA templates amplified with primers from Oka et al. (2006) and forward-primers from KS.

Extracted RNA from fecal samples were amplified using both RT-PCR assays. As for the DNA templates, no notable difference in C_t-values was observed between the two assays (No data shown).

3.3 Monoplex assay

To test the specificity of the multiplex real-time RT-PCR, a monoplex assay was set up by using three different master mixes and each monoplex reaction was tested for the possibility to amplify the three different DNA templates.The master mixes for SaVGI.2 included primers SaV124F, SaV1245R and probe SaV124TP. The master mix for SaVGI.2 included primers SaV1F, SaV1245R and probe SaV124TP, and master mix for SaVGV included primer SaV5F, SaV1245R and probe SaV5TP.

The monoplex reaction indicated that the master mix containing SaV5F and SaV5TP was specific for DNA template SaVGV, thus only wells with SaVGV combined with GV specific master mix were positive (Tab 6).

Wells with master mix containing SaV124F and SaV124TP were positive for both SaVGI.2 and SaVGII. The same applied for master mix with SaVF1 and SaV124TP, that also was positive for both SaVGI.2 and SaVGII (Tab 6).

Table 6. Detection of DNA templates in a monoplex reaction

DNA templates	SaV1F[a]	SaV124F[a]	SaV5F[a]
SaVGI.2	+	+	-
SaVGII	+	+	-
SaVGV	-	-	+

[a] (+) positive and (-) negative result

3.4 Screening fecal samples for GV

All eleven fecal samples were screened for GV. The PCR assay was made with one GV specific master mix including primers SaV5F, SaV1245R and probe SaV5TP, and one master mix specific for the other genogroups I, II and IV including primers SaV124F, SaV5F, SaV1245R and probe SaV124TP.

The PCR assay with GV specific master mix indicated that sample 13519 belonged to genogroup V. All other samples were negative for GV, but positive for GI, GII and GIV.

Table 7. Confirmation of fecal samples for SaV genogroups

Sample name	GV[a]	GI, GII, GIV[a]	Age-group
11504	-	+	> 65 year
11510	-	+	25-60 year
11520	-	+	< 5 year
12507	-	+	> 65 year
12511	-	+	< 5 year
12512	-	+	25-60 year
13507	-	+	< 5 year
13519	+	-	> 65 year
14500	-	+	< 5 year
14502	-	+	> 65 year
14504	-	+	25-60 year

[a] (+) positive and (-) negative result

3.5 Troubleshooting of DNA templates

Heterogeneity was observed in some standard curves, meaning an amplification curve from a well with the concentration 10^2 would come up the same time as one with 10^4. Self-aggregation of synthetic templates, creation of secondary or tertiary structures, was suspected to be the sources of error due to the inconsequent standard curves. Experiments to prove the hypothesis was established.

Dilution series made with water, as well as a heating step for half of the series, were set up as standard curves using 10^5 to 10^0 DNA copies and analyzed by the RT-PCR method published by Oka *et al*. (2006). In total were twelve standard curves were analyzed. All dilutions were analyzed in duplicates. From the dilution series with QuantiTect nucleic acid dilution buffer (Qiagen), 10^5 DNA copies were used, in duplicate wells.

The amplification curves for heated DNA templates came up later than for the non-heated. This was a trend throughout all three DNA templates and dilution series.

DNA template SaVGV in buffer gave rise to near exact same curves as for using water as diluent. Amplification curves by SaVGI.2 and SaVGII varied more, but no major difference was seen.

No data is shown for these experiments.

4 Discussion

The real-time RT-PCR assay developed in this work did not have the same sensitivity as for Oka *et al.* (2006), although no statistic measurements of significance could be made due to low number of test runs. As generally the C_t value can vary between comparable runs, generating a standard deviation of almost 1 C_t. To determine an improved assay, the C_t value should clearly deviate from the standard deviation of 1 C_t.

The T_m of the primers should ideally be as close as possible to gain a good amplification. The T_m of the reverse-primer SaV1245R was much lower than for the other forward primers, and therefore TINA modified primers were tested. We were expected to see an improved RT-PCR assay when using TINA modified primers, but no such improvement was seen.

The decision to use 60°C was not solely founded on the exact temperature optimum but also the fact that other virus PCR methods used in the lab uses 60°C which opens the possibilities to examine both sapovirus and other viruses in the same PCR assay.

The RT-PCR assay was able to amplify all DNA templates and fecal samples used. If it will detect all human SaV of all genogroups and clusters is still too early to tell. It would have been good to validate the assay by screening samples of known genogroups since there are strains within the genogroups with sequence variations that slightly differ from the sequences in the used DNA templates.

The monoplex RT-PCR showed that primers SaV124F and SaV1F target both DNA templates SaVGI.2 and SaVGII. If it is possible to use only one of these primers and still be able to detect SaV from GI, GII, GIV, a simplified primer mix in the reaction might improve the sensitivity of the assay.

All extracted fecal samples were screened for GV by using a GV specific master mix. Sample 13519 was positive for genogroup GV, which is interesting since genogroup V is rather unusual, compared to GI and GII. No other genogroup was tested, due to the lack of specific primers and probes. The development of specific primers and probes for the other genogroups would be beneficial for diagnostic purposes, contact tracing, and trace back of contaminated food sources.

As already mentioned, the sensitivity of the PCR was determined to approximately 50-500 ssDNA copies. Problems with the synthetic DNA templates occurred during the study, and troubleshooting analyzes resulted in more questions rather than answers. Neither heating the templates, nor changing the diluents to buffer or water, worked though the standard curves remained its heterogeneity. If the single-stranded templates had self-aggregated orcreated secondary structures in the tube, they should have disassembled when heated to melting temperature.

These problems indicate that the measured sensitivity could be questioned and further experiments with for instance modified plasmids or in vitro transcribed RNA should be performed.

As synthetic DNA templates are very pure there is a risk that they will bind to the plastic of the test tube. Using buffer as diluents should theoretically decreased the binding capacity, but no difference between diluents was seen. The stock solution of DNA templates were dissolved in nuclease free water, although the manufacturer recommended Tris-EDTA buffer. Using water as diluent for stock solutions may have affected the outcome of using the stocks for working solutions.

Other resolutions that might generate enhanced standard curves and contribute to accurate quantification are the use of RNA carriers or nucleic acid coated tubes.

5 Conclusion

In many cases of food-poisoning, the causative agent is often unknown. SaV is one increasingly discussed agent causing food-poisoning, but are not currently tested for in food items, and as of today there is no standard method for detection. An improved method for SaV detection may aid the screening of potentially contaminated foods,and thereby trace back the source of contamination in case of an outbreak.

The aim of this study was to optimize a real-time RT-PCR assay for the detection of human sapoviruses in foods. The sensitivity of the assay achieved in this study must be improved in order to be used for screening foods for human SaV. I hope this project may serve as a pilot study for further investigation and development of an RT-PCR assay for detection of human sapoviruses in foods.

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