Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Veterinary Medicine and Animal Sciences

Molecular characterization of a murine norovirus isolate from Sweden and detection of noroviruses in artificially contaminated raspberries

Sofia Persson

Department of Biomedical Sciences and Veterinary Public Health
Degree project/Master thesis 30 hp Level A2E
Uppsala, 2013

Molecular characterization of a murine norovirus isolate from Sweden and detection of noroviruses in artificially contaminated raspberries

Sofia Persson

Supervisor: Shaman Muradrasoli

Assistant Supervisor: Ronnie Eriksson

Examiner: Eva Tydén

Credits: 30 hec
Level: Advanced/A2E
Course title: Master thesis in biology
Course code: EX0648

Place of publication: Uppsala, Sweden

Year of publication: 2013

Online publication: http://stud.epsilon.slu.se

Keywords: Murine norovirus, norovirus, food borne viruses, raspberries, affinity magnetic separation, process control

Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Veterinary Medicine and Animal Science
Department of Biomedical Sciences and Veterinary Public Health
Microbial Food Safety

Abstract

Frozen, imported raspberries have been linked to several outbreaks of human norovirus (NoV) gastroenteritis in Sweden. Noroviruses are highly infectious and are often presented in low numbers in contaminated foods. Detection with RT-PCR must therefore be preceded by a method that extract and concentrate viruses from the tested food samples, but most available methods are laborious and/or inefficient. Studies on noroviruses are further complicated by the fact that human noroviruses are unable to grow in routinely used cell culture models. The murine norovirus (MNV), a common pathogen of immunocompromised mice, can be cultivated in macrophage-like cells and is often used as a model for studies on human noroviruses.

In the present study, a previously unidentified MNV isolate from Sweden was sequenced and molecularly characterized. This isolate also served as a surrogate for testing and further developing a method to concentrate noroviruses from raspberries. Pathatrix (Life Technologies) is an automated magnet separation system that allows concentration of viruses or bacteria from large sample volumes. This system was tested together with cationic paramagnetic beads that attract the negatively charged surface of NoV particles. Results from this study shows that few modifications of the Pathatrix protocol might enhance viral recovery. For further evaluation, the Pathatrix method was compared with polyethylene glycol (PEG) precipitation for concentrating MNV, NoV GI, and GII from artificially contaminated raspberries. PEG precipitation was clearly more efficient but displayed a high degree of inhibition in RT-PCR.

Altogether, this study shows that the Pathatrix method is a convenient and quick alternative. However, it needs to be further optimized before it can be used to concentrate noroviruses from raspberries.

Keywords: Murine norovirus, norovirus, food borne viruses, raspberries, affinity magnetic separation, process control

Sammanfattning

Frysta, importerade hallon utgör en vanlig källa till utbrott av norovirusinfektion i Sverige. Norovirus (NoV) är mycket smittsamma och förekommer vanligtvis i låga nivåer i kontaminerade livsmedel. Detta innebär att detektion med RT-PCR måste föregås av en metod som effektivt separerar och koncentrerar virus från det testade livsmedlet, men de flesta metoder som finns tillgängliga är tidskrävande och/eller inte tillräckligt effektiva. Studier på humana norovirus försvåras ytterligare av det faktum att de inte kan odlas i etablerade cellkultursystem. Det murina noroviruset (MNV) är ett relativt nyupptäckt virus som vanligen infekterar immunförsvagade möss och som kan odlas i makrofagliknande celler. Det senare gör MNV till ett passande modellsystem för studier på humana norovirus.

I denna studie sekvenserades och identifierades ett tidigare okänt MNV-isolat från Sverige. Detta isolat fungerade senare som surrogat för att testa och utveckla en ny metod att koncentrera norovirus från kontaminerade hallon. Pathatrix (Life Technologies) är ett automatiserat magnetiskt separationssystem som är utvecklat för att koncentrera virus och bakterier från stora provvolymer. I denna studie testades systemet tillsammans med positivt laddade paramagnetiska kulor som attraherar negativt laddade noroviruskapsider. Resultaten visar att ett antal modifikationer av tillverkarens standardprotokoll kan ge ökad extraktionseffektivitet. För att ytterligare utvärdera Pathatrixmetoden gjords en jämförelse med polyetylenglykol (PEG) - precipitering för att koncentrera MNV, NoV GI och GII från artificiellt kontaminerade hallon. PEG-precipitering gav avsevärt högre extraktionseffektivitet men visade samtidigt på mycket inhibition i RT-PCR.

Sammantaget visar resultaten att Pathatrixmetoden kan vara ett snabbt och enkelt alternativ. Dock krävs ytterligare utveckling och optimering innan metoden kan användas för att koncentrera norovirus från hallon.

Nyckelord: murint norovirus, norovirus, livsmedelsburna virus, hallon, affintetsmagnetisk separation, processkontroll

Table of contents

Abbreviations

BLAST	Basic local alignment search tool
CEN	European Committee for Standardization
DNA	Deoxyribonucleic acid
FAO	Food and Agriculture Organization of the United Nations
FCV	Feline calicievirus
G	Genogroup
IMS	Immunomagnetic separation
LNA	Locked nucleic acid
MNV	Murine norovirus
NoV	Norovirus
ORF	Open reading frame
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
RNA	Ribonucleic acid
RT	Reverse transcriptase
RT-PCR	Reverse-transcription polymerase chain reaction
SLV	Swedish National Food Agency
SMI	Swedish Institute for Communicable Disease Control
SVA	Swedish National Veterinary Institute
WHO	World Health Organization

1 Introduction

1.1 Background

Virus transmission via food and water is increasingly recognized as a health risk to humans. The relevant food borne viruses are those that infect via the gastro-intestinal tract and are excreted in feces and occasionally, in vomits. There are several groups of viruses that infect via the fecal-oral route, but noroviruses (NoV) are currently recognized as the most important food borne pathogens with respect to the number of outbreaks and individuals affected (Koopmans & Duizer, 2004; FAO/WHO, 2008). They are the single most common cause of acute gastroenteritis in Sweden and around 1 in 10 Swedes are estimated to fall ill in norovirus infection every year (SMI, 2011). Noroviruses typically transmit from person-to-person or through ingestion of contaminated food or water. Common transmission vehicles are bivalve mollusks from contaminated waters, or vegetables, berries, and fruits that have been irrigated with sewage polluted water. Contamination is also common later in the food chain when ready-to-eat foods are prepared by an infected food handler (FAO/WHO, 2008). However, the significance of virus transmission via foods was not properly realized until quite recently, and an important reason is that it has been challenging to develop effective methods to extract, concentrate, and to detect viruses from various food matrices (Widén *et al.,* 2010). Furthermore, research on noroviruses is complicated by the fact that human noroviruses are unable to grow in routinely used cell culture models (Duizer *et al.,* 2004).

1.2 Noroviruses

Noroviruses belong to the family *Calicieviridae* and are a diverse group of non-enveloped, positive sense single stranded RNA viruses. They are classified in five different genogroups (GI to GV) that altogether contain at least 30 genotypes. GI,

GII, and GIV can infect humans whereas GIII and GV have been observed to infect cattle and mice, respectively (Zheng *et al.,* 2006; Morillo & Timentsky, 2011). Recent epidemiological investigations have shown that approximately 70 % of the norovirus outbreaks in humans are caused by the variant GII.4 (Morillo & Timentsky, 2011).

The genomes of noroviruses are around 7.3-7.8 kb in size and contain three open reading frames (ORFs). ORF1 encodes non-structural proteins involved in viral replication, ORF2 encodes the major capsid protein VP1, and ORF3 encodes the minor structural protein VP2 (Zheng *et al.,* 2006; Morillo & Timenetsky, 2011).

Human noroviruses cause acute gastroenteritis and outbreaks of the so-called winter vomiting disease. The incubation period is 1-2 days and the symptoms typically include a rapid onset of nausea, vomiting, and diarrhea, sometimes together with headache, myalgia, and low grade fever. The infectious dose is 10-100 particles and the virus is normally shed in large numbers in feces up to 72 h after the last symptom (Morillo & Timenetsky, 2011; SMI, 2011).

Although quite promising efforts have been made (Straub *et al.,* 2007), there is currently no reliable and widely available cell culture system for human noroviruses (Duizer *et al.,* 2004). This has clearly limited the knowledge of these viruses and the molecular mechanisms that promote norovirus pathogenesis are often studied using the murine norovirus (MNV) as a model. MNV belongs to genogroup V and is the only norovirus that be cultivated in a routinely used cell culture system (Wobus *et al.,* 2004; Wobus *et al.,* 2006). The virus was first discovered in research mice in 2003 and causes everything from asymptomatic or mild infections to lethality in mice deficient in different parts of the innate immune system (Karst *et al.,* 2003).

1.3 Detection of viruses in food

Traditional microbial food safety guidelines have mainly been focusing on preventing and detecting pathogenic bacteria, but the characteristics of food borne viruses differ a lot from those of the common food borne bacterial pathogens. Viruses depend solely on their host(s) to replicate and many of them (including human noroviruses) cannot be enriched in culture methods. Additionally, viruses do not grow in food and will therefore not cause deterioration or any other changes in the sensory characteristics of the food product. Many food borne viruses are very stable in the environment since they lack envelope, and show resistance to a wide range of pH, drying, radiation, etc. This means that traditional food safety guidelines are not always reliable for sensing and preventing viral contamination. Food samples that have previously been declared safe by for instance absence of fecal

indicator organisms have later been revealed to contain high viral loads (Koopmans & Duizer, 2004; Newell *et al.,* 2010; FAO/WHO, 2008).

Nucleic acid based techniques such as PCR have greatly enhanced the ability to detect viruses and have become the gold standard for virus detection in food. However, PCR is a very sensitive method and most food matrices contain substances that can inhibit the enzymatic reactions and thereby cause false negative results. In addition, viruses are often presented in low numbers in food, and thus remain below the detection limit of most diagnostic assays. Still, most food borne viruses are highly infectious, meaning that even low levels of viruses can pose a significant health risk to humans (Atmar, 2006; FAO/WHO, 2008). PCR methods must for these reasons be preceded by methods that concentrates viruses as well as separates the inhibitors from the tested food samples. Various approaches have been developed and include ultracentrifugation, polyethylene glycol (PEG) precipitation, adsorption/elution, and immunomagnetic separation (IMS) (Tian *et al.,* 2011; Atmar, 2006). Drawbacks with these methods are that they can be laborious and time consuming (e.g. PEG precipitation, adsorption/elution), or too narrow in specificity to include different genotypes (Tian *et al.,* 2011).

1.4 Process controls

There are several steps involved in testing food for viruses and the overall efficiency of virus recovery and presence of potential inhibitors for RT and PCR-reactions must be monitored with a process control. A process control is a viral sample that is added in a known amount to the food matrix and is extracted and handled in exactly the same way as the target samples. The process control should exhibit similar morphological and physiochemical properties, and have the same persistence in foods as the target virus (Lees & CEN/WG 06, 2010). Ideally it should also be unlikely to naturally contaminate the tested food sample (Baert *et al.,* 2011). Feline calicievirus (FCV) is often being used as a process control for detection of RNA viruses in food and water samples (Mattinson *et al.,* 2009; D'Souza *et al.,* 2006), but it has been observed that FCV is less stable in food matrices and has different physical properties compared to human noroviruses. FCV is inactivated at relatively low pH and may therefore not reflect the stability or inactivation of human noroviruses in food products; especially since many outbreaks originate from acidic foods (frozen raspberries have a pH around 3). The murine norovirus (MNV) has quite recently been addressed as suitable process control and surrogate for human noroviruses (Cannon *et al.,* 2006). MNV belongs to the norovirus genus and has a similar size, shape, buoyant density, and genomic structure as human noroviruses (Wobus *et al.,* 2006; Kim *et al.,* 2010). It is more stable at low pH since it is predominantly transmitted fecal-orally between mice,

in contrast to FCV that spread via the respiratory route among cats (Cannon *et al.,* 2006; Karst *et al.,* 2003).

1.5 Aim

This project had two specific aims: (1) to sequence and genetically characterize a MNV isolate from Sweden, and (2), to test a commercialized affinity magnetic separation system (Pathatrix, Life Technologies), in combination positively charged paramagnetic beads to concentrate noroviruses from raspberries. Raspberries are one of the most common sources of food borne norovirus gastroenteritis outbreaks in Sweden (Lund & Lindqvist, 2004), and the Pathatrix method has previously been applied to viruses and various food matrices with promising results (e.g. Plante *et al.,* 2005; Papafragakou *et al.,* 2008; Mattinson *et al.,* 2009). In the present study, the Swedish MNV isolate was used as a surrogate for the initial testing and development of the methodology. The Pathatrix method was also compared with PEG precipitation for concentrating norovirus GI, GI, and MNV from raspberries.

2 Materials and methods

2.1 Molecular characterization of an MNV isolate from Sweden

2.1.1 Collection of specimens and cultivation of cells for viral propagation

The test isolate of MNV was obtained from the Swedish National Veterinary Institute (SVA), where it had been isolated from a research mouse from Southern Sweden and passaged two times in RAW 264.7 cells (Wobus *et al.,* 2004). The isolate was cultivated in RAW 264.7 cells at the National Veterinary Institute (SVA).

2.1.2 RNA extraction and RT-PCR

Viral RNA was extracted from a cell culture using QIAamp Viral RNA Mini Kit (Qiagen), and eluted in 30 µl EB containing 50 ng/µl carrier RNA. PCR products were generated using SuperScript III One-Step RT-PCR System with Platinum *Taq* High Fidelity polymerase (Invitrogen). The genome was amplified in five separate reactions with different primer pairs (**Table 1**). RT-PCR reactions were performed with cDNA synthesis and pre-denaturation at 55°C for 30 min, heat inactivation of reverse transcriptase and activation of the *Taq*-polymerase at 94°C for 5 min, 40 cycles of PCR amplification at 94°C for 1 min, 50-65°C (depending on primer pair) for 1 min and 68°C for 4 min, followed by a final extension at 68°C for 5 min. Five µl of RNA-template was used in a total volume of 25 µl for each reaction. Amplicons were separated by gel electrophoresis (1 % agarose gel) and purified using QIAquick gel extraction kit (Qiagen), according to the manufacturer's instructions.

Table 1. *Primers used to generate MNV amplicons.*

Primer	Pair	Sequence	Location	Amp. Temp
MNVFW-5'	1	GTGAAATGAGGATGGCAACGC	1-21	60°C
MNV-Rev1		CASCCRATMGCTGCCATYTT	2198-2218	
MNV-FW2	2	CTATGACTTTGATGCYGGCAA	2129-2149	53-57°C
MNV-Rev2		CYTCGACRACGATCTTRTAG	4412-4431	
MNV-FW2	3	CTATGACTTTGATGCYGGCAA	2129-2149	54°C
MNV-Rev3		TCRTGCTTGAAAGAGTTGGY	6882-6901	
MNV-FW3	4	ACTAYAAGATCGTYGTCGAR	4411-4431	50°C
MNV-3'-end		AAAATGCATCTAACTACCAC	7363-7382	
MNV-FW4	5	CAARCCAACTCTTTCAAGCA	6879-6898	53-57°C
MNV-3'-end		AAAATGCATCTAACTACCAC	7363-7382	

Genome locations are based on the MNV reference strain (GenBank accession number NC_008311). Y: C, T wobble R: A, G wobble W: A, T wobble.

2.1.3 Sequencing and sequence analysis

Purified PCR-products were sequenced in both directions with Sanger's dideoxy chain termination method at the Macrogen Europe Laboratory, the Netherlands. Primers used for genome sequencing are listed in **Table 2**. Obtained nucleotide sequence data were analyzed using CLC Main Workbench 6.7.1 with default parameters. The consensus nucleotide sequence was aligned with selected reference isolates from GenBank (**Figure 1**) using the ClustalW algorithm. A phylogenetic tree was constructed using the neighbor-joining method in MEGA 5 (Saitou & Nei, 1987; Tamura *et al.,* 2011).

Table 2. *Primers used for genome sequencing.*

Primer	Sequence (5'-3')	Location
MNV A_Fw	GTGAAATGAGGATGGCAAC	1-19
MNV A_Rev	ACGCACTTCCTCAACTCA	596-613
MNV B_Rev	TGATGATGATGACTTGGGA	1782-1800
MNV D_Fw	TTGATGATTACCTCGCTG	2740-2757
MNV E_Fw	TGGATCCGCTTATGTTTCT	5994-6012
MNV E_Rev	TGTTTGTTTGCCTGAAGGT	6757-6775
MNV_F456	ACTACTCTGTCTACATCGG	454-472
MNV_F6677	TCAAACAATAATGGCTGGTGC	6671-6691
MNV_R2624	TTGCCCTCAGAGTGGTACC	2602-2620
MNV_R4559	TCAGATTCTTGCATCACAATGT	4534-4555
MNV_R6369	CATGTAGGTCCGGAACCTC	6345-6363
MNV-FW2	CTATGACTTTGATGCYGGCAA	2129-2149
MNV-FW3	ACTAYAAGATCGTYGTCGAR	4411-4431
MNV-Rev1	CASCCRATMGCTGCCATYTT	2198-2218
MNV-Rev2	CYTCGACRACGATCTTRTAG	4412-4431
MNV-Rev3	TCRTGCTTGAAAGAGTTGGY	6882-6901

Genome locations are based on the MNV reference strain (GenBank accession number NC_008311). Y: C, T wobble R: A, G wobble.

2.2 Generation of a MNV plasmid standard for real-time PCR

2.2.1 Plasmid construction

A purified and sequenced PCR product (position 4413-7382 of the MNV genome) was ligated into a pJET1.2/blunt cloning vector according to a protocol from CloneJET Cloning kit (Fermentas). The plasmid was transformed into One Shot TOP10 chemically competent *E. coli* cells (Invitrogen), and cultivated on LA plates with ampicillin (50 µg/ml) over-night at 37˚C. Positive clones were selected with PCR and plasmids with inserts were purified using Plasmid Miniprep Kit (Qiagen) and sequenced at the Macrogen Europe Laboratory, the Netherlands.

2.2.2 Analysis with quantitative real-time PCR

Plasmid DNA was determined spectrofotometrically (NanoDrop ND-1000 UV/Vis), serially diluted from $5 \cdot 10^5$ to approximately 0.5 copies/reaction in a nucleic acid dilution buffer (Qiagen) and analyzed in real-time PCR. PCR reactions occurred in a CFX 96 system (Biorad) using a TaqMan probe and QuantiTect Virus Kit (Qiagen). Thermal cycling occurred at 95°C for 5 min, followed by 50 cycles of 94°C for 15 s and 60°C for 45 s. Five µl of purified plasmid was used as a template in a total reaction volume of 25 µl, containing 500 nM of forward pri-

mer, 900 nM of reverse primer, and 250 nM of probe. Primers and probe (**Table 3**) targeted the capsid region of ORF2.

Table 3. *Primers and probe used for detection of MNV.*

Primer/probe	Sequence (5'-3')	Location
MNV-forward	TTGGGAACATGGAGGTTCAR	5363-5382
MNV-reverse	GGRAAATAGGGTGGTACAAGG	5430-5450
MNV-probe	6-FAM-CCACCTTGCCAGCAGT-DABCYL	5407-5422

Genome locations and primer sequences are based on the MNV reference strain (GenBank accession number NC_008311). Underlined positions indicate LNA nucleotides. FAM: 6-carboxyfluorescein. DABCYL: 4-(4-dimethylaminophenyl) diazenylbenzoic acid. R: A,G wobble.

2.3 Detection of noroviruses in artificially contaminated raspberries

2.3.1 Preparation of process control and sample viruses

MNV was obtained from SVA (described in section 2.1.1), and NoV GI and GII were collected from fecal samples obtained from the Swedish Institute for Communicable Disease Control. The virus stocks were serially diluted in PBS and quantitated as plasmid equivalents by quantitative real-time RT-PCR.

2.3.2 Virus elution and clarification

Twenty five grams of thawed raspberries (obtained from a local supermarket) were added into a 500 ml stomacher sample filter (Seward) placed in a beaker. Raspberry samples were artificially contaminated by pipetting viruses onto different areas on the surface and left at room temperature for 10 min. Uninoculated raspberry samples served as negative process controls. Different buffer conditions, were tested in this study (**Table 5**), and the following protocol gave the highest viral recovery: Viruses were eluted by adding 40-45 ml of glycine buffer (0.05 M glycine, 0.14 M NaCl, 1 M tris, 0.26 % Tween-20, pH 9) onto the samples, pH was adjusted to 9.0-9.5 with 1 M NaOH, and the samples were put onto a shaking platform for 10 min. Filtrates were successively transferred to 50 ml falcon tubes and centrifuged for 10 min at 10 000 x g, 5°C, in order to pellet remaining raspberry particles that could potentially interfere with virus concentration and RT-PCR. Supernatants were collected and pH was subsequently adjusted in to 7.2-7.4 with 5 M HCl. The above described procedure is a slightly modified protocol by Mattinson *et al.,* 2010.

2.3.3 Separation and concentration of viruses using Pathatrix

Pathatrix (Life Technologies) is a commercialized recirculating affinity magnetic separation system that utilizes coated paramagnetic beads to capture bacteria or viruses from food, water, or environmental samples. The system is fully automatic and allows relatively large sample volumes (up to 50 ml) to be analyzed. The Pathatrix system was tested together with positively charged paramagnetic beads that attract the negatively charged virus capsid.

Some modifications were made to enhance virus-bead interaction and to reduce loss of magnetic beads. Supernatants from the raspberry eluates were placed in the sample vessel of generic Pathatrix consumable systems and 100 µl of paramagnetic beads from Pathatrix Cationic/General Viral Capture Kit (Life Technologies) were added directly to the sample vessels and mixed by brief vortexing, instead of applying them through the lid as recommended by the manufacturer. Moreover, the samples were pre-incubated on a rotating platform for 10 min to further enhance bead mixing and virus-bead interaction. Thirty-five µl of PBS pH 7.4 was added to the wash vessel of each sample, and the systems were assembled and placed into the Pathatrix work station. Viruses were concentrated using program 1 in the Pathatrix instrument. Elution chambers were placed onto a magnet for 2 min after finishing each run, and the magnetic beads were subsequently resuspended in 500 µl of PBS.

2.3.4 Nucleic acid extraction and real-time RT-PCR analysis for detection of NoV GI and GII

RNA was extracted using NuckiSENS MiniMAG extraction kit (Biomérieaux), according to the manufacturer's protocol. Positive and negative extraction controls were included in each reaction. Positive extraction controls consisted of MNV, NoV GI, and GII (added in the same amounts as the inoculum of each raspberry sample), and negative extraction controls consisted of PBS pH 7.4 (500 µl). The magnetic beads from Pathatrix remained in the samples through the extraction process in order to minimize loss of viral RNA. Potential inhibitors of the PCR reactions were removed using OneStep PCR Inhibitor Removal Kit (Zymo Research). Real-time RT-PCR reactions were performed in a volume of 25 µl using QuantiTect Virus Kit (Qiagen) and TaqMan probes in a CFX 96 system (Biorad). Five µl of purified RNA was used as template. MNV was detected in monoplex, using the primers and probe listed in **Table 3,** in the same concentrations as described previously. Detection of NoV GI and GII occurred in duplex, and primers and probes were added to a final concentration of 400 nM and 200 nM, respectively (**Table 4).** Reverse transcription occurred at 50°C for 20 min, followed by inactivation of RT and heat activation of *Taq* at 95°C for 5 min. Thermal cycling occurred with 50 cycles of 95°C for 15 s, and 60°C for 45 s.

Table 4. *Primers and probe used for detection of NoV GI and GII.*

Virus	Primer/probe	Sequence (5'-3')
NoV GI	IFRGI (F)	CGCTGGATGCGNTTCCAT
	NV1LCR (R)	CCTTAGACGCCATCATCATTTAC
	NVGGIp (P)	6- FAM-TGGACAGGAGAYCGCRATCT-BHQ1
NoV GII	QNIF2 (F)	ATGTTCAGRTGGATGAGRTTCTCWGA
	COG2R (R)	TCGACGCCATCTTCATTCACA
	QNIFS (P)	HEX-AGCACGTGGGAGGGCGATCG-BHQ1

FAM: 6-carboxyfluorescein, HEX: 6-carboxy-2′,4,4′,7,7′hexachlorofluoresceinsuccinimidyl ester, BHQ1: Black Hole Quencher 1. Y: C,T wobble R: A,G wobble.

2.3.5 Determination of virus recovery and overall efficiency

Standard curves were generated for quantitation of NoV GI, GII, and MNV. NoV GI and GII plasmids were kindly provided by the Centre for Environment, Fisheries and Aquaculture Science (Cefas), United Kingdom. Dilution series of $5 \cdot 10^5$ to 50 plasmid copies were included in RT-PCR reactions to quantitate RNA. The results from each PCR were plotted to standard curves, and the overall recovery efficiency was calculated by dividing the number of plasmid equivalents in the sample by the number of plasmid equivalents in the original virus preparation (positive extraction control). The potential loss of template in RNA extraction was not taken into account.

2.3.6 Elution and concentration of viruses using PEG precipitation

MNV, NoV GI and GII were eluted by pouring 40 ml tris-glycine beef extract buffer (pH 9) onto 25 g of artificially contaminated raspberries placed in a stomacher bag inside a glass beaker. pH was adjusted to 9-9.5 and the samples were put onto a shaking platform for 40 min. Filtrates were subsequently centrifuged at 10 000 x g for 30 min, at 5°C. Supernatants were collected and pH was adjusted to 7.2 with HCl. Viruses were concentrated by adding 5x PEG solution to a volume corresponding to ¼ of the sample volumes. Samples were placed onto a rotating platform for 1 h at 5°C, followed by centrifugation at 10 000 x g for 30 min, 5°C. Supernatants were discarded and the samples were centrifuged once more (10 000 x g for 5 min at 5°C at this time). Pellets were diluted in 500 µl PBS, and five-hundred µl of chloroform:1-butanol (1:1) was added to each sample. Samples were incubated for 5 min at room temperature, followed by centrifugation at 10 000 x g, 5°C for 15 min. The water phase was transferred to eppendorf tubes, and RNA was in this case extracted using BioRobot EZ1 (Qiagen), according to the manufacturer's instructions. Inhibitors were removed using OneStep PCR Inhibitor Removal Kit (Zymo Research).

3 Results

3.1 Molecular characterization of an MNV isolate from Sweden

The obtained genetic sequence of the Swedish MNV isolate was compared with several other full length genomes of human and murine noroviruses from GenBank. **Figure 1** shows a phylogenetic tree comprising the Swedish MNV isolate together with selected reference strains of MNV, FCV, and norovirus GI, II, III, and IV. A BLAST search revealed that MNW/Sweden is most closely related to a MNV isolate from Berlin (GenBank accession number EF531290.1) with a nucleotide sequence similarity of 93 %. The lowest similarity between MNV/Sweden and the full length MNV isolates available at GenBank was 87 %.

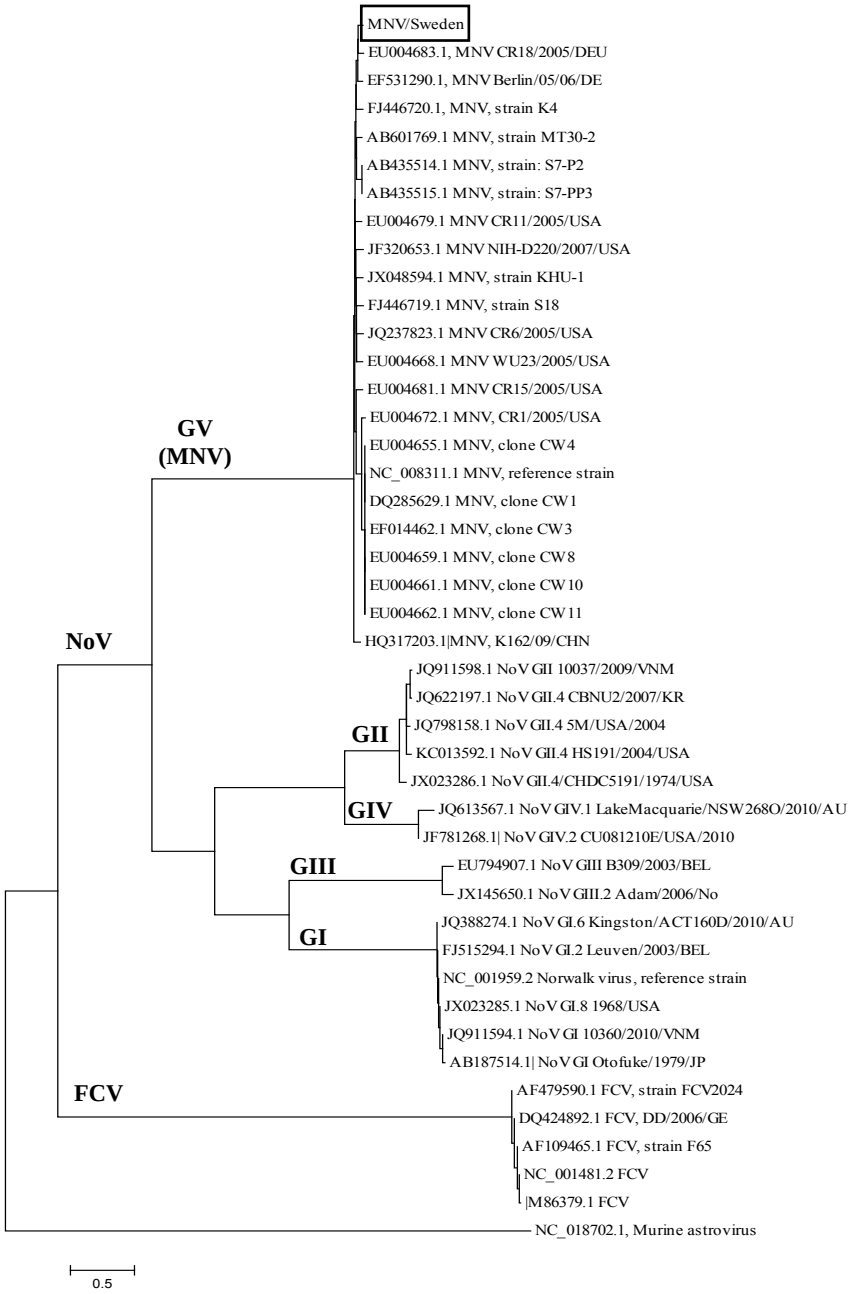

Figure 1. Phylogenetic analysis based on full-length genomes. The sequence of the Swedish MNV isolate (MNV/Sweden) was aligned with 42 selected full-length reference isolates of MNV, FCV, and norovirus GI, II, III and IV using the software MEGA 5 (Tamura *et al.,* 2011). The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The branch lengths of the tree are in the same units as those of the evolutionary distances used to surmise the phylogenetic tree. Evolutionary distances are in the units of the number of base substitutions per site and were calculated using the Maximum Composite Likelihood method (Tamura *et al.,* 2004).

3.2 Generation of a plasmid standard for MNV quantitation

A plasmid standard containing a part of the MNV genome was generated for quantitation of MNV RNA in order to evaluate recovery efficiency of methods to detect MNV from raspberries. The sensitivity of the MNV PCR was roughly estimated by testing the plasmid in 10-fold dilutions from $5 \cdot 10^5$ to 50 plasmids/reaction (two observations/dilution), followed by 2-fold dilutions from 50 to 0.8 plasmids/reaction (three observations per dilution). A negative result was observed in 1 out of 3 wells at 12.5 plasmids/reaction. Completely negative results (3 out of 3 reactions) were seen at 6.25 plasmids/reaction. **Figure 2** shows the amplification plot of the dilution series from $5 \cdot 10^5$ to 50 plasmids/reaction. The standard curve displayed a linear relationship (R^2 = 0.997) with an efficiency of 98.8 % (**Figure 3**).

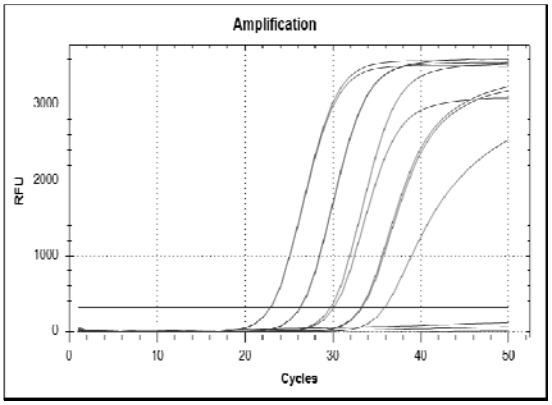

Figure 2. Amplification plot of the MNV plasmid standard, showing a serial dilution from $5 \cdot 10^5$ to 50 plasmid copies/reaction. RFU: relative fluorescence units.

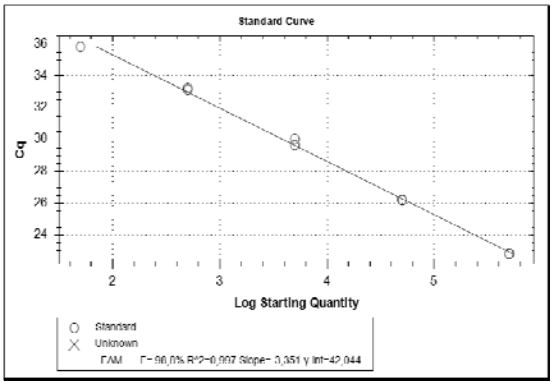

Figure 3. Standard curve showing the MNV-plasmid in 10-fold dilutions from $5 \cdot 10^5$ to 50 copies/reaction. The correlation coefficient (R^2) was 0.997 and the efficiency (E) was 98.8 %. Cq: crossing point.

3.3 Detection of noroviruses from artificially contaminated raspberries

3.3.1 Optimization of a sample concentration process using Pathatrix

This work focused on optimizing a protocol for sample concentration with Pathatrix. Samples were inoculated with 10^7 plasmid equivalents of MNV. Various volumes of cationic beads (50 and 100 µl), and different buffers (PBS, TGBE, glycine, tris-glycine, etc, pH 7.2-7.4 at the Pathatrix concentration step) were evaluated. Different modifications of the Pathatrix standard protocol were also investigated. Highest recovery was obtained with a buffer containing 0.05 M glycine, 0.14 M NaCl, 1 M tris, and 0.26 % Tween-20, and if 100 µl of cationic beads were added to the samples (50 µl is recommended by the manufacturer). A better recovery was acquired when beads were added directly to the sample vessel (instead of through the lid as recommended by the manufacturer), followed by brief vortexing. A slight enhancement in recovery was also achieved when a 10 min pre-incubation step was added prior to concentration by Patharix. However, addition of pectinase (Sigma Aldrich) to the glycine buffer did not result in any better recovery (**Table 5**).

Nucleic acid extraction was performed in a MiniMAG system (Biomérieaux) that utilizes magnetic silica beads to bind nucleic acids during the different wash steps. It was investigated whether it was better to remove the Pathatrix-beads after lysis of viral particles, or to maintain the beads through the extraction procedure and thus remove them together with the MiniMAG beads at the nucleic acid elution step instead. Neither of the two options resulted in any substantial loss of RNA, and there was no significant difference between the two options (data not shown). For ease of use, the Pathatrix beads were therefore retained through the nucleic acid extraction processes.

Application of a commercial PCR inhibitor removal kit (Zymo research) resulted in less inhibitory substances in undiluted samples (**Table 5**).

Figure 4 summarizes the most efficient method developed in this study.

Table 5. *Different buffers and conditions for concentration of MNV using Pathatrix.*

Buffer	Beads (µl)	Program[1]	Modification[2]	Virus recovery (%)	
				Buffer only (undil/1:10)[3]	+Raspberries (undil/1:10)[3]
PBS	50	2		3.5/3.5	0.02/0.04
TGBE[4]	50	2		Not tested	0.03/0.3
Glycine	50	2		Not tested	0.02/0.02
Tris-glycine	50	2		5.1/4.9	0.04/0.07
	100	2		3.8/5.4	0.02/0.02
	100	2	A	21.2/22.7	0.02/0.4
Tris-glycine-tween-20	100	1	A, B	21.1/27.7	0.5/1.2
	100	1	A, B,C		0.2/1.8
	100	1	A,B,C,D		1.2/1.8
	100	1	A,B,C,D,E		1.0/1.4

Samples were inoculated with 10^7 plasmid equivalents of MNV. Virus recovery was calculated as (plasmid equivalents in sample*100)/(plasmid equivalents in the original virus inoculum).

1. Program selected in the Pathatrix instrument. Program 2 was first suggested by representatives from the manufacturer. However, after discussing the issue at an additional meeting, the protocol was changed to program 1.

2. A: beads were added directly in sample vessel, B: pH was adjusted from 9-9.5 to 7.2-7.4 after centrifugation of raspberry particles instead of before centrifugation, C: a pre-incubation step of 10 min at a rotating platform at room temperature was included before concentration with Pathatrix, D: inhibitors were removed from purified nucleic acids, E: pectinase was added to the buffer.

3. RNA template dilution in RT-PCR.

4. TGBE: tris-glycine beef extract.

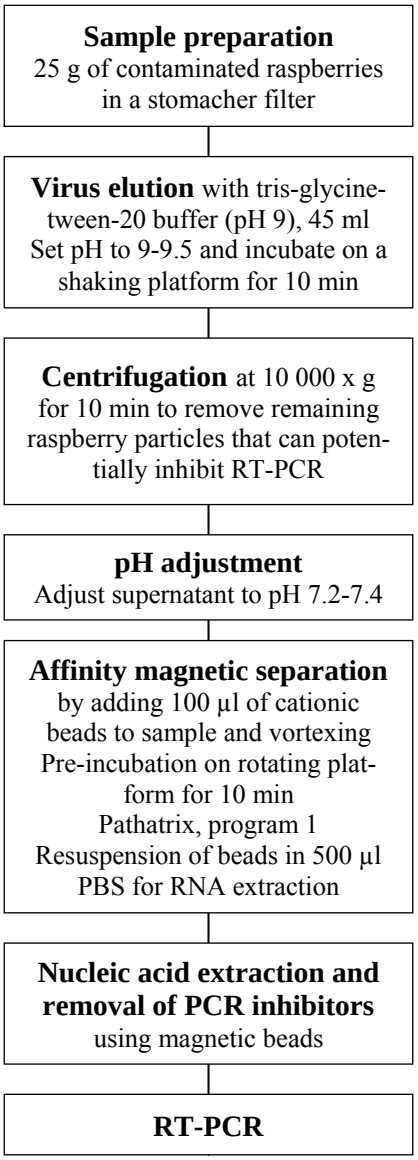

Figure 4. Flow chart over the most efficient virus extraction method tested in the present study.

3.3.2 Comparison between Pathatrix and PEG precipitation for concentration of NoV GI, GII, and MNV from raspberries

The Pathatrix method was compared with PEG precipitation for concentrating viruses from raspberries. Raspberry samples (25 g) were inoculated with 10^5 plasmid equivalents of NoV GI and GII, and 10^6 plasmid equivalents of MNV. Uninoculated raspberry samples served as negative process controls. Viruses were eluted and concentrated using the two different methods, and the efficiency of recovery was measured with quantitative real-time RT-PCR.

PEG precipitation was clearly the most efficient method for concentrating viruses but displayed a higher degree of inhibition in RT-PCR than the Pathatrix method. MNV, NoV GI, and GII were detected in all tested samples for PEG precipitation at 1:10 dilution in PCR, with recoveries of 17.7±4.6 %, 10.9±5.2 %, and 13.9±9.4 %, respectively. The relatively low recoveries obtained for undiluted samples indicate that there is a lot of inhibition in RT-PCR. The Pathatrix method successfully detected MNV and NoV GI, but failed to detect GII in 1 of 6 times at both undiluted and 10-fold diluted RNA template. The Pathatrix method displayed recoveries of 0.4±0.1 %, 1.1±0.6 %, and 0.9±0.1 % at 1:10 template dilution for MNV, NoV GI, and GII, respectively (**Table 6**). The recoveries of MNV from the Pathatrix method were generally lower in these experiments compared to previous experiments where raspberries were inoculated with MNV only (0.4 % compared to a maximum of 1.8 %, **Table 5**).

Table 6. *Detection of NoV GI, GII, and MNV after separation and concentration from raspberries.*

Virus	RNA template dilution in RT-PCR	No. of samples positive in PCR/no. of tested samples		Mean virus recovery [standard deviation] (%)	
		PEG precipitation	Pathatrix	PEG precipitation	Pathatrix
MNV	Undiluted	3/3	6/6	1.2 [±0.2]	0.3 [±0.1]
	1:10	3/3	6/6	17.7 [±4.6]	0.4 [±0.1]
NoV GI	Undiluted	2/3	6/6	0.4 [±0.3]	1.2 [±0.2]
	1:10	3/3	6/6	10.9 [±5.2]	1.1[±0.6]
NoV GII	Undiluted	2/3	5/6	0.4 [±0.2]	0.4 [±0.4]
	1:10	3/3	2/6	13.9 [±9.4]	0.9 [±0.1]

Raspberry samples were inoculated with 10^5 plasmid equivalents of NoV GI and GII, and 10^6 plasmid equivalents of MNV. Percent recovery was calculated as (plasmid equivalents in the sample*100)/(plasmid equivalents in the original virus inoculum). Mean virus recovery was calculated as the sum of the percent recovery for each positive sample divided by the number of positive samples.

4 Discussion

The aims of this work were to genetically characterize a murine norovirus isolate from Sweden and to test and further develop a method to concentrate noroviruses from raspberries.

MNV can be cultivated *in vitro* and is therefore used as a model system for studying human noroviruses (Wobus *et al.,* 2006). It is also suitable to use as a process control or surrogate in methods to detect noroviruses from food (Cannon *et al.,* 2006). However, infection with MNV is problematic since it can cause a wide range of symptoms in laboratory mice (Karst *et al.,* 2003). Previous studies from United States, Canada, and South Korea demonstrate a high prevalence of MNV in research facilities; approximately 20 % of the tested mice were seropositive for MNV (Kim *et al.,* 2010, Hsu *et al.,* 2005). These infections seems to be highly persistent (Kastenmayer *et al.,* 2008), and the effects can potentially influence on the results of studies on other diseases or infectious agents (Hsu *et al.,* 2005; Kim *et al.,* 2010).

The MNV from this study was previously isolated from a research mouse in Southern Sweden, and bioinformatic analyses revealed that this isolate was previously unidentified. The situation in Sweden is as of this moment unknown, but preliminary results from Swedish National Veterinary Institute show that a relatively large proportion of tested research mice are both seropositive and PCR positive for MNV (SVA, unpublished). Frequent screening for MNV in research facilities is necessary in order to prevent diseases and interference with experimental results (Kim *et al.,* 2010).

Transmission of human noroviruses through food and water is relatively common and constitutes a problem for public health. It is therefore important to have rapid, sensitive, and robust methods for detection of viruses from these matrices. Food samples often contain a lot of inhibitors for RT and PCR, and human noroviruses are usually presented in low numbers in contaminated foods, which means that viruses need to be concentrated and separated from the tested food prior to detection with RT-PCR. Several approaches for virus concentration have

been developed, but many of them are inefficient, laborious, or time-consuming (Tian *et al.,* 2011).

In the present study, the Swedish MNV-isolate was used as a surrogate for testing and developing a method to concentrate human noroviruses from raspberries. Cationic paramagnetic beads in combination with the Pathatrix system is an easy and relatively quick method for virus concentration from large sample sizes, and has been applied previously to various food matrices (e.g. Plante *et al.,* 2005; Papafragakou *et al.,* 2008; Mattinson *et al.,* 2009). In this study, we used this method to concentrate MNV, norovirus GI, and GII from raspberries. GI and GII are the genogroups mainly associated with food borne outbreaks, and raspberries have been linked to several outbreaks of norovirus gastroenteritis in Sweden (Lund & Lindqvist, 2004).

The isoelectric points for the capsid proteins of norovirus GI and GII range between 5.9-6.0 and 5.5-6.9, respectively (Goodridge *et al.,* 2004), meaning that their surfaces are negatively charged at neutral and basic pH, which will allow the viral particles to interact with the positively charged beads. However, the exact mechanism of virus-bead interaction has not been investigated. Studies on hepatitis A virus suggest that the interaction is not solely or primarily electrostatic since altered pH and ionic strength does not seem to influence on virus-bead interaction. The same study also suggests that the charge density on the virus capsid may affect binding stability (Papafragakou *et al.,* 2008).

Different buffers were tested for eluting and concentrating viruses. First, a tris-glycine beef extract buffer (TGBE) was tested in combination with the Pathatrix for concentrating NoV GI and GII from raspberries with completely negative results in RT-PCR (data not shown). The TGBE buffer was also tested for concentrating MNV and revealed 0.03 % recovery at undiluted samples and 0.3 % recovery at one observation but was undetected at the other at 1:10 dilution (**Table 5**). These unsatisfying results could be due to the fact that the negatively charged proteins in TGBE may potentially bind to the cationic beads and thereby out-compete the virus particles. For this reason, we changed to a glycine buffer without beef extract, and tris was later added in order to enhance the buffer capacity.

A few modifications of the generic Pathatrix protocol enhanced viral recovery slightly. However, we experienced a high degree of bead loss when using Pathatrix. This was partly overcome by increasing the bead volume from 50 to 100 µl, but we also investigated whether we could obtain better bead mixing and higher viral recovery by manually mixing the beads with the food sample instead of using Pathatrix. We added cationic beads directly to the sample supernatant (45 ml) in a 50 ml Falcon tube and simply mixed the sample on a rotating platform for 20 min. Beads were collected after brief centrifugation for 2 min, the sample liquid was discarded while holding the tube onto a magnet, and beads were subsequently

washed with PBS. This method gave higher recovery when only buffer was used (up to 70 %, compared to a maximum of 27 % with the Pathatrix), but a similar recovery with raspberries (data not shown).

In order to further evaluate the methodology, the Pathatrix was compared with PEG precipitation for concentrating noroviruses from raspberries. PEG precipitation is currently suggested to become the standard method for concentrating viruses from raspberries and lettuce according to the Centre of European Standardization Committee (CEN), and is used routinely at SLV. However, a major drawback is that the method is time-consuming.

The PEG method gave much higher efficiency than Pathatrix at 1:10 template dilution in RT-PCR, but displayed a high degree of inhibition in undiluted samples. The efficiencies of PEG precipitation (**Table 6**) are in this case comparable to previous observations and published results that demonstrate recoveries between 1-28 % from raspberries (Summa *et al.,* 2012; unpublished observations from SLV). The Pathatrix method gave recoveries slightly above and slightly below 1 %, which is the lowest acceptable extraction efficiency according to CEN (CEN/TC 275/WG 06, 2011). Morales-Rayas and colleagues (2010) evaluated the Pathatrix for concentration of norovirus GII from raspberries in a fairly similar way as we did, and showed similar extraction efficiency (0.8 %) as in this study (0.9 %).

Notably, the recovery of MNV was less during the experiment with norovirus GI, GII, and MNV, than with MNV alone. The low recovery can potentially be explained by a high virus:bead ratio, i.e. that beads get saturated by the high levels of virus in the sample. Moreover, binding and separation of viruses upon charge is highly unspecific, and food matrices may presumably contain other negatively charged substances that also interact with the beads and thereby influence on RT-PCR.

In this study we inoculated our raspberries with high viral titers (10^5-10^7 plasmid equivalents per sample), which of course does not reflect the reality of food borne viruses. A good method should be able to detect viral levels down to the infectious dose, which are 10-100 particles for noroviruses (Morillo & Timenetsky, 2011). Thus, further investigation is needed in order to evaluate the lower detection limit of the Pathatrix method.

To summarize, concentration of viruses by magnetic capture (Pathatrix) and cationic beds is a simple and relatively quick technique, but is also highly unspecific and needs to be further optimized before it can be used for concentrating viruses from raspberries. It should, however, be mentioned that raspberries are recognized as a particularly challenging food matrix to work with due to their low pH and high presence of inhibitors (Le Guidader *et al.,* 2004; Summa *et al.,* 2012).

References

Atmar, R.L. (2006). *Molecular methods of virus detection in foods.* Food Microbiology and Food Safety, 121-149.

Baert, L., Mattison, K., Loisy-Hamon, F., Harlow, J., Martyres, A., Lebeau, B., Stals, A., Van Coillie, E., Herman, L. & Uyttendaele, M. (2011). Review: Norovirus prevalence in Belgian, Canadian and French fresh produce: A threat to human health? *International Journal of Food Microbiology* 151(3), 261-269.

Baert, L., Uyttendaele, M., Vermeersch, M., Van Coillie, E. & Debeverei, J. (2008). Survival and transfer of murine norovirus 1, a surrogate for human noroviruses, during the production process of deep-frozen onions and spinach. *Journal of Food Protection* 71(8), 1590-1597.

Cannon, J.L., Papafragkou, E., Park, GW., Osborne, J., Jaykus, L.A. & Vinjé, J. (2006). Surrogates for the study of norovirus stability and inactivation in the environment: a comparison of murine norovirus and feline calicivirus. *Journal of Food Protection* 69(5), 2761-2765.

CEN/TC 275/WG 06. (2011). *Microbiology of food and animal feed - Horizontal method for detection of hepatitis A virus and norovirus in food using real-time RT-PCR.* Part 1: Method for quantitative determination.

D'Souza, D.H., Sair, A., Williams, K., Papafragkou, E., Jean, J., Moore, C. & Jaykus, L. (2006). Persistence of caliciviruses on environmental surfaces and their transfer to food. *International Journal of Food Microbiology* 108(1), 84-91.

Duizer, E., Schwab, K.J., Neill, F.H., Atmar, R.L., Koopmans, M.P. & Estes, M.K. (2004). Laboratory efforts to cultivate noroviruses. *Journal of General Virology* 85(Pt 1), 79-87.

Food and Agriculture Organization of the United Nations/World Health Organization. (2008). *Viruses in food: scientific advice to support risk management activities.* Meeting report. Microbiological Risk Assessment Series, 13.

Goodridge, L., Goodridge, C., Wu, J.Q., Griffiths, M. & Pawliszyn, J. (2004). Isoelectric point determination of norovirus virus-like particles by capillary isoelectric focusing with whole column imaging detection. *Analytical Chemistry* 76(1), 48-52.

Hsu, C.C., Wobus, C.E., Steffen, E.K., Riley, L.K. & Livingston, R.S. (2005). Development of a microsphere-based serologic multiplexed fluorescent immunoassay and a reverse transcriptase PCR assay to detect murine norovirus 1 infection in mice. *Clin Diagn Lab Immunol* 12(10), 1145-51.

Karst, S.M., Wobus, C.E., Lay, M., Davidson, J. & Virgin, H.W.t. (2003). STAT1-dependent innate immunity to a Norwalk-like virus. *Science* 299(5612), 1575-8.

Karstenmeyer, R.J., Perdue, K.A. & Elkins, W.R. (2008). Eradication of murine noroviruses from a mouse barrier facility. *J Am Assoc Lab Anim Sci* 47(1), 26-30.

Kim, M., Lee, H., Chang, K.O. & Ko, G. (2010). Molecular characterization of murine norovirus isolates from South Korea. *Virus Res* 147(1), 1-6.

Koopmans, M. & Duizer, E. (2004). Foodborne viruses: an emerging problem. *International Journal of Food Microbiology* 90(1), 23-41.

Le Guyader, F.S., Mittelholzer, C., Haugarreau, L., Hedlund, K.O., Alsterlund, R., Pommepuy, M. & Svensson, L. (2004). Detection of noroviruses in raspberries associated with a gastroenteritis outbreak. *International Journal of Food Microbiology* 97(2), 179-186.

Lees, D. & Tag4, C.W. (2010). International Standardisation of a Method for Detection of Human Pathogenic Viruses in Molluscan Shellfish. *Food and Environmental Virology* 2(3), 146-155.

Le Gyader, F.S., Mittelholzer, C., Haugarreau, L., Hedlund, K.O., Asterlund, R., Pommepuy, M. & Svensson, L. (2004). Detection of noroviruses in raspberries associated with a gastroenteritis outbreak. *International Journal of Food Microbiology* 97(2), 179-186.

Lund, F. & Lindkvist, R. (2004). *Virus in food and drinking water in Sweden – norovirus and hepatitis A virus.* Risk profile, report 22. National Food Agency, Sweden.

Mattison, K., Brassard, J., Gagne, M.J., Ward, P., Houde, A., Lessard, L., Simard, C., Shukla, A., Pagotto, F., Jones, T.H. & Trottier, Y.L. (2009). The feline calicivirus as a sample process control for the detection of food and waterborne RNA viruses. *International Journal of Food Microbiology* 132(1), 73-77.

Mattinson, K., Plante, M. & Bidawid, S. (2010). *Concentration and detection of hepatitis A virus from contaminated strawberries by the Pathatrix system and reverse transcription polymerase chain reaction.* Government of Canada, health products and food branch.

Morales-Rayas, R., Wolffs, P.F.G. & Griffiths, M.W. (2010). Simultaneous separation and detection of hepatitis A virus and norovirus in produce. *International Journal of Food Microbiology* 139(1-2), 48-55.

Morillo, S.G. & Timenetsky, M.C.S.T. (2011). Norovirus: an overview. *Revista da Associacao Medica Brasileira* 57 (4), 453-8.

Newell, D.G., Koopmans, M., Verhoef, F., Duizer, E., Kidara-Kane, A., Spong, H., Opsteegh, M., Langelaar, M., Turefall, D., Scheutz, F., van der Giessen, J. & Kruse, H. (2010). Food-borne diseases – The challenge of 20 years ago still persist while new ones continue to emerge. *International Journal of Food Microbiology* 139, 3-15.

Papafagkou, E., Plante, M., Mattinson, K., Bidawid, S., Karthikeyan, K., Farbrer, J.M., & Jaykus, L.A. (2008). Rapid and Sensitive Detection of Hepatitis A Virus (HAV) in Representative Food Matrices. *Journal of Virological Methods* 147(1), 177-187.

Plante, M., Karthikeyan, K., Bidawid, S., Mattinson, K. & Farbrer, J.M. (2005). Development of methods for norovirus detection from various outbreak foods. Health Canada, Ottawa, Canada. Presented at the IAFP annual meeting, Baltimore, August 14-17.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4(4), 406-25.

Stals, A., Baert, L., Van Coillie, E. & Uyttendaele, M. (2011). Evaluation of a norovirus detection methodology for soft red fruits. *Food Microbiol* 28(1), 52-8.

Straub, T.M., Honer zu Bentrup, K., Orosz-Coghlan, P., Dohnalkova, A., Mayer, B.K., Bartholomew, R.A., Valdez, C.O., Bruckner-Lea, C.J., Gerba, C.P., Abbaszadegan, M. & Nickerson, C.A. (2007). In vitro cell culture infectivity assay for human noroviruses. *Emerg Infect Dis* 13(3), 396-403.

Summa, M., von Bonsdorff, C.H. & Maunula, L. (2012). Evaluation of four virus recovery methods for detecting noroviruses on fresh lettuce, sliced ham, and frozen raspberries. *Journal of Virological Methods* 183(2), 154-60.

Swedish Institute for Communicable Disease Control. (2010). *Sjukdomsinformation om calicivirus (noro- och sapovirus).* http://www.smittskyddsinstitutet.se/sjukdomar/calicivirus-noro-och-sapovirus/. [2012-10-31].

Swedish Institute for Communicable Disease Control. (2011). *Statistik för norovirus.* http://www.smittskyddsinstitutet.se/statistik/norovirus/. [2012-10-31].

Tamura, K., Nei, M. & Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences of the United States of America* 101(30), 11030-11035.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution* 28(10), 2731-2739.

Tian, P., Yang, D. & Mandrell, R. (2011). A simple method to recover Norovirus from fresh produce with large sample size by using histo-blood group antigen-conjugated to magnetic beads in a recirculating affinity magnetic separation system (RCAMS). *International Journal of Food Microbiology* 147(3), 223-227.

Widen, F., Vagsholm, I., Belak, S. & Muradrasoli, S. (2011). Achievement V - Methods for breaking the transmission of pathogens along the food chain Detection of viruses in food. *Trends in Food Science & Technology* 22(1), S49-S57.

Wobus, C.E., Karst, S.M., Thackray, L.B., Chang, K.O., Sosnovtsev, S.V., Belliot, G., Krug, A., Mackenzie, J.M., Green, K.Y. & Virgin, H.W. (2004). Replication of Norovirus in cell culture reveals a tropism for dendritic cells and macrophages. *PLoS Biol* 2(12), e432.

Wobus, C.E., Thackray, L.B. & Virgin, H.W.t. (2006). Murine norovirus: a model system to study norovirus biology and pathogenesis. *J Virol* 80(11), 5104-12.

Zheng, D.P., Ando, T., Fankhauser, R.L., Beard, R.S., Glass, R.I. & Monroe, S.S. (2006). Norovirus classification and proposed strain nomenclature. *Virology* 346(2), 312-23.

Acknowledgements

I am deeply thankful to my supervisors, Shaman Muradrasoli and Ronnie Eriksson, for their valuable and patient support during this project.

Parts of this project were conducted at the National Food Agency (Livsmedelsverket). Thanks to Karin Jacobsson, Magnus Simonsson, and Hans Lindmark for giving me the opportunity to work there.