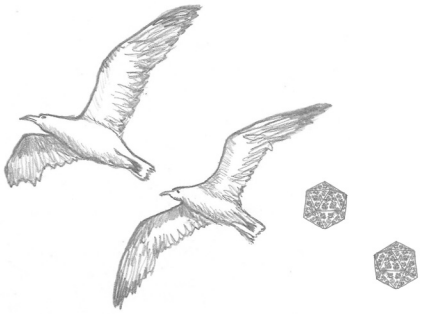
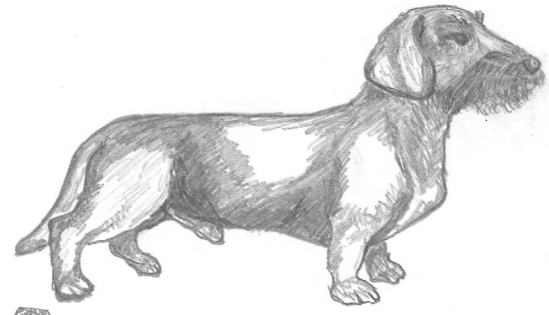


DISSERTATIONES SCHOLA DOCTORALIS SCIENTIAE CIRCUMIECTALIS, ALIMENTARIAE, BIOLOGICAE. UNIVERSITATIS HELSINKIENSIS

MAIJA SUMMA

HUMAN NOROVIRUSES: DETECTION IN FOOD AND NEW TRANSMISSION ROUTES



DEPARTMENT OF FOOD HYGIENE AND ENVIRONMENTAL HEALTH
FACULTY OF VETERINARY MEDICINE
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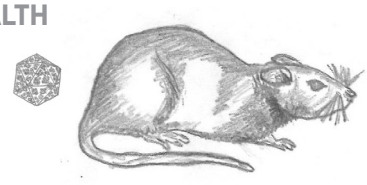

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HUMAN NOROVIRUSES: DETECTION IN FOOD AND NEW TRANSMISSION ROUTES

Maija Summa

DOCTORAL DISSERTATION

To be presented for public examination with the permission of the Faculty of Veterinary Medicine of the University of Helsinki, in the Walter Auditorium, EE-building, on 16th August 2019, at 12 o'clock.

Helsinki 2019

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ABSTRACT

Human noroviruses (HuNoVs) are yearly responsible for a large number of acute human gastroenteritis cases globally in all age groups. Typically, the virus transmits via the fecal-oral route from person to person, causing strong symptoms such as nausea, vomiting, and diarrhea, which usually disappear in a few days. However, HuNoVs cause also numerous food-related illnesses in developed countries, including Finland, inducing gastroenteritis outbreaks through contaminated water and foodstuffs. According to the reports of the European Commission, both in Europe and in Finland the most common foods causing HuNoV outbreaks are shellfish, berries (especially frozen raspberries), vegetables, and mixed foods, which most likely became contaminated by a sick food handler.

Noroviruses belong to the *Caliciviridae* family and are classified into seven genogroups. HuNoVs belong to genogroups I (GI), II (GII), and IV (GIV). Other genogroups contain only animal noroviruses. Noroviruses are generally regarded as host-species-specific, but the possibility of zoonotic transmission and infections has been discussed for over a decade for several genotypes.

The purpose of this study was to develop a simple and rapid method for detection of HuNoVs in food. The potential zoonotic nature of HuNoVs, particularly whether animals can serve as transmitters for these viruses, was also investigated.

In the past two decades, numerous methods for detecting HuNoVs in food have been developed. However, many of these are time-consuming and the sensitivity of the methods has been highly variable. In this work, four published extraction methods for detection of HuNoV in food (lettuce, ham, and frozen berries) were compared. The method based on alkaline elution and polyethylene glycol (PEG) precipitation was found to be the most reliable detection method for all three food matrices tested. The recovery efficiency of the method with frozen raspberries was on average 28%. Two rapid methods for detection of HuNoV in frozen raspberries were also presented. The rapid method based on direct RNA extraction yielded the same recovery levels (32%) as the PEG precipitation method. The method proved to be sensitive because it detected HuNoV also with a virus level of 10^2 genome copies in a 25 g sample. Moreover, the method detected HuNoV in naturally contaminated berry samples that were linked to outbreaks of disease.

A treatment with either a chloroform-butanol mixture or dilution of the food samples for the RT-PCR reaction was efficient in reducing the effect of PCR inhibitors. The same effect was achieved with PEG as a supplement in the food samples.

Thirty-nine frozen berry samples purchased from local stores in 2010, 2014, and 2017 were screened. All berries tested negative for HuNoVs GI and GII.

The possibility of zoonotic transmission of HuNoVs was investigated by analyzing fecal samples of birds, rats, mice, and pet dogs for HuNoVs. HuNoV genome was detected in the feces of 31 birds, two rats, and four dogs. The genotypes found in six bird samples and all dog samples were the same as those commonly found in human samples at the time of sampling.

HuNoVs can be detected in food samples also in small numbers using the rapid method presented in this study. The use of PEG as a supplement was found to reduce inhibition of the RT-PCR reaction in the two rapid methods, and therefore, the commonly used chloroform-butanol treatment, which easily loses viruses during processing, could be omitted. The results of animal samples strongly indicate that wild birds, pet dogs, and possibly also rats may be involved in the transmission of HuNoVs to food, water, and surfaces.

ACKNOWLEDGMENTS

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First and foremost, I thank Docent Leena Maunula and Professor Emeritus Carl-Henrik von Bonsdorff for supervising my thesis and introducing me to the world of noroviruses. I am deeply grateful for all of the support and advice received over the years. I am also grateful to Leena Maunula for creating such a comfortable working environment and for giving me freedom to implement my own ideas in the laboratory work.

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This thesis is dedicated to my beloved Mother who passed away during this long process.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their Roman numerals:

I 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, 154-160.

II Summa M, Maunula L. 2018. Rapid Detection of Human Norovirus in Frozen Raspberries. Food and Environmental Virology, 10, 51-60.

III Summa M, Henttonen H, Maunula L. 2018. Human noroviruses in the faeces of wild birds and rodents - new potential transmission routes. Zoonoses and Public Health, 65, 512-518.

IV Summa M, von Bonsdorff C-H, Maunula L. 2012. Pet dogs - A transmission route for human noroviruses? Journal of Clinical Virology, 53, 244-247.

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The author's contribution to these publications was as follows:

I Main responsibility for designing the study. Performed laboratory work and interpreted results. Main responsibility for writing the paper.

II Main responsibility for designing the study. Collected samples, performed laboratory work, and interpreted results. Main responsibility for writing the paper.

III Main responsibility for designing the study. Collected bird samples and most of the rat samples, performed laboratory work, and interpreted results. Main responsibility for writing the paper.

IV Main responsibility for designing the study. Collected samples and performed laboratory work. Main responsibility for interpreting the results and writing the paper.

ABBREVIATIONS

bp	base pair
CB	chloroform-butanol
Cq	quantification cycle
DNA	deoxyribonucleic acid
EC	external control
gc	genome copy
GITC	guanidine thiocyanate
IEM	immune electron microscopy
IMS	immuno magnetic separation
ISO	International Organization for Standardization
HGBA	histo blood group antigen
HuNoV	human norovirus
kb	kilobase
MM	miniMAG nucleic acid extraction system
MRSA	methicillin-resistant *Staphylococcus aureus*
MuNoV	murine norovirus
NC	naturally contaminated
nm	nanometer
nt	nucleotide
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PCRU	PCR unit
PEG	polyethylene glycol
PEGP	PEG precipitation method
ppm	parts per million
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
RT reaction	reverse transcription reaction
SD	standard deviation
SDS	sodium dodecyl sulphate
SRSV	small round structured virus
TGBE	tris glycine beef extract
VLP	virus like particle

1 INTRODUCTION

Human noroviruses (HuNoVs) have globally been one of the most significant causes of acute gastroenteritis in all age groups. Typically, HuNoV infection causes strong symptoms such as nausea, vomiting and diarrhea, which usually appear suddenly within 12-48 hours of virus ingestion and pass after one to three days. Most commonly (88% of all HuNoV infections), virus transmission occurs via the fecal-oral route from person to person. However, HuNoVs have for years been on the list of the top four pathogens causing food-related illness in developed countries, including Finland, inducing gastroenteritis outbreaks through contaminated water (1.5% of all infections) and various foodstuffs (10% of all infections) (Koopmans et al. 2008, Mathijs et al. 2012, Maunula et al. 2005, Niskanen et al. 2011). Quickly spreading noroviruses can easily cause large outbreaks through multiple countries or in closed environments, such as cruise ships, where large numbers of people gather (Anderson et al. 2006, Verhoef et al. 2008).

According to the European Commission (European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC) 2006-2017), both in Europe and in Finland the most common foods causing HuNoV outbreaks are shellfish, berries, vegetables, and mixed foods that have likely become contaminated by an infected food handler. Of the berries, particularly frozen raspberries, have caused numerous HuNoV outbreaks in Europe (Cotterelle et al. 2005, European Commission 2014, 2015, Falkenhorst et al. 2005, Le Guyader et al. 2004a, Hjertqvist et al. 2006, Korsager et al. 2005, Maunula et al. 2009, Müller et al. 2014, Sarvikivi et al. 2012, Tavoschi et al. 2015). In recent years, several research groups have focused on how food items become contaminated by HuNoVs during food handling (Mokhtari and Jaykus 2009, Rönnqvist et al. 2014; Stals et al. 2013; Tuladhar et al. 2013; Verhaelen et al. 2013), harvesting, and irrigation processes (Kokkinos et al. 2012, Maunula et al. 2013).

In a literature review by Stals et al. (2012), several methods for detecting noroviruses in various food matrices have been presented. A number of sensitive methods are available for norovirus-contaminated oysters, which usually contain viruses in high loads. For other high-risk foodstuffs, such as berries, salads, and cold cuts, numerous methods have been developed based on e.g. ultrafiltration, ultracentrifugation, cationic separation, and polyethylene glycol (PEG) precipitation, but the virus recovery efficiencies have been highly variable, ranging from 3% to 72% (Stals et al. 2012). Because viral contamination levels in food are usually low, the sensitivity of the method is a key factor. The unstable sensitivity of the different methods was one of the reasons that led to the need for a standardized method. In 2013, the first technical specification for HuNoV detection in foods (ISO/TS 15216) was published, and a few years later, in 2017, the section concerning quantitative detection was published as ISO standard 15216-1:2017. The application of the standard method for soft fruit, however, consists of numerous steps, such as time-consuming virus concentration, and it often requires two working days to obtain the results, especially if the analysis involves several samples. In an outbreak situation, noroviruses spread quickly and to prevent

dissemination, the results of virus analysis are needed as soon as possible emphasizing the need for a rapid method.

The objectives of the two first studies presented here were to compare four published methods and, based on the results of the comparison, to create a simple method for detecting noroviruses in food. Another aim was to perform a screening study on high-risk foods for noroviruses.

Both wild and domestic animals are known to transmit many zoonotic bacteria and viruses (Chomel and Sun 2011, Heredia and García 2018, Kruse et al. 2004). The best-known zoonotic viral diseases include rabies, influenzas, severe acute respiratory syndrome (SARS), West Nile fever, and ebola (Heeney 2006). In Asia, up to 48% of domestic animals, including dogs, cats, and cattle carry rabies, which is a lethal disease for both animals and humans (Thiptara et al. 2011). According to Chomel and Sun (2011), up to 45% of pet dogs in Europe and the USA sleep in their owner's bed and as many as half of the owners let the dogs lick their faces or kiss the pets themselves. Several zoonotic diseases, such as plague, pasteurellosis, *Capnocytophaga canimorsus* septicemia, MRSA infection, rabies, and giardiasis, have been reported to transmit to the owner from the pet. De Grazia et al. (2007) proposed that dogs are a potential source of human viral pathogens after finding an animal-like rotavirus strain in a child with acute gastroenteritis. Heredia and García (2018) have reviewed that the typical bacteria causing foodborne outbreaks, i.e. *Salmonella* spp., *Campylobacter* spp., Shiga-toxin producing *E. coli*, and *Listeria* spp., are frequently carried by poultry, cattle, small ruminants, and also dogs and cats.

Small rodents, especially rats, spread various bacterial human diseases, such as salmonellosis and plague, but they also carry zoonotic viruses like hepatitis E virus and hantaviruses (Kosoy et al. 2015, Leibler et al. 2016, Vaheri et al. 2013). In addition, wild birds have transmitted to humans at least salmonella, mycobacteria, West Nile virus, and influenza viruses (Tsiodras et al. 2008). Many food- or water borne pathogens, such as salmonella, *Listeria monocytogenes*, and campylobacteria, can also be transmitted by birds, especially by gulls and corvids (including crows and jackdaws) (Butterfield et al. 1983, Tsiodras et al. 2008).

Although noroviruses are generally regarded as host species-specific, the possibility of zoonotic transmission and infections has been discussed (Bank-Wolf et al. 2010, Mathijs et al. 2012, Mattison et al. 2007, Mesquita et al. 2010, Scipioni et al. 2008, Wang et al. 2005, Wilhelm et al. 2015). Genogroup II noroviruses resembling closely HuNoVs and even GII.4-like HuNoVs have been found in pigs and cattle (L'Homme et al. 2009a, 2009b, Mattison et al. 2007, Wang et al. 2005). Genogroup IV noroviruses found in dogs and a lion were closely related to GIV HuNoVs (Martella et al. 2007, Martella et al. 2008, Mesquita et al. 2010), and some evidence of HuNoVs occurring in dogs (Caddy et al. 2015) has been presented. An old report also suggested that a sick dog caused an HuNoV outbreak in a retirement home in the UK (Humphrey et al. 1984). All of these findings suggest the possibility of species-to-species transmission.

The objective of the two last studies presented in this thesis was to investigate the possibility of animals having a role in the transmission of HuNoVs to food and humans.

2 REVIEW OF THE LITERATURE

2.1 History of noroviruses

The prototype virus of noroviruses, Norwalk virus, was discovered in 1972 (Kapikian et al. 1972, Kapikian 2000). Before this discovery, there had been studies in the 1940's of an agent causing a non-bacterial acute gastroenteritis. The studies were carried out with human volunteers. Gordon et al. (1947) found that the causative agent caused illness through stool and throat washings of symptomatic persons when the pathogen was taken by mouth. In the late 1960's there were large outbreaks in the US. One of these outbreaks, which later became the reference outbreak of noroviruses, occurred in Norwalk, Ohio. At that time, the illness was called the "winter vomiting disease", a name that Zahorsky had used for a similar disease in 1929. In the early 1970's, new volunteer studies were carried out with the same pathogen that had caused outbreaks in Norwalk. During these studies Kapikian succeeded in viewing for the first time the "small round structured viruses" (SRSVs) by immune electron microscopy (IEM) using convalescent human sera (Figure 1).

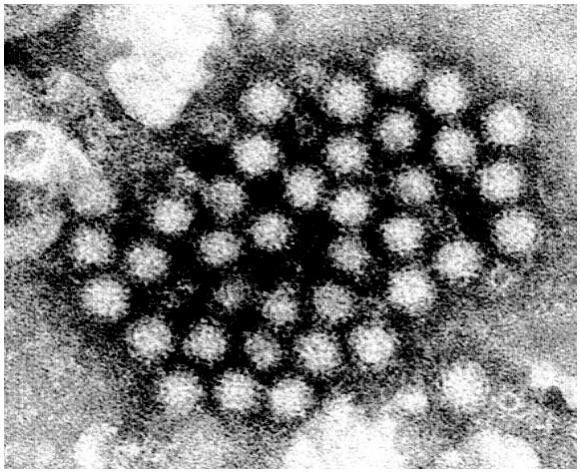

Figure 1. Picture of norovirus particles in electron microscopy (Charles D. Humphrey, USCDCP, PIXNIO)

Before and after its discovery, norovirus has had many names. At first, it was thought that "viral gastroenteritis" was caused by one particular virus, "Norwalk agent". However, the virus was not easy to detect using electron microscopy, and it did not replicate in cell cultures or in animal models. It subsequently turned out that the SRSV or SSRV (small spherical round virus) was a group of different viruses or virus strains that caused the

viral gastroenteritis, and these were named after the location in which the strain was detected. The viruses were called e.g. Snow Mountain virus, Lordsdale, Hawaii, Southampton, and also in Finland 98-Riihimäki, 97-Helsinki, 97-Kuopio etc. (Maunula et al. 1999, Monroe et al. 2000). In 1990's, the PCR techniques became more general in the field of virus research, and the genome sequences of two norovirus strains were solved. After this, the classification of noroviruses became clearer and the viruses were placed in the family of *Caliciviridae* and were usually known as "Norwalk-like viruses" (NLVs) (Monroe et al. 2000). At the turn of the 2000s, noroviruses were classified as human caliciviruses, and in Finland the virus causing large outbreaks was also called "kalikivirus". However, the International Committee of Taxonomy of Viruses confirmed the ubiquitous name, Norovirus, in 2002 (www.norovirus.com). The noroviruses were then divided into six genogroups and dozens of genotypes (Zheng et al. 2006). After the new classification, history started to repeat itself and the new variants of genogroup II noroviruses were again named after the year and location of the first full-length capsid sequence in the public domain, e.g. Den Haag_2006b, NewOrleans_2009, and Sydney_2012 (Kroneman et al. 2013) (Figure 2).

In the early 1980's, when the diagnosis of norovirus infection was based on insensitive (for this purpose) electron microscopy, Kaplan et al. (1982) created the criteria to distinguish the outbreaks caused by norovirus from outbreaks caused by a bacterial source. The Kaplan criteria include four conditions: more than half of the affected persons vomit, mean incubation period is 24-48 h, mean duration of illness is 12-60 h, and no bacterial pathogen can be detected in stool culture.

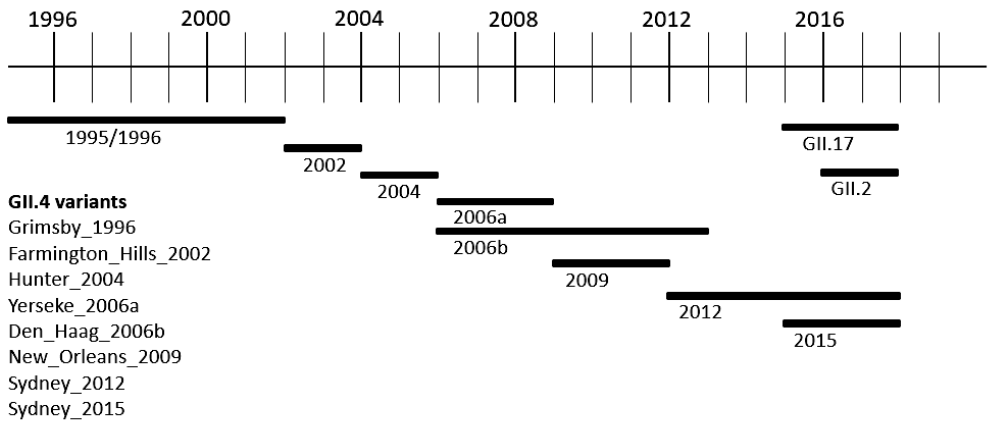

Figure 2. Norovirus GII.4 variants and some other globally circulating genotypes (Atmar et al. 2018).

2.2 Classification, taxonomy, and genotypes of norovirus

Noroviruses belong to the family of *Caliciviridae* along with four other virus genera, namely Sapovirus (Sapporo virus, human gastroenteritis), Lagovirus (Rabbit hemorrhagic disease virus, European brown hare syndrome virus), Nebovirus (Newbury-1 virus, bovine gastroenteritis), and Vesivirus (Vesicylar exanthema virus of swine, Feline calicivirus). Noroviruses are further classified into seven genogroups (Gs) as presented in Figure 3 (Atmar et al. 2018, Vinjé 2015). Genogroup I contains only human noroviruses, including the Norwalk virus GI.1. Genogroup II comprises human and porcine noroviruses. Genogroup III noroviruses can infect cows and sheep, while genogroup V infects rats and mice. Genogroup IV contains human, feline, and canine noroviruses, and genogroups VI and VII only canine noroviruses. Within the genogroups, noroviruses are further divided into genotypes, and within the genotypes into different variants, especially in genotype GII.4, which has been the most common genotype circulating globally, causing 62% of all norovirus outbreaks in 2001-2007. Studies of the genetic variation of noroviruses have led to the conclusion that evolution of new variants happens mostly by recombination mechanisms in mixed infections in the same host and point mutations, which are caused by errors during the replication process (Green 2013, Bull and White 2011, Siebenga et al. 2009, White 2014).

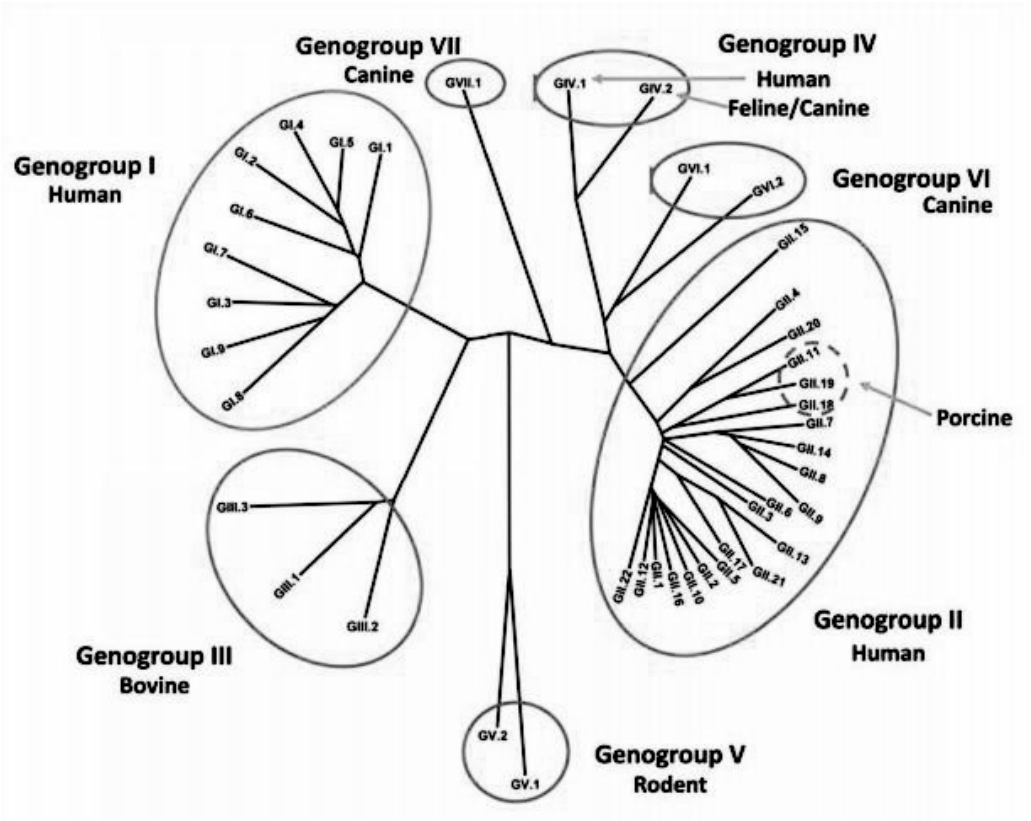

Figure 3. The seven genogroups of noroviruses (Atmar et al. 2018).

2.3 Structure and physical features of norovirus

Round norovirus particles have a non-enveloped icosahedral capsid that is 27-40 nm in diameter. Norovirus particles resemble enterovirus particles, which also replicate in the human intestines. The genome of noroviruses is approximately 7.3-8.5 kilobases (kb) long, linear single-stranded positive-sense RNA, which contains three open reading frames (ORFs) (Figure 4). ORFs 2 and 3 encode the major and minor structural proteins, respectively (VP1 and VP2), towards the polyadenylated 3' end of the genome, which forms the virus capsid. The capsid proteins form structures that are seen as cups on the surface of virus particles (Latin word calyx means cup -> calicivirus). ORF1 encodes non-structural proteins beginning from the 5' end of the genome. Non-structural proteins, NS1-NS7, are needed in the interaction with the host cell and in the replication process of the viruses. Uniquely, the genome of murine noroviruses in genogroup V contain also fourth ORF area, ORF4 (Green 2013).

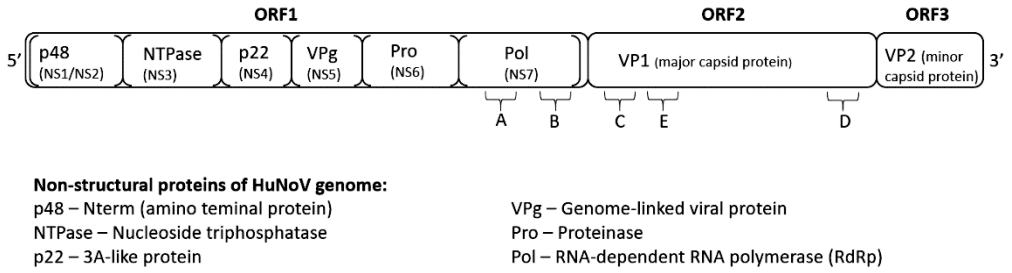

Figure 4. The HuNoV genome (Robilotti et al. 2015).

Noroviruses are among the most stable viruses against environmental stress. The stability has, however, been challenging to test because for decades the only way to confirm the infectivity of the virus particles was volunteer tests. Early studies revealed that noroviruses can withstand pH as low as 2.7 for three hours at room temperature (Dolin et al. 1972). In foods having a low pH (noodle salad pH 5.0 and tomato ketchup pH 4.5) and stored at 6°C, norovirus RNA, probably protected by virus capsid, can remain stable for 24-58 days. Neither cooling nor freezing damaged norovirus RNA in apple, lettuce, minced meat, or pizza during a storage of 2-14 days (Mormann et al. 2010). Washing with cold or warm water did not remove virus contamination from berries and some herbs (Butot et al. 2008). Viruses remain infectious for 61 days at room temperature in ground water, and RNA can be detected even after three years (Seitz et al. 2011). Heating at 60°C for 30 minutes did not destroy noroviruses (Dolin et al. 1972). Chlorine concentrations of less than 6.25 ppm did not inactivate noroviruses, but the concentration of 10 ppm, which is commonly used for disinfection of water supply systems, has been shown to inactivate also noroviruses in drinking water (Keswick et al. 1985). Treatments with chlorine concentrations of 50 ppm or more completely inactivated HuNoVs in fecal filtrates (Costantini et al. 2018). However, according to Tuladhar et al. (2012) for contaminated surfaces, a minimum of 1000 ppm of free chlorine containing solutions should be used to reduce norovirus contamination significantly.

HuNoVs are detectable in RT-PCR on different surfaces for at least 21-28 days, but GII HuNoV was significantly more persistent (42 days) than GI HuNoV (Liu et al. 2009). However, both Bitler et al. (2013) and Matthews et al. (2012) suggest that GI HuNoVs are more stable in the environment than GII HuNoVs because they cause significantly more waterborne outbreaks. Still, according to the report of Maunula et al. (2005), GI and GII noroviruses were found equally in water samples from waterborne outbreaks in Finland in 1998-2003. Richards et al. (2012) concluded that HuNoV GII.4 is highly resistant to repeated freezing and thawing and long-term storage as frozen. Teunis et al. (2008) have also shown that HuNoV GI stays infectious for even decades when stored frozen. The commonly used waste water treatments that contain no disinfection chemicals or radiation do not completely remove HuNoVs from wastewater (Courault et al. 2017). Alcohol-based disinfectants or hand sanitizers may reduce the number of HuNoV particles, but after alcohol treatment infectious viruses will remain (Costantini et al. 2018, Blaney et al. 2011, Tuladhar et al. 2015, Vogel 2011).

2.4 Human noroviruses

Human noroviruses (HuNoVs) infect people globally in both developed and developing countries (Ahmed et al. 2014). Even though HuNoVs are divided into dozens of genotypes within the three genogroups, one genotype, GII.4, has dominated in humans worldwide. It has caused at least six global epidemics starting from the 1990's with its variants US95_1996, Farmington_Hills_2002, Hunter_2004, Den_Haag_2006b, Yerseke_2006a, New_Orleans_2009, and Sydney_2012 (Atmar et al. 2018, Siebenga et al. 2009, Vinjé 2015) (Figure 2). Apart from genotype GII.4, genotypes GII.3, GII.6, and GII.2 have been found to infect especially children under 5 years of age (Hoa Tran et al. 2013).

2.4.1 Norovirus infection

Noroviruses are one of the most important causes of acute gastroenteritis worldwide, causing about 20% of all acute gastroenteritis cases (Ahmed et al. 2014).
Noroviruses enter the host via the oral route (Green 2013). After ingestion, noroviruses are presumed to bind to the host cell with the help of histo-blood group antigen (HGBA) carbohydrates, which are present in saliva and mucosal secretions of epithelial cells in the intestine of individuals who carry the fucosyltransferase 2 gene (*FUT2*) (Marionneau et al. 2002, Shirato, 2011). Several research groups have concluded that individuals who do not express the *FUT2* gene, known as non-secretors, do not become infected by noroviruses (Kambhampati et al. 2016, Lindesmith et al. 2003, Rodríguez-Díaz et al. 2017, Shirato 2011). However, Currier et al. (2015) showed that secretor status was important only with GII.4 and GII.6 infections in children; only secretors got infected. With other norovirus genotypes, secretor specificity could not be seen. According to Lindesmith et al. (2003), not all secretors become infected with norovirus.

Virus replication occurs in the intestinal cells, presumably in the small intestine after the virus has entered the cell (Atmar et al. 2018, Dolin et al. 1975, Green 2013, Karandikar et al. 2016). The RNA genome is released and translated by the host cell, which also constructs the new virions. After the replication process, noroviruses are released from lysed cells in the gastrointestinal tract i.e. in feces or vomitus of the host (Green 2013).

In some cases, norovirus may also be present in blood. Viremia has been shown in young children with acute norovirus infection (Huhti et al. 2016). However, Newman et al. (2015) did not find noroviruses in blood samples of immunocompetent adults with norovirus infection.

The infectious dose of norovirus is low; an estimated 10-20 virus particles are needed to cause the disease (Teunis et al. 2008). In an acute phase of infection, humans can secrete 10^5-10^8 virus particles per gram of stool. The incubation period is typically 24-48 hours, but symptoms can occur as early as 8 hours after virus ingestion (Atmar et al. 2008, Robilotti et al. 2015). Lee et al. (2013) have separated incubation periods according to genogroups and estimated that GI noroviruses have a slightly shorter incubation period than GII noroviruses. Noroviruses infect humans in all age groups. Typical symptoms are sudden nausea, diarrhea, vomiting, abdominal discomfort, headache, myalgias, and fever. Children have vomiting more often than diarrhea and adults the reverse. Symptoms usually disappear in 24-48 hours. Interestingly, Devasia et al. (2015) have concluded based on numerous published studies that food-borne norovirus infections have shorter symptomatic periods than other transmission routes. Strong norovirus infection can cause severe dehydration, especially in elderly or the very young. For immunocompromised persons, norovirus infection can even be life-threatening or cause chronic gastroenteritis (Atmar et al. 2008, Green 2013, Harris et al. 2008, Robilotti et al. 2015). Asymptomatic infections with viral shedding also occur in about 5-7% of adult population and even more in children under the age of 5 years (Ahmed et al. 2014, Currier et al. 2015, Phillips et al. 2010). Norovirus tends to have an infection peak during the cold winter months, but its seasonality is not as clear in children as in adults (Kroneman et al. 2008, Maunula and von Bonsdorff 2005, Patel 2009, Phillips et al. 2010).

Infected persons secrete norovirus in their feces 2 weeks or more after recovery (Atmar et al. 2008). In children, old persons, and in individuals with underlying disease or immunodeficiency, the shedding time can be several weeks or even months (Kirkwood and Streitberg 2008, Pringle et al. 2015, Siebenga et al. 2008). Schorn et al. (2010) described a viral shedding over 2 years for a patient with a kidney transplant.

Immunological investigations have been a challenge because tissue cultures have not been available for norovirus research. Immunity or resistance to norovirus infection likely consists of both genetic (secretor status) and immunological susceptibility of the person. According to early volunteer studies, there is a short-term immunity that is virus-specific, possibly even variant-specific, and lasts at least 14 weeks. However, norovirus antibody levels in serum do not correlate with resistance to illness (Lindesmith et al. 2003, Pringle et al. 2015, Robilotti et al. 2015), and for this reason serological tests are not used for norovirus diagnostics. Simmons et al. (2013) have suggested, based on epidemiological studies, that a genotype-specific immunity could last up to 9 years. This could explain why certain genotypes are more common in children under 5 years of age who get multiple infections with different genotypes before the genotype-specific immunity has been developed. Blazevic et al. (2016) also suggest that maternal antibodies can protect children from norovirus infections for up to 2 years. However, Parra et al. (2017) have grouped human noroviruses into 12 “immunotypes”. They suggest that viruses belonging

to the same immunotype do not re-infect the same individual, with the exception of GII.4 viruses, which could re-infect individuals due to the numerous new variants.

2.5 Foodborne norovirus infections and presence of noroviruses in foods

Shellfish-associated viral diseases were reported already in the 1980's (Richards 1987). In the 1990's, other foods, especially berries, were strongly suspected as a cause of norovirus outbreaks (Pönkä et al. 1999), but the first foodborne HuNoV infection where HuNoV was detected in food other than shellfish was described by Daniels et al. (2000). At the end of the 1990's, 12 laboratories in nine European countries, including Finland, decided to form a network to collect harmonized data of food borne outbreaks caused by viruses. In 2001, a web-based database was established for systematic collection of data of these outbreaks (Koopmans et al. 2003). In the early 2000's, foodborne norovirus infections accounted for 10% of all reported norovirus transmissions in Europe (Kroneman et al. 2008). Globally, noroviruses have been one of the leading causes of food-related outbreaks for several years (European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC) 2006-2017, Scallan et al. 2011).

In Finland, norovirus has been the most important cause of food-related outbreaks from the beginning of the 2000's (Niskanen et al. 2006, 2007, 2010a, 2010b, 2011, Pihlajasaari et al. 2012, 2016). According to the outbreak rating in Finland, the food borne outbreaks are classified into classes A to D according to the strength of evidence. If there is no evidence of a food source for the outbreak, the classification is E. Strong evidence status (classes A and B) usually requires that in addition to appropriate epidemiological findings the virus has been detected in the food sample or in the fecal sample of a food handler (Pihlajasaari et al. 2016). About 2000-3000 persons in Europe (strong evidence cases) and a few hundred persons in Finland yearly become ill via norovirus-contaminated food. More detailed information is presented in Table 1, which includes strong evidence food-borne norovirus outbreaks in Europe (and in parentheses all food borne norovirus outbreaks if the information was available) and all food borne norovirus outbreaks in Finland in 2005-2016. In 2012, over 10 000 persons became ill in Germany via contaminated Chinese strawberries. In 2009, there was a marked increase in food-related norovirus outbreaks in Finland. The majority (69%) of these outbreaks was caused by frozen raspberries.

The most common individual foods causing norovirus outbreaks are shellfish, berries, and vegetables in both Europe and Finland. However, a considerable portion of all outbreaks is caused by mixed foods that most likely became contaminated by a sick food handler (European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC) 2006-2017, Niskanen et al. 2006, 2007, 2010a, 2010b, 2011, Pihlajasaari et al. 2012, 2016, personal communication, Finnish Food Safety Authority Evira, currently Finnish Food Authority). In a 40-year period (1973-2012) in the USA, of all food-borne outbreaks connected to fresh leafy vegetables that had a confirmed etiology, the most common cause was norovirus, inducing 55% of the outbreaks (Herman et al. 2016). In a 30-year period (1983-2013) in Europe, of foodborne diseases associated with frozen berries, 95% were caused by norovirus (Tavoschi et al. 2015).

Mathijs et al. (2012) reviewed 58 food-related HuNoV outbreaks over the period 2000-2010. In 19 of these outbreaks, the raw material (10 raspberries, 3 salads, 11 shellfish) of the food was contaminated. In 18 outbreaks, the food handler had contaminated the food (sandwiches, salads, mixed foods), and in 16 outbreaks there was a suspicion of food handler-based contamination. Only in 17 of these 53 outbreaks was HuNoV detected in a food sample. Norovirus strains found in human samples in these outbreaks were GI, GI.I, GI.2, GI.3, GI.4, GI.5, GI.12, GII.I, GII.2, GII.4, GII.6, GII.7, GII.8, GIIb. According to Verhoef et al. (2010), bivalve mollusks were more often contaminated with GI noroviruses.

The contamination of food products can happen in many production or handling stages. The individual foods (raw material) are usually contaminated at the pre-harvest stage or during harvesting. Contaminated irrigation water for fruits and vegetables and HuNoV-contaminated growing water for shellfish have been demonstrated to cause contaminated food products (Lowther et al. 2008, Wei and Kniel 2010). Kokkinos et al. (2012) have also shown the presence of HuNoV in irrigation water and swabs taken from harvester's hands when examining the food supply chain for lettuce. Irrigation water was contaminated with HuNoV in a berry supply chain investigated by Maunula et al. (2013).

In the material of Mathijs et al. (2012), the majority (59%) of food-borne outbreaks were due to contamination caused by a food handler. In the studies of Kokkinos et al. (2012) and Boxman et al. (2009), HuNoV was detected in a swab sample taken from a food handler's hand. Boxman et al. (2009) also detected HuNoV on food contact surfaces (knife used for cutting bread). Several studies (Rönnqvist et al. 2014, Stals et al. 2013, Tuladhar et al. 2013, Verhalen et al. 2013) have later demonstrated that HuNoV transfers to food from contaminated hands and food contact surfaces, and vice versa, leading to the possibility of cross-contamination. Rönnqvist et al. (2014) have also demonstrated that HuNoV transfers to the outer surface of protective gloves from contaminated hands. However, the number of transferring viruses may be low, hindering the detection of viruses from contaminated food samples, as concluded also by Mathijs et al. (2012).

The presence of HuNoV in food products not related to outbreaks has been demonstrated by many research groups; most often the studies concern HuNoV contamination in shellfish, as reviewed by Mathijs et al. (2012). Although HuNoV is present in oysters relatively often (Bellou et al. 2013), Lowther et al. (2012) suggest that low contamination levels (<100 copies per gram) decrease the risk for illness relative to higher contamination levels (>1000 copies per gram). Mattison et al. (2010), Loutreul et al. (2014), and Pérez-Rodríguez et al. (2014) screened almost 300, 200, and 120 samples of leafy greens, respectively, and 6%, 12%, and 10% of the lots tested were positive for HuNoV GI or GII. The screening studies concerning fruits and berries have shown the presence of HuNoV in 24% (18/75) (Stals et al. 2011c), 16% (32/200 samples) (Loutreul et al. 2014), and 9% (6/70) (De Keuckelaere et al. 2015) of samples. However, two studies of berry fruit and leafy green vegetable supply chains in several European countries did not find any HuNoVs in tested 120 berry samples, and only 1% of the tested 275 salad samples were positive for HuNoVs (Bouwknegt et al. 2015, Kokkinos et al. 2012, Maunula et al. 2013).

Table 1. Reported food-related norovirus outbreaks in Europe and Finland in 2005-2016 according to EFSA and Finnish food authorities. The numbers of strong evidence outbreaks are presented without parentheses, and all norovirus outbreaks in parentheses if the information was available. The Finnish data contain numbers of all norovirus outbreaks.

	Year											
	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016
in Europe												
HuNoV outbreaks	(192)	(358)	82	31	44	88	88	98	77	78(423)	36(330)	94(376)
Human cases in outbreaks	(5060)	(11253)	3384	1090	2309	2653	2475	13766	1773	3560	(12591)	(11706)
in Finland												
HuNoV outbreaks	16	12	5	11	32	13	17	12	13	8[a]	12[a]	22[a]
Human cases in outbreaks	582	820	199	278	1386	502	567	454	309	203	370	784

[a]personal communication, Finnish Food Safety Authority Evira, currently Finnish Food Authority

2.6 Other transmission routes of HuNoVs

The most common transmission route for human noroviruses has always been from person to person, forming 88% of all reported HuNoV transmissions at the beginning of the 2000's. Waterborne outbreaks can also easily infect a high number of people in a short time, and they comprised 1.5% of all norovirus transmissions (Kroneman et al. 2008). The first descriptions of waterborne norovirus outbreaks, where norovirus was detected in drinking water samples, are from the end of the 1990's (Kukkula et al. 1999). In Finland, norovirus has caused multiple outbreaks also via bathing water (Kauppinen et al. 2017, Maunula et al. 2004).

Other transmission vehicles, such as contaminated surfaces, have made up 1% of reported transmissions (Kroneman et al. 2008). Maunula and von Bonsdorff (2005) suggested that the majority of norovirus outbreaks in Finland during 1998-2002 occurred in places where people gather together, e.g. hospitals, restaurants, nursing homes, parties, and military bases. In these places, the transmission can happen from person to person, but also via contaminated food and surfaces. Weber et al. (2010) reviewed the challenges of norovirus contamination on hospital surfaces. The most common site with a HuNoV contamination was the toilet lid, and through the contaminated surfaces the virus spreads easily among patients and health-care workers. Wadl et al. (2009) and Oristo et al. (2017) found HuNoV in several surface swabs taken from garrisons where people had suffered from acute gastro enteritis.

Animals as a potential source of HuNoV transmission are discussed later in this text.

The known and hypothetical transmission routes are gathered in Figure 5 and presented with arrows.

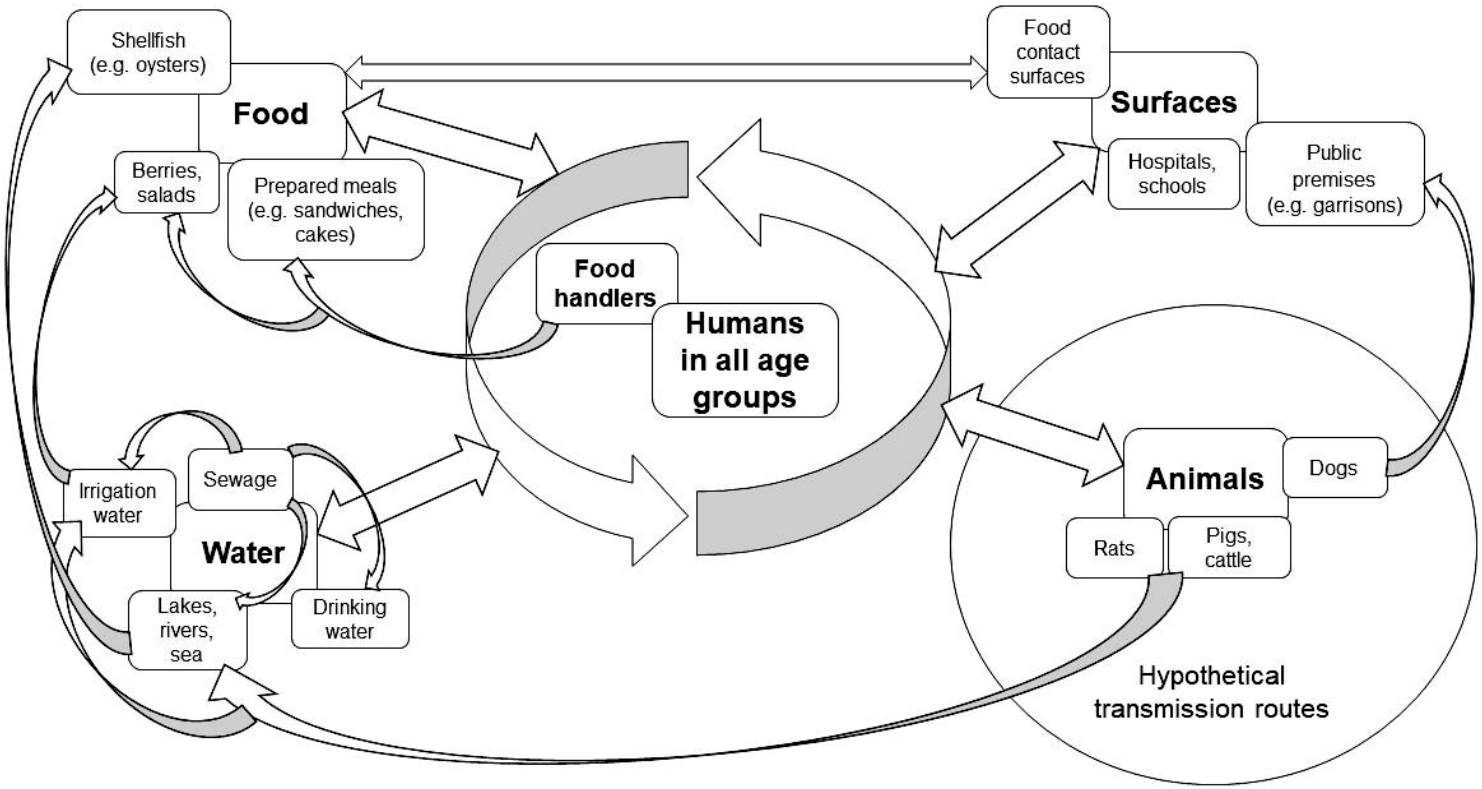

Figure 5. Known and hypothetical transmission routes for HuNoVs.

2.7 Detection of HuNoVs and prevention of norovirus infections

Despite efforts for many years (Duizer et al. 2004, Lay et al. 2010), no widely used method is available to cultivate human noroviruses in cell cultures, although some reports of successful replication in stem-cell derived enteroids and human B cells have emerged (Ettayebi et al. 2016, Jones et al. 2015). The review of Robilotti et al. (2015) considers a broad selection of different detection methods for HuNoVs and presents a variety of antigen detection methods available for clinical samples. However, the sensitivity of these methods is not very high. Antibody assays are not either suitable in clinical diagnostics. The most commonly used technique, i.e. the golden standard, to detect noroviruses in fecal, food, water, and surface samples is reverse transcription polymerase chain reaction (RT-PCR), which replicates a part of the genome of the virus particle exponentially. The large amount of replicated genome can be visualized by gel electrophoresis in older applications or by an increase in fluorescence in newer applications. In brief, the RNA genome of the virus particle must be released from the capsid and purified by RNA extraction before the initial RT-PCR. The RNA genome is transcribed to complementary DNA (cDNA) for the PCR replication. In addition to rapid detection, newer applications of real-time quantitative RT-PCR or digital RT-PCR allow quantitation of virus particles based on a standard curve or absolute number of target RNA and statistics, respectively.

There are, however, some limitations of the RT-PCR techniques. RT-PCR reveals only the presence of viral genome in the sample but provides no information on infectivity of the virus. Some pretreatments before RT-PCR have been used to remove RNA released from damaged non-infective HuNoV particles. Combined proteinase and RNase treatments or propidium monoazide (PMA) pretreatments reduced the level of inactivated virus particles but positive RT-PCR signals were not completely removed (Oristo et al. 2018, Rönnqvist et al. 2014). So far, the pretreatments have not offered a sufficient solution for this issue. The RT-PCR reaction is also sensitive to inhibitors, which both fecal and food samples may contain in high numbers. The genetic diversity of noroviruses creates also an important role for the primer choice. The primers used for general screening of human noroviruses target highly conserved regions of the viral genome, usually the polymerase region. If the new variant is, however, converted in that region, the primers may not recognize the virus and the test gives a false-negative result (Green 2013).

At the moment, there is no widely used vaccine available for human noroviruses. High diversity, rapid evolution, and short-term variant-specific immunity create a great challenge for vaccine development. However, the research with vaccines is ongoing, and in recent years some progress has been made. Several vaccine candidates have been introduced in the pre-clinical phase; some of them have even reached the clinical phase (Cortes-Penfield et al. 2017, Heinimäki et al. 2018, Leroux-Roels et al. 2018, Lucero et al. 2018, Pringle et al. 2015). No specific preventative medicine for norovirus infections exists. According to the review of de Graaf et al. (2016), some antivirals, such as nitazoxanide and ribavirin, have been successfully used with chronically infected patients to reduce the symptoms. However, in some cases these treatments were also unsuccessful. In this case, preventing the spread of the virus by careful hand hygiene

and efficient disinfectants is critical. Alcohol-based disinfectants or hand sanitizers do not destroy noroviruses, and thus, mechanical hand washing with soap is necessary (Blaney et al. 2011, Tuladhar et al. 2015, Vogel 2011). Contaminated environmental surfaces must be disinfected effectively with hypochlorite (1000-5000 ppm depending on contact time and cleaning technique) or hydrogen peroxide containing cleaners (Anonymous 2011, Barker et al. 2004, Green 2013, Tuladhar et al. 2012). The United States Environmental Protection Agency (EPA) maintains a list of registered antimicrobial products that are effective against noroviruses. The list updated in 2018 that includes only the brand names is available in internet (https://www.epa.gov/sites/production/files/2018-04/documents/list_g_ disinfectant_list_3_15_18.pdf).

2.7.1 RT-PCR detection in fecal samples

Norovirus detection in fecal samples is most commonly based on direct RNA extraction combined with RT-PCR. The fecal sample is usually diluted in a neutral buffer solution as a 10% suspension. After a short centrifugation, the supernatant is separated for RNA extraction (Nishimura et al. 2010). Fecal samples usually contain PCR inhibitors, but in human samples, especially during the acute phase of the infection, the virus content is so high that any special processing is only rarely needed to remove the inhibitors.

2.7.2 RT-PCR detection in food samples

Norovirus detection in food samples is usually more complicated than detection in fecal samples because foods contain viruses often in low numbers and release components that inhibit the RT-PCR reaction (Wilson 1997). The first RT-PCR methods for detection of noroviruses in food have been presented for shellfish and other foods in the 1990's (Atmar et al. 1993, Atmar et al. 1995, Gouvea et al. 1994). A decade later, dozens of different virus extraction methods emerged for norovirus in foods (Tables 2, 3, and 4), followed by RT-PCR detection. After many years of waiting, also a standardized method was published by ISO at first as a technical specification in 2013 and later as an ISO standard (ISO 15216-1:2017). The standard method consists of both the real-time RT-PCR method for detection of HuNoVs and different virus extraction methods for specific food matrices.

Butot et al. (2014) have grouped virus extraction methods into elution-concentration methods and direct viral RNA extraction methods. Elution-concentration methods typically entail washing of the food with a large volume (tens of milliliters) of elution buffer, which must then be concentrated to a small volume (microliters) for RNA extraction. Direct RNA extraction methods usually involve washing the food sample directly with a guanidine thiocyanate (GITC) -based lysis buffer for the RNA extraction. The methods described for extraction of HuNoV and published in 2000-2018 for foods other than shellfish along with their main features have been introduced in Tables 2, 3, and 4 according to this grouping.

Table 2 introduces the elution-concentration methods using the most common concentration for viruses with polyethylene glycol (PEG). The variation of PEG content

used in different studies is wide, 6-50%; the final concentration of 10% is, however, the most commonly used. Some study groups have used even two separate PEG concentration steps (Baert et al. 2008, Parada-Fabian et al. 2016) or combined PEG precipitation with ultracentrifugation (Boxman et al. 2007) or immunomagnetic separation (Park et al. 2008). Table 3 gathers together the elution-concentration methods that use some other concentration method such as filtration or ultracentrifugation. In all of the methods described in the two tables, the most often used washing/elution solutions have been alkaline (pH >9) and have contained Tris or glycine or a combination of these. Table 4 introduces the methods based on direct RNA extraction. In the method presented by Scherer et al. (2009), the viruses are eluted from the swabs into phosphate-buffered saline (PBS) before the direct RNA extraction.

The elution-concentration protocols often include some steps for removing the PCR inhibitors. As Tables 2 and 3 show, in PEG precipitation methods, the most commonly used step for inhibition removal has been a chloroform-butanol treatment. The treatment with a low-speed centrifugation causes sedimentation of cell debris and coagulated plant material into the bottom with the organic phase, leaving the virus in the upper aqueous phase, and the denatrured protein at the interface (Hull 2014). The fat content of the sample also likely settles to the bottom along with the organic phase. The methods using some other virus concentration protocols use also a wide variation of inhibitor removal compounds. In recent years, it has become more common to use an additional inhibitor removal step after RNA extraction with some commercial kits that remove plant contaminants such as polyphenolic compounds, polysaccharides, and humic and fulvic acids.

As seen in Tables 2, 3, and 4, the most commonly used commercial kits for RNA extraction have been the QIAamp Viral RNA Mini Kit by Qiagen and MiniMAG/EasyMAG system with NucliSens reagents by BioMerieux. Both kits use silica with guanidine thiocyanate as a chaotropic salt, as published originally by Boom et al. (1990). The QIAamp kit features silica on a membrane in a column, and the MiniMAG/EasyMAG system on the magnetic beads.

The virus levels detected using different methods reviewed in Tables 2, 3, and 4 have varied from 1 PCR units (PCRU) or genome copies (gc) to 10^7 genome copies. However, there is no consistent method to determine the viral content of a fecal suspension used for artificial contamination of food samples. Different laboratories have used their own methods for determination, and thus virus levels may not be fully comparable between laboratories.

The success of the virus extraction in food is generally controlled by using a process control virus. The commonly used process control viruses are murine norovirus (MuNoV) and mengovirus strain MC_0. Le Guyader et al. (2009) recommended use of mengovirus as a control for norovirus extraction efficiency. Hennechart-Collette et al. (2015), however, concluded, that MuNoV is better for HuNoV GII analysis and mengovirus for GI analysis.

Table 2. Summary of elution-concentration based virus extraction methods for HuNoVs in food that used PEG / NaCl (various concentrations: 6-50% / 0.3-1.5M) precipitation for virus concentration.

Food matrix	Elution	Other additives / treatments	Nucleic acid extraction	Virus load tested positive	Reference
Methods tested for naturally contaminated (NC) or both naturally contaminated and spiked samples					
deli ham roast beef salami	Neutral PBS wash	freon extract	phenol-chloroform	10^1-10^4 PCRU	Schwab et al. 2000
raspberries	Glycine / NaCl pH 9.5	Cat-Floc T chloroform-butanol		NC	Le Guyader et al. 2004a
dairy products mixed food	PBS pH 7.2 and Vertrel		TRIzol	NC	Rutjes et al. 2006
raspberry berry mix strawberry raspberry cherry tomato strawberry fruit salad	Tris-HCl / glycine pH 9.5	beef extract, pectinase, chloroform-butanol	RNeasy Mini Kit	10^4 gc NC	Stals et al. 2011a Stals et al. 2011c
frozen raspberries				NC	de Keuckelaere et al. 2015
berries	TGBE pH 9.5	pectinase, chloroform-butanol	NucliSens MiniMAG	NC	Maunula et al. 2013
berries lettuce				NC	Loutreul et al. 2014
vegetables				NC	Terio et al. 2017
frozen raspberries		pectinase, chloroform-butanol, Plant RNA Isolation Aid		NC	Müller et al. 2014
strawberries		pectinase, chloroform-butanol MobiSpin S-400 column	NucliSens EasyMAG	10^2-10^5 gc	Bartsch et al. 2016
lettuce green onion strawberry		pectinase, chloroform-butanol, KCl incubation on ice 30 min	QIAamp Viral RNA Mini Kit	NC	Marti et al. 2017

vegetables berries	Glycine pH 9.0	filter paper, filter through nitrocellulose membrane		NC	Parada-Fabian et al. 2016
Methods tested only for spiked samples					
lettuce hamburger	Glycine / saline pH 9	filter through three layers before concentration + Viraffinity	GITC / phenol-chloroform	10^3-10^5 PCRU	Leggitt and Jaykus 2000 Sair et al. 2002
berries lettuce tomatoes	Tris-HCl / glycine pH 9.5	beef extract, $MgCl_2$, pectinase, chloroform-butanol	heating 90°C or RNeasy mini kit	10^3 PCRU	Dubois et al. 2002
lettuce raspberry ham		beef extract, pectinase, chlofoform-butanol	QIAamp Viral RNA Mini Kit	10^2-10^3 PCRU	Scherer et al. 2010
watercress radish green onion		beef extract	NucliSens MiniMAG	10^5-10^7 gc	El-Senousy et al. 2013
lettuce	A) PBS and Vertrel B) glycine / NaCl pH 9.5	B) Cat-Floc T, chloroform-butanol	A) TRIzol, RNAMatrix B) RNeasyPlant Minikit	10^5 PCRU	Le Guyader et al. 2004b
green onion	Glycine / tryptose phosphate pH 9.5		TRIzol	10^0-10^4 PCRU	Guévremont et al. 2006
deli ham	Tris / glycine pH 9.5	Na_2HPO_4	Boom method	10^0-10^3 PCRU	Boxman et al. 2007
lettuce strawberry		Plant RNA Isolation Aid	QIAamp Viral RNA Mini Kit	10^1-10^3 PCRU	Cheong et al. 2009
grape strawberry raspberry	Beef extract pH 7.1	chloroform extraction		10^4 PCRU	Kim et al. 2008
lettuce ham	Beef extract 3%			10^3 and 10^6 PCRU	Park et al. 2010
ready-to-eat foods	Glycine / NaCl pH 9	Proteinase-K chloroform-butanol	Phenol/ chloroform/ iso-amylalcohol + ethanol	10^4 PCRU	Baert et al. 2008
parsley spinach salad	Buffered peptone water pH 7.2	Plant RNA Isolation Aid	NucleoSpin RNA virus kit	10^1-10^3 PCRU	Sánchez et al. 2012

lettuce	TGBE pH 9.5	pectinase, chloroform-butanol (digital RT-PCR)	NucliSens MiniMAG	10^3-10^6 gc	Coudray-Meunier et al. 2015
raspberry strawberry red fruit mix		pectinase, chloroform-butanol, OneStep PCR Inhibitor Removal Kit	NucliSens EasyMAG	10^5-10^6 gc	Fraisse et al. 2017
raspberry lettuce green onion		pectinase, chloroform-butanol	NucliSens	10^4-10^5 gc	Lowther et al. 2017

TGBE: Tris-Glycine and Beef Extract (pH 9.5, beef extract 1%)
PCRU: PCR-unit
gc: genome copy

Table 3. Summary of elution-concentration based virus extraction methods for HuNoVs in food that used a method other than PEG / NaCl precipitation for virus concentration.

Food matrix	Elution	Concentration	Other additives / treatments	Nucleic acid extraction	Virus load tested positive	Reference
Methods tested for naturally contaminated (NC) or both naturally contaminated and spiked samples						
dairy products mixed food	PBS pH 7.2 and Vertrel	ultracentrifugation		RNeasy Mini Kit	NC	Rutjes et al. 2006
lettuce raspberry ham	PBS	ultrafiltration	pectinase, filtration	QIAamp Viral RNA Mini Kit	10^2-10^3 PCRU	Scherer et al. 2010
frozen strawberries					NC	Mäde et al. 2013
strawberry	glycine / NaCl pH 7.5	filter through Whatman GD/X membrane, ultrafiltration	pectinase, Plant RNA Isolation Aid	RNeasy Viral Mini Kit	NC	Brassard et al. 2012
Methods tested only for spiked samples						
Lettuce	A) beef extract 3% pH 9.5 B) PBS and Vertrel C) glycine pH 8.5	A and B) ultracentrifugation C) ultrafiltration		A) GITC, CsCl B and C) RNeasy Plant Minikit	10^5 PCRU	Le Guyader et al. 2004b
fresh raspberries	$NaHCO_3$	ultracentrifugation	Catfloc TL, pectinase, soy protein	QIAamp Viral RNA Mini Kit	not specified	Rzezutka et al. 2005
berries vegetables	Tris / glycine pH 9.5	ultrafiltration	Beef extract, pectinase, filtration through a cell strainer, Plant RNA Isolation Aid		10^0-10^2 PCRU	Butot et al. 2007
lettuce strawberry		negatively charged filter or ultrafiltration	Plant RNA Isolation Aid		10^1-10^3 PCRU	Cheong et al. 2009

strawberries	Beef extract 3% pH 7.4	PEG/NaCl and IMS	chloroform, PBS/BSA wash 3x		10^3-10^4 PCRU	Park et al. 2008
lettuce strawberries green onion deli ham	Citrate buffer pH 4	carbohydrate-coated magnetic beads	PBS wash		10^2-10^4 PCRU	Morton et al. 2009
lettuce cheese	PBS pH 7.2 (and Vertrel for cheese)	ultrafiltration	negatively charged membrane		10^0-10^3 PCRU	Fumian et al. 2009
lettuce ham	Glycine/ NaCl pH 9.5	hollow fiber ultrafiltration (HUF)			10^3, 10^6 PCRU	Park et al. 2010
cabbage lettuce ham	PBS pH 7.4	ACP (amorphous calcium phosphate) particles	filtration through a polyester mesh		10^3-10^5 gc	Shinohara et al. 2013
lettuce	PBS pH 7.2 or glycine / NaCl pH9.5 or Tris-HCl / glycine pH 9.5	Filtration (negatively charged membrane) and ultrafiltration			10^5-10^6 gc	de Abreu Corrêa et al. 2013
celery	Tris-HCl / glycine pH 9.2	ultracentrifugation	beef extract, polyvinylpyrrolidone, pectinase		10^3-10^6 gc	Yang et al. 2017
fruit salad vegetable salad	KNT (KH_2PO_2, NaCl, Tritox X-100 1%) pH 9.2	filtration (negatively charged HA membrane)	TRI Reagent, pectinase, chloroform		10^1-10^4 PCRU	Cheng et al. 2018
strawberry raspberry green onion lettuce	Tris-HCl / NaCl pH 7	nanoalumina filter (24 mm) or cationic beads		QIAamp MinElute virus spin kit	10^2, 10^3, 10^5 PCRU	Morales-Rayas et al. 2010

penne salad deli sandwich soups composite meal	Tri Reagent	Chloroform, isopropanol	ethanol wash, acetone wash	RNeasy Mini Kit and NucliSens EasyMAG	10^3-10^6 gc	Stals et al. 2011b
tomatoes blueberry fruit salad	Glycine / NaCl pH 9.5 or distilled water	porcine gastric mucin conjugated magnetic beads	PBS wash three times for the beads	heat denaturation combined to TIANamp viral RNA extraction kit	10^1-10^2 PCRU	Pan et al. 2012
lettuce	Tris-HCl / glycine pH 9.5	IMS (protein G magnetic beads)	beef extract, 2 x PBS wash for beads	TRIzol-LS	10^0 PCRU	Lee et al. 2013
watercress radish green onion		organic flocculation (pH 3.5)	beef extract	NucliSens MiniMAG	10^5-10^7 gc	El-Senousy et al. 2013
lettuce		electrochemical biosensor, concanavalin A	beef extract	none	10^2-10^6 gc	Hong et al. 2015
milk cottage cheese		no concentration (ISO TS 15216 for shellfish)	beef extract, proteinase K	NucliSens EasyMAG	10^1-10^6 gc	Hennechart-Collette et al. 2017
potato salad noodles various foods	Tris-HCl / NaCl / Tween 0.1% pH 8.4	PANSORBIN, antibody	filter bag, sonication, alpha-amylase powder	TRIzol LS and chloroform, ethanol	10^1-10^6 gc	Saito et al. 2015

GITC: guanidine thiocyanate

Table 4. Summary of direct RNA extraction-based methods for HuNoVs in food.

Food matrix	Elution	Nucleic acid extraction	Virus load tested positive / naturally contaminated samples	Reference
deli ham roast beef salami	TRIzol wash two times	phenol-chloroform	10^3-10^5 PCRU and naturally contaminated	Schwab et al. 2000
lettuce deli turkey	glycine / NaCl / Tween 0.2% pH 8.5 two times	Boom commercial kit	10^3 PCRU	Jean et al. 2004
deli ham kebab, spareribs meat, salami ham	TRIzol wash	Boom	10^0-10^3 PCRU and naturally contaminated	Boxman et al. 2007
apple pepper sliced ham salami	cotton swab with PBS three times / surface	QIAamp Viral RNA Minikit	10^4-10^5 PCRU	Scherer et al. 2009
penne salad deli sandwich soups composite meal	Tri Reagent	RNeasy Mini Kit and NucliSens EasyMAG	10^3-10^6 gc	Stals et al. 2011b
blue cheese sauces soup butter	TRIzol suspension	chloroform, isopropanol, ethanol	naturally contaminated	Morillo et al. 2012

2.8 Animal noroviruses

After the use of electron microscope had become more popular in the 1980's, small round viruses were found also in animal stool samples, as reviewed by Scipioni et al. (2008). RT-PCR methods enabled further characterization and classification of animal noroviruses in the 1990's (Scipioni et al. 2008).

Bovine noroviruses (GIII) have been found in feces from calves and adult cows suffering from diarrhea, but mainly in fecal samples of asymptomatic cattle and sheep (Mattison et al. 2007, Oliver et al. 2003, van der Poel et al. 2000, Scipioni et al. 2008, Wolf et al. 2009). Porcine noroviruses (GII.11, GII.18, GII.19) have been found only in fecal samples of asymptomatic pigs (L'Homme et al. 2009a, 2009b, Mattison et al. 2007, van der Poel et al. 2000, Scipioni et al. 2008, Wang et al. 2005, Wolf et al. 2009).

Murine noroviruses (GV, MuNoV) have been detected for the first time in laboratory mice, and they have not caused clinical symptoms for immunocompetent animals (Hsu et al. 2006, Karst et al. 2003). However, for immunocompromised mice, MuNoVs have caused signs of encephalitis, vasculitis, pneumonia, hepatitis, and other systemic infections (Karst et al. 2003, Scipioni et al. 2008). Later, Smith et al. (2012) found MuNoVs from pet and show mice, but also in the intestinal content of wild wood mice. At the same time, Tsunesumi et al. (2012) and Tse et al. (2012b) detected MuNoV in fecal samples of the Japanese field mouse and in a few fecal samples of brown and black rats.

The feline norovirus (GIV) was first found in a lion cub with severe diarrhea, but it remained unclear whether norovirus was causing the symptoms (Martella et al. 2007). Later, feline norovirus was detected several times in young kittens with diarrhea (Di Martino et al. 2016, Pinto et al. 2012, Soma et al. 2015), but also in a rectal swab from a cat with no clinical signs (Takano et al. 2015).

Canine noroviruses (GIV, GVI, and GVII) have been detected in dogs with diarrhea, but also in asymptomatic dogs (Martella et al. 2008, Mesquita et al. 2010, Tse et al. 2012a). Diarrhea symptoms have been seen especially in young dogs and puppies with canine norovirus infection (Bodnar et al. 2017, Soma et al. 2015). Ntafis et al. (2010) even described a diarrhea outbreak in puppies. However, in the study of Moreno et al. (2017), which examined the fecal virome of adult and young dogs with acute diarrhea, canine norovirus was detected in only one sample. Di Martino et al. (2010) detected norovirus GIV antibodies in serum samples of 34 (of 211) cats and five (of 103) dogs, indicating that noroviruses circulate among domestic carnivores.

2.9 Zoonotic potential of norovirus

Traditionally, noroviruses have been strictly regarded as host species-specific. Animal noroviruses found in cattle (GIII), sheep (GIII), and mice (GIV) are genetically clearly different from HuNoVs (Mathijs et al. 2012, Oliver et al. 2003, Scipioni et al. 2008, Wolf et al. 2009). However, in the early 2000's, discussion focused on the possibility of zoonotic transmission of noroviruses (Bank-Wolf et al. 2010, Mathijs et al. 2012, Mattison

et al. 2007, Mesquita et al. 2010, van der Poel et al. 2000, Scipioni et al. 2008, Wang et al. 2005, Wilhelm et al. 2015).

In 2000, van der Poel et al. (2000) suggested a cattle reservoir for HuNoVs after finding GI clustering noroviruses in bovine samples. Wang et al. (2005) and L'Homme et al. (2009a, 2009b) then found porcine noroviruses in swine samples that were closely related to HuNoVs both genetically and antigenically. Furthermore, Mattison et al. (2007) detected GII.4-like HuNoVs in swine and cattle samples. Sisay et al. (2016) observed noroviruses closely resembling HuNoV GII.1 in swine samples. In addition, Cheetham et al. (2006) demonstrated that GII HuNoV can infect gnotobiotic pigs, causing mild diarrhea for the majority of infected pigs.

Wang et al. (2005) and Martella et al. (2009) showed recombination between porcine and canine noroviruses. Costantini et al. (2006) and Zakhour et al. (2010) detected human, porcine, and bovine noroviruses in the same oyster samples. These findings raise a concern about recombination between human and animal noroviruses followed by new recombinant strains that can infect both humans and animals.

Martella et al. (2007, 2008) and Mesquita et al. (2010) found GIV noroviruses in lion and canine samples that were fairly closely related to HuNoV GIV strains. Caddy et al. (2015) presented some evidence of HuNoV infection in dogs with an immune response for HuNoV virus like particles (VLPs) in serological samples. Peasey et al. (2004) concluded that a dog in the household increased norovirus seropositivity among children under 15 years. Even decades ago, a report suggested that a sick dog had caused a norovirus outbreak in a retirement home in the UK (Humphrey et al. 1984). Some proposals of animal norovirus infections in humans have also been tendered when Widdowson et al. (2005) found antibodies to GII bovine noroviruses and Mesquita et al. (2013) to GVI canine noroviruses in humans.

The zoonotic aspect of noroviruses in wild animals is less known. Wolf et al. (2013) detected HuNoV GI in the intestinal content of a rat trapped in a sewer system. No other zoonotic risks of HuNoVs have been described concerning wild animals (Leibler et al. 2016, Lõhmus et al. 2013).

3 AIMS OF THE STUDY

The objectives of this work were to create a simple method for detecting noroviruses in food, to perform a screening study for high-risk foods for noroviruses, and to explore the possibility of animals having a role in transmission of norovirus to food and humans.

Specific goals were as follows:

1. To compare four published norovirus extraction methods for foods that differ in their elution and concentration steps with three food matrices (lettuce, ham, and frozen raspberries), and to identify the strengths and weaknesses of the four methods (I),
2. To create and evaluate a rapid and simple norovirus extraction method for risk foods, especially frozen raspberries (I, II),
3. To test the new method for naturally contaminated berries using frozen berries connected to old suspected or confirmed norovirus outbreaks, and to screen frozen raspberries in retail for human norovirus using the new method (II),
4. To investigate the possibility of zoonotic transmission of human noroviruses in wild birds and rodents and in pet dogs (III, IV).

4 MATERIALS AND METHODS

4.1 Viruses used in the study (HuNoV, mengovirus, MuNoV)

The HuNoV strain GII.4-2006b used for inoculation of food samples (I, II) was originally from a fecal sample obtained from HUSLAB, Helsinki, Finland. The stool sample was diluted to a 10% suspension in phosphate-buffered saline (PBS) and then used as a stock solution for the inoculation dilutions. The endpoint dilution method showed a virus concentration of 2 x 10^9 PCR units (PCRUs)/ml in the stool suspension used for the trials in Study I. For the trials in Study II, a newly prepared 10% suspension of the same fecal sample, which showed virus concentration of 1.7 × 10^8 genome copies (gc)/ml of the suspension with the end-point dilution method, was used. The same norovirus strain was also used as a positive control in studies with animal fecal samples.

The original strain of mengovirus (virus strain MC_0) was received from Dr. A. Bosch, University of Barcelona, Spain. It was used as an external control (EC) to estimate the degree of RT-PCR inhibition in food samples in Study I, and in dog fecal samples in Study IV. It was also used as an internal process control to estimate the success of the virus extraction for the analysis in food samples in Study II, and in mouse and rat fecal samples in Study III. The process and the external control were used as loads of 10^5 and 10^4 PCRUs, respectively.

The original strain of murine norovirus (MuNoV, strain MNV-1) was obtained from Herbert W. Virgin of the Washington University School of Medicine, St. Louis, Missouri, USA. MuNoV served as an EC in food samples in Study II, and it was used as a load of 10^4 PCRUs.

4.2 Artificially inoculated food samples and virus inoculation (I, II)

In Study I, fresh lettuce (*Lactuca sativa var. crispa*) grown in a pot and sliced cooked ham stored in a vacuum pack (Huhtahyvat, Finland) were purchased from a local store. All raspberries used in these studies (I, II) as inoculated originated from Finland and were obtained fresh from a wholesaler of berries.

Spiking solutions were prepared from the stock solution (10% stool suspension) by diluting the stock solution in water. Spiking solutions containing virus loads of 10^6 and 10^4 PCRUs in 100 µl in Study I and virus loads from 10^4 to 10^1 gc in tenfold dilutions (in 100 µl) in Study II were used.

All food samples as 25 g portions were spiked by evenly spreading 100 µl of spiking solutions as small droplets onto the food surface. After inoculation, the samples stayed in a fume chamber until the droplets had dried, ham for 30 min, raspberries for up to 2 h, and lettuce samples for 4 h. Raspberries were frozen after the inoculation procedure, lettuce samples were stored overnight in a refrigerator, and ham samples were analyzed on the same day.

The remaining spiking solutions were treated the same way as the corresponding samples and used as positive controls for the samples.

4.3 Untreated food samples (II)

In Study II, 11 naturally contaminated berry samples related to nine suspected norovirus outbreaks in Finland and one outbreak in Sweden were used. The samples had been stored frozen at −20°C for 1-8 years before this study. Previous analysis of these samples during the outbreak investigations showed seven of these samples to be positive for HuNoV GI or GII genome or both; four samples had remained negative.

For screening the berries in Study II, 39 packages of frozen raspberries or mixed berries containing raspberries were purchased in several local grocery shops in 2010, 2014, and 2017.

4.4 Fecal samples of birds (III)

Avian fecal samples for Study III were collected during the norovirus epidemic season, at the end of March, in 2009 (50 samples), 2010 (30 samples), and 2011 (35 samples) from a dump site where the household waste from the metropolitan area of southern Finland was stored. The samples were collected on clear days aseptically from the untouched snow surface at sites where bird flocks, consisting mainly of gulls, crows, and jackdaws, awaited the new waste deposits. After collection, the fecal samples were frozen at −20°C.

4.5 Fecal samples of rodents (III)

Rat (susp. *Rattus norvegicus*) samples, 8 carcasses and 92 droppings, were collected in late winter 2012 (18 samples) and 2013 (82 samples) from three dump sites in southern Finland. The dead rats were found around the dump site and the majority of the droppings were collected near or inside rat poison bait stations. Three dead mice were also found and collected from the dump sites. All of the samples were frozen at -20°C. The fecal samples from the carcasses were collected from the dissected gut after thawing the carcasses at room temperature.

The other mouse samples used in Study III (85 samples, yellow-necked mouse *Apodemus flavicollis*) were received from the Finnish Forest Research Institute, currently the Natural Resources Institute Finland. The mice were trapped inside human dwellings in late autumn 2008 and 2009 in two localities in southern Finland. After trapping, the mice were frozen, and later the guts were dissected from thawed carcasses and frozen again at -20°C. For this study, the feces were excised from the guts and used to prepare a fecal suspension.

4.6 Fecal samples of dogs (IV)

In Study IV, 92 fecal samples were collected from pet dogs that potentially had been in contact with human noroviruses. The request for sample donations was distributed in

internet sites, via facebook, and in nearly all dogparks in the capital region of Finland. The main criterion for donating the sample was that the dog or humans in the household had suffered from symptoms typical of norovirus infection, mainly vomiting and/or diarrhea. If the dog owner was in some other way in close contact with humans with a confirmed norovirus infection, e.g. working in a hospital with an outbreak, the samples were also accepted. The fecal samples were collected outdoors in a plastic bag by the dog owners who were instructed to collect the samples only if the ground was visually clean. The samples were stored in a refrigerator and after arriving to the laboratory, they were frozen at −20°C. All samples were collected between February and April in 2009 (56 samples) and 2010 (36 samples). Background information on the dogs, their families, and symptoms of humans or dogs was collected with a questionnaire.

4.7 Virus extraction

4.7.1 Method evaluation (I)

In Study I, four virus extraction methods were evaluated to recover two levels (10^6 or 10^4 PCRUs) of HuNoV GII.4 from artificially inoculated lettuce, sliced ham, and frozen raspberries. Three and six samples were analyzed for each food with all four methods with the higher and lower virus load, respectively.

Method 1 was based on an ultrafiltration method originally described in a round robin study by Le Guyader et al. (2004b). Method 2 was a commercial NoroCheck IMS kit (Kim Laboratories, USA) based on immunomagnetic separation. Method 3 was based on an ultracentrifugation method described by Rzezutka et al. (2005). There was no proper ultracentrifuge available for this study, so the ultracentrifugation was performed at 50 000 x g for 3.3 h. The needed centrifugation time was calculated according to basic physical laws for time (t), distance (d), and velocity (v); the latter value was based on the Svedberg value for caliciviruses: 170 S (Murphy et al. 1995). Method 4 was a combination of two PEG precipitation-based methods described by Butot et al. (2007) and Dubois et al. (2002). The method was meant to resemble the planned ISO standard method, which was published as a technical specification the year after this study. The details of the methods are presented in Figure 6.

All methods had an elution step to release the viruses from the food surface, but all with different elution buffers, glycine (pH 8.5), wash buffer of the kit, 1M $NaHCO_3$ with 1% soy protein, and TGBE buffer (pH 9.5) (100 mM Tris, 50 mM glycine, 1% beef extract), in numerical order. Each method had also a different concentration step, each described above in the methods descriptions. Method 1 did not have a specific step for removal of detrimental food material or the PCR inhibitors from the sample. Method 2 included one washing step for the magnetic beads with the wash buffer of the kit. In both Methods 3 and 4, the elution buffer contained pectinase to decompose pectin, which is a constituent of berries that can easily hinder the extraction process. The protocol of Method 3 included also addition of Catfloc to the sample before concentration, and Method 4 a chloroform-butanol treatment for the concentrated sample.

Method 4 was used as a reference method for new Methods 1 and 2 in Study II.

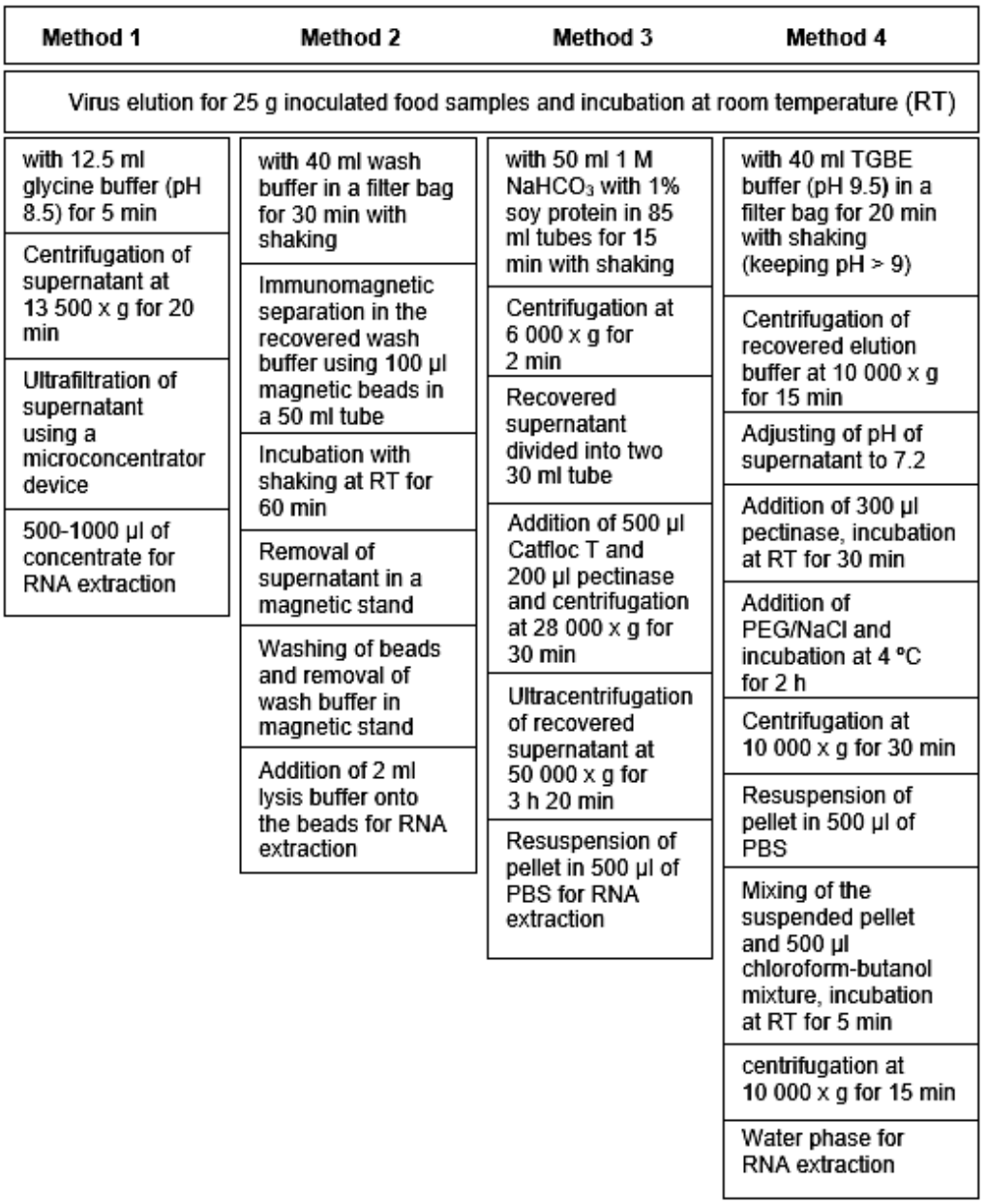

Figure 6. Flow charts of the virus extraction methods used in Study I.

4.7.2 New Methods 1 and 2 (II)

In Study II, Methods 1 and 2 were developed and evaluated using frozen raspberries artificially contaminated with HuNoV loads of 10^4, 10^3, 10^2, and 10^1 gc per 25 g sample in 9, 9, 9, and 6 replicates, respectively.
Before the final procedure of new Method 1, five elution fluids were tested for raspberry samples contaminated with 10^4 gc of HuNoV GII.4. The fluids were (1) untreated tap water (pH 7), (2) salt solution (pH 7, 1.04 mM NaHCO3, 0.59 mM K_2CO_3, 0.25 mM $CaCl_2$, 0.37 mM $MgCl_2$) prepared in the laboratory, (3) commercial sparkling mineral water (pH < 7, water, carbon dioxide, and salts corresponding to the salt solution in (2)), (4) sparkling water prepared in the laboratory (untreated tap water pH 7, carbon dioxide), and (5) TGBE buffer (pH 9.5, 100 mM Tris, 50 mM glycine, 1% beef extract).

In new Method 1, thawed berries were briefly (1–5 min) washed in a plastic bag with 27 ml of sparkling water, which was then supplemented with 3 ml of 10 x TGBE buffer (pH 11, 1 M Tris, 500 mM glycine, 5% beef extract) to prevent a marked decrease in pH during the washing step. The wash buffer was decanted to a 50 ml tube and centrifuged at 10 000 x g for 15 min. The supernatant was then combined with 10 g of guanidine thiocyanate (GITC) powder and 3 ml PEG/NaCl (50% (w/v) PEG 8000, and 1.5 M NaCl) solution in another 50 ml tube. The tube was incubated in a water bath (at about 55°C) until the GITC had completely dissolved before beginning the lysis step of RNA extraction. Completion of the whole procedure from a frozen berry sample to the nucleic acid took about 2.5 h with 1 h thawing time.

New Method 2 included three steps: thawing the berries, combining 1 ml of juice from the defrosted berries with 250 µl PEG/NaCl solution in a tube, and RNA extraction. After a 1 min manual shaking of the tube containing the juice and PEG/NaCl, 2 ml lysis buffer was inserted into the tube to start the lysis step of RNA extraction. The procedure from thawing the berries to extracted RNA took about 1.5 h, including a 1 h thawing time. The main features of new Methods 1 and 2 are presented and compared with a PEG precipitation-based method in Figure 7.

New Method 2 was also used for analysis of untreated berry samples.

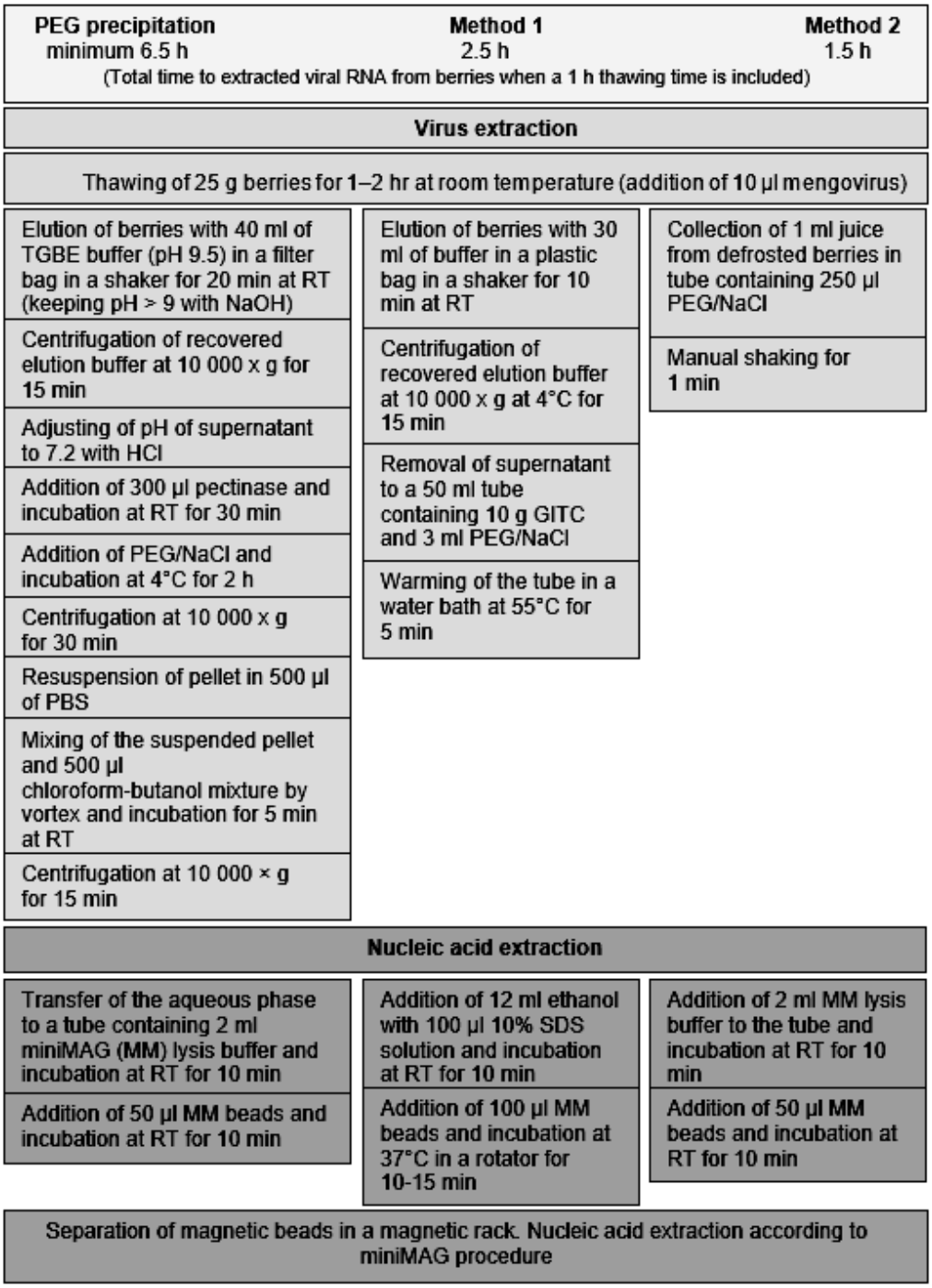

Figure 7. Flow charts of the virus and viral nucleic acid extraction methods used in Study II.

4.7.3 Fecal samples (III, IV)

All of the fecal samples used in these studies were diluted to a 10% suspension in phosphate-buffered saline (PBS). The suspension was carefully mixed and then centrifuged at 10 000 x g for 2 min before collecting the supernatant for RNA extraction.

4.8 Nucleic acid extraction (I-IV)

RNA extraction for viruses in food samples was performed using a miniMAG (MM) nucleic acid extraction system and a NucliSENS Magnetic Extraction Reagents kit primarily according to the instructions of the manufacturer. Briefly, 500-1000 µl of the sample was added to a tube containing 2 ml of lysis buffer and incubated at room temperature for 10 min, then 50 µl magnetic beads were added to the tube and after a short shaking the tube was incubated at room temperature for 10 min. The tube was centrifuged for 2 min at 1500 x g and then the supernatant was discarded. Beads were transferred to a smaller tube with wash buffer 1 and the beads were washed by the MM machine for 30 s. After washing, the supernatant was discarded. One 30 s wash with wash buffer 1 and two washes with wash buffer 2 were repeated. After the last 15 s wash with wash buffer 3, the supernatant was again discarded and 50-70 µl of wash buffer 3 was added to the tube, which was then incubated at 60°C for 5 min. After this elution step, the supernatant containing the viral RNA was collected and frozen at −20°C.

However, for Method 2 in Study I and for new Method 1 in Study II some modifications were made. In Study I, the IMS magnetic beads were washed directly with 2 ml of the MM lysis buffer, and to unify the reaction volume in all samples, 500 µl of water was added to the tube. In Study II new Method 1, 12 ml of ethanol (Etax A 96.1 vol%) and 100 µl of sodium dodecyl sulphate (SDS, solution of 10% (w/v)) were added for the lysis step in the 50 ml tube containing the supernatant of the sample, PEG/NaCl and GITC from the virus extraction. After a 10 min incubation, 100 µl of the MM magnetic beads were added to the tube, which was briefly manually shaken and incubated at 37°C in a rotator for 10–15 min. The beads were then separated from the supernatant in a magnetic rack and moved to the MM tubes with wash buffer 1. After this, the MM procedure was continued according to the manufacture's instructions.

The extraction was performed in groups that included the contaminated food samples, the spiking controls with the corresponding load of HuNoV (10^4 or 10^6 PCRU or 10^4–10^1 gc in 100 µl) and at least one blank sample (water) to rule out the possibility of cross-contamination between the samples.

For all of the fecal samples, 140 µl of supernatant of fecal suspension was used in RNA extraction that was carried out with a QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions.

For bird species identification, DNA extraction for the avian fecal samples was performed using 150 µl of the supernatant of the fecal suspension in a miniMAG system as described above.

Also with fecal samples, each extraction group included positive controls and at least one blank sample.

4.9 Detection of viral nucleic acid by real-time RT-PCR (I-IV)

For all of the real-time RT-PCR runs, a LightCycler system (Roche) and a QuantiTect Probe RT-PCR kit (Qiagen) were used. RT reaction for 25 min at 50°C started each run and was followed by preheating at 95°C for 15 min. The 50 amplification cycles consisted of a denaturation step at 95°C for 3 s, an annealing step at 54°C for 25 s, and an extension step at 72°C for 25 s. The run was completed by a final incubation at 40°C for 30 s. All viruses were detected in separate reactions.

Each real-time RT-PCR reaction used a volume of 20 µl (or 21 µl when external control was used) containing 10 µl of QuantiTect Probe RT-PCR Master Mix, 0.2 µl of QuantiTect RT mix, and 5 µl of extracted RNA from the samples. In HuNoV GII, mengovirus and MuNoV detection, 0.4 µl of RNAse-free water, 1 µM of both primers, and 0.2 µM of probe were included in the mastermix. For HuNoV GI and GIV, 0.6 µl of RNAse-free water, 0.9 µM of both primers, and 0.3 µM of probe were used. For estimations of the degree of RT-PCR inhibition in samples, 1 µl portion of EC RNA was included in the mastermix along with the sample. The EC quantitation control reactions included 5 µl of water instead of the sample.

All food samples were analyzed in duplicate real-time RT-PCR reactions for the neat sample and its tenfold dilution. In addition to the food samples, each real-time RT-PCR run included the corresponding spiking control or for untreated samples the preparation of the internal process control, and a standard. For fecal samples and internal and external controls, the sample and its tenfold dilution were analyzed in single reactions. All RT-PCR runs contained also a positive control for the target virus and 1-2 blank samples to control for cross-contamination.

For the food samples, the minimum detectable quantity of viruses in the entire sample was 10 PCRUs or 10 gc in Study I and II, respectively.

The spiked food samples were tested for the HuNoV GII used for spiking. The untreated food samples were screened for HuNoV GI and GII. The dog and mouse fecal samples were analyzed for HuNoV GI, GII, and GIV. Bird and rat samples were tested for HuNoV GI and GII.

The primers and probes used in real-time RT-PCR are described in Table 5.

Table 5. Primers and probes used in the RT-PCR reactions

Target	Primer F	Primer R	Probe	Target region	Reference
Real-time RT-PCR					
HuNoV GII	QNIF2	COG2R	QNIFS	89 bp, nucleotides 5012–5100 of Lordsdale virus, at the 5' end of ORF2	Loisy et al. 2005
HuNoV GI	QNIF3[A]	QNIF4	JJV1P	86 bp, nucleotides 5291–5376 of Norwalk virus	Da Silva et al. 2007, Jothikumar et al. 2005
HuNoV GIV	Mon4F	Mon4R	Ring4	98 bp, nucletides 718-815 of Saint Cloud virus	Trujillo et al. 2006
Mengovirus	Me110	Me209	Me147pro	100 bp, nucleotides 110–209 of the deletant mengovirus strain MC_0	Pinto et al. 2009
MuNoV	MNVfor	MNVrev	MNV	nucleotides 6520-6645 of Murine norovirus MNV	Hewitt et al. 2009
Conventional RT-PCR					
HuNoV GII	QNIF2	G2SKR		location 5401 of the 5' end of the primer in the nucleotide residue of Lordsdale virus	Kojima et al. 2002
	MJV12	RegA		nucleotides 4552–4572, region A in ORF1	Vinjé et al. 2004
HuNoV GI	JJVMF	G1SKR		Position 5283-5300 and location 5671 of the 5' end of the primer in the nucleotide residue of Norwalk virus, respectively	Hill et al. 2010 Kojima et al. 2002
Bird species	BirdR2	BirdF1		cytochrome c oxidase I (COI) -gene	Hebert et al. 2004

A: 5'-gtc ctt aga cgc cat cat cat tt-3', designed by F.S. Le Guyader, unpublished data
bp: base pair
ORF: open reading frame

4.9.1 Quantitation of viruses (I, II) and inhibition of RT-PCR reaction (I-IV)

Quantitative analysis was used for all of the inoculated food samples, and it was based on a standard curve formed by a series of tenfold dilutions of HuNoV GII.4 viral RNA of the virus strain used for inoculations. The quantification was performed by placing the results of each food sample on the standard curve. Recovery efficiency for the inoculated virus was calculated for the higher virus loads by dividing the number of PCRUs (Study I) or gc (Study II) in the sample by the number of PCRUs or gc in the spiking control and multiplying by 100. The calculations did not take into account the possible loss of template in RNA extraction.

Mengovirus (Study I) and MuNoV (Study II) were used as external controls to estimate the degree of inhibition in the real-time RT-PCR runs caused by the food samples by comparing the quantification cycle (*C*q) value of the EC RNA in the food samples against the corresponding value of the EC quantitation controls. The amplification efficiencies of mengovirus, MuNoV, and HuNoV GII.4 were adequate to estimate the effect of PCR inhibitors in HuNoV analysis.

The inhibition of RT-PCR reaction in fecal samples was tested for each animal species separately. The level of inhibition was variable between the individual samples tested, but the tenfold dilution of the samples was estimated to be sufficient to reduce the harmful effect of the inhibitors. Therefore, the EC was not used in the fecal samples, except with the dog samples. However, for the most challenging samples regarding RT-PCR inhibition (rat, mouse), an internal control (mengovirus) was used to ensure the success of the detection process.

4.10 Sequencing and phylogenetic analysis (III, IV)

From the nucleic acid extracts prepared from fecal samples of birds and dogs and testing positive for HuNoV GII with real-time RT-PCR, a conventional RT-PCR was performed with primers QNIF2 and G2SKR and a Qiagen One Step RT-PCR kit to amplify a 377 nt portion of the viral genome in the capsid region (region C). For two dog samples, also a portion of 320 nt in polymerase gene (region A) was amplified using primers MJV12 and RegA. For the few bird fecal samples testing positive for HuNoV GI in real-time RT-PCR, amplification using conventional RT-PCR with primers GOG1NF and JJVMF was performed.

The nucleic acid sequences of the RT-PCR products were determined at the DNA Sequencing Service, Institute of Biotechnology, University of Helsinki (www.biocenter.helsinki.fi/bi/dnagen/index.htm). Norovirus genotyping tool (www.rivm.nl) was used to determine the HuNoV genotypes. Clustal W software was used to align the sequences of viral genomes, and NJplot software was applied to construct the phylogenetic trees by the neighbor-joining method with Kimura's correction.

The sequences of viral genomes in bird and dog fecal samples were saved in GenBank, and their accession numbers are presented in Table 8 in the Results section.

4.10.1 Double RT-PCR for weak samples (used in III, IV, unpublished)

There were some challenges in obtaining a positive signal in conventional RT-PCR with some of the fecal samples testing positive for HuNoV with real-time RT-PCR with a high Cq value. Because of rather weak positivity of these samples, it was supposed that they would need a boost to provide a visual positive signal also in conventional RT-PCR.

To boost the samples, a double RT-PCR was used. In the first run, 30 min RT reaction at 50°C was followed with preheating for 15 min at 95°C. The 30 amplification cycles consisted of 1 min denaturation at 94°C, 1 min annealing at 50°C, and 1 min extension at 72°C. The run was completed with final extension of 10 min at 72°C. The second run was the same, but with 40 amplification cycles.

In each RT-PCR reaction, a 25 µl (first run) or 24 µl (second run) volume was used. The mastermix of one reaction in both runs included 5 µl of OneStep 5 x RT-PCR buffer, 0.4 µM of dNTP, 0.6 µM of each primer, 1 µl of Qiagen OneStep PCR mix, 0.15 of µl RNAse inhibitor, and 14.25 µl of RNAse-free water. In the first run, 3 µl of RNA of the sample was added to the mastermix. In the second run, 2 µl of the product of the first run was added to a new mastermix.

4.11 Identification of bird species (III)

After DNA extraction of the fecal samples, the conventional PCR was performed using primers BirdR2 and BirdF1 (Hebert et al. 2004) and the Qiagen HotStarTaq Plus DNA Polymerase kit (Qiagen). The run included preheating for 10 min at 94°C, five starting cycles consisting of 1 min at 94°C, 1.5 min at 45°C, and 1.5 min at 72°C, amplification of 30 cycles at 94°C for 1 min, at 51°C for 1.5 min, and at 72°C for 1.5 min, and final incubation for 15 min at 72°C. For samples that were negative or only weakly positive (hardly visible band on a gel) after this run, a double PCR modification was used, where the first run consisted of the same parameters as mentioned above, but only 20 amplification cycles. The second run included the preheating step, which was directly followed by 30 amplification cycles as mentioned above, but with the annealing temperature of 57°C. For each PCR reaction, the following volumes were used: 2.5 µl of PCR buffer (10x), 0.5 µl of dNTP (10 mM), 2.5 µl of each primer (10 µM), 0.5 µl of DNA polymerase (2.5 units), 13.5 µl of water, and 3 µl of the sample. When using the double PCR, 1 µl of the sample volume (product of the first run) was used for the second run. Bird species were identified using BLAST (U.S. National Library of Medicine, http://blast.ncbi.nlm.nih.gov) by the nucleic acid sequences determined at the DNA Sequencing Service.

5 RESULTS

5.1 Virus detection in food samples

5.1.1 Evaluation of extraction methods for HuNoV in three food matrices (I, II)

In Study I, four virus extraction methods were compared with three food matrices, including lettuce, ham, and frozen raspberries, containing two inoculation levels of HuNoV GII.4. With higher virus load (10^6 PCRU), all methods succeeded in detecting HuNoV in each sample replicate, except for Method 3 (ultracentrifugation), which failed with one raspberry sample. With the lower virus load (10^4 PCRU), all methods succeeded with lettuce samples. Methods 1 (ultrafiltration) and 3 detected HuNoV in each ham sample, but Methods 2 (IMS) and 4 (PEG precipitation) failed with one sample. Method 4 was the only one able to detect HuNoV in all raspberry samples. Based on these results, Method 4 seemed to be the most reliable of these four methods for all food matrices.

In Table 6, the results of all raspberry analyses are gathered together from Studies I and II. In Study II, two newly developed rapid viral extraction methods were evaluated for frozen raspberries spiked with HuNoV GII.4 levels of 10^4, 10^3, 10^2 and 10^1 gc per 25 g sample. A method based on PEG precipitation (PEGP) and chloroform-butanol (CB) purification that was used as Method 4 in Study I served as a reference method to scale the results of the two new methods. All three methods successfully detected HuNoV GII.4 in all berry samples with virus loads of 10^4 and 10^3 gc. New Method 2 yielded four (of nine) positive results with virus load of 10^2 gc, whereas New Method 1 and PEGP succeeded with only one sample. None of the methods was able to detect HuNoV in berry samples with the lowest inoculation level. The condition for the acceptance of each result of the HuNoV analysis was a positive signal for mengovirus as a process control in the neat sample or its tenfold dilution.

5.1.2 Effect of food matrix and extraction method on recovery efficiency (I, II)

The viral recovery efficiencies were evaluated more closely in Studies I and II only for virus load of 10^4 PCRU or gc because the recovery efficiencies were generally lower with higher contamination levels and unstable with lower virus levels. In Study I, the highest virus recovery efficiencies for different methods were obtained when analyzing lettuce and ham samples. In lettuce analysis, the recoveries were 20%, 3%, 19%, and 19% for Methods 1-4, respectively. In ham analysis, the corresponding figures were 5%, 5%, 70%, and 47%. Generally, the virus yields were higher for tenfold dilutions of the viral RNA than for neat RNA templates. For Method 3, the recovery efficiencies were highly variable (maximum standard deviation SD 0.38), while the results of Method 4 were rather consistent across all food matrices (SD 0.03-0.19).

In raspberry analysis, the virus recovery efficiencies were generally lower than for other food matrices in Study I, with Method 4 yielding the highest recovery (28%, SD 0.19) (Table 6). In Study II, new Method 2 produced the highest virus yields, with an overall mean of 32% (SD 0.16). New Method 1 produced slightly lower yields, with a mean recovery of 9% (SD 0.05), but the recovery efficiencies showed less variation than for new Method 2. PEGP had an efficiency of 24% (SD 0.02), which is at the same level as for new Methods 1 and 2, and the PEG precipitation method in Study I (Method 4).

5.1.3 Selection of washing fluid for eluting viruses from food surfaces (II)

In Study II, five fluids (untreated tap water, salt solution, sparkling water, sparkling mineral water, and TGBE buffer) were tested for their ability to wash viruses from raspberries spiked with 10^4 gc HuNoV GII.4. Virus detection in all six replicate samples succeeded only when washing the samples with the sparkling water. Water, salt solution, and TGBE wash seemed to be insufficient with their several negative results, with mengovirus as the process control showing success of the overall process in all samples. The variation of RT-PCR inhibitor levels between the elution fluids was not significant, less than one log difference (ΔCq < 3 cycles). Based on these results, sparkling water without minerals was selected to elute viruses from the berries in new Method 1.

5.1.4 RT-PCR inhibition caused by different food matrices (I, II)

Mengovirus and MuNoV were used as external controls to estimate the degree of RT-PCR inhibition after the virus extraction methods in Studies I and II, respectively. The inhibitors released from lettuce samples were efficiently removed by all methods, excluding Method I, in Study I. Ham did not release a significant amount of inhibitors, and the inhibitors were rather efficiently removed by all four methods in Study I. None of the methods in either Study I or Study II completely removed inhibitors released from the raspberries (Table 6), which turned out to be the most challenging food matrix concerning the inhibitors. However, a tenfold dilution of RNA extracted from the food samples clearly reduced the level of RT-PCR inhibition for each method. The most efficient steps for removing the RT-PCR inhibition caused by raspberries were in new Methods 1 and 2 (using PEG/NaCl as a supplement) in Study II and PEGP (using CB treatment) in both studies when only minor RT-PCR inhibition was seen for each method in tenfold dilution of samples.

5.1.5 Screening of retail berries for HuNoVs (II)

In study II, 11 berry samples obtained during earlier HuNoV outbreak investigations and stored as frozen were now analyzed for HuNoV GI and GII using new Method 2. Seven of these samples had been positive for HuNoV genome during the investigations. Now, in two of the berry samples stored for 5 and 8 years (outbreaks in 2006 and 2009, respectively) HuNoV GI genome was still detected. All frozen berry samples from 39 packages purchased from local stores in 2010, 2014, and 2017 tested negative for HuNoVs GI and GII.

Table 6. Detection of HuNoV GII.4 by real-time RT-PCR in frozen raspberries inoculated with virus at various concentrations by five virus extraction methods.

	HuNoV positive samples[a]						
Virus load (PCRU / gc)	UF[b]	IMS[c]	UC[d]	PEGP[e]	M1[f]	M2[g]	PEGP[e]
10^6	3 / 3	3 / 3	2 / 3	3 / 3	-	-	-
10^4	3 / 6	2 / 6	5 / 6	6 / 6	9 / 9	9 / 9	2 / 2
10^3	-[i]	-	-	-	9 / 9	9 / 9	2 / 2
10^2	-	-	-	-	1 / 9	4 / 9	1 / 9
10^1	-	-	-	-	-	0 / 6	0 / 2
10^4 av. recovery %	1%	4%	4%	28%	9%	32%	24%
10^4 RT-PCR inhibition (ΔCq)[h]	>4/>4	>4/1.5-2	1-4/0.5-2	1-4/<0.5	3-4/<0.5-1	2/<0.5	3-4/<0.5

a: Number of positive replicates / number of spiked samples tested (negative results were accepted only when positive signal was obtained by mengovirus used as a process control in Study II)
b: Ultrafiltration-based method, Method 1 in Study I
c: Immunomagnetic separation-based method, Method 2 in Study I
d: Ultracentrifugation-based method, Method 3 in Study I
e: PEG precipitation-based method, Method 4 in Study I and PEGP in Study II
f: Elution - direct RNA extraction-based method, Method 1 in Study II
g: Direct RNA extraction-based method, Method 2 in Study II
h: Difference between the mengovirus (Study I) or MuNoV (Study II) (10^4 PCRU in 1 µl of mengovirus or MuNoV RNA was added) Cq value in neat sample/1:10 dilution of the sample and the mengovirus or MuNoV Cq value in H_2O (range between the replicates)
i: - not done

5.2 Virus detection in animal fecal samples

5.2.1 HuNoVs in bird samples, bird species carrying HuNoVs (III)

Altogether 115 avian fecal samples were analyzed for HuNoV GI and GII; of these 31 (27%) tested positive for HuNoVs, 25 for GII, and six for GI (Table 7). HuNoVs were found in all three years that samples were collected. Positive results were confirmed by another real-time RT-PCR run for 12 samples and also by sequencing for six samples. For 13 weakly positive samples, the positive signals could not be repeated. Based on the genome sequence, four samples contained genotype GII.4 and two samples genotype GII.3 HuNoVs (Table 8). All other GII and GI isolates remained without a specific genotype.

Bird species carrying HuNoV-positive feces could be identified in 10 fecal samples according to the nucleic acid sequence of their cytochrome c oxidase I (COI) genes (Table 8). Eight samples originated from gulls (*Larus hyperboreus*, *Larus fuscus*, and *Larus argentatus*) and two from crows (*Corvus corone cornix*).

GenBank accession numbers of HuNoV sequences from bird feces are presented in Table 8.

5.2.2 HuNoVs in rodent samples (III)

Two (one from 2012 and one from 2013) of the 100 fecal samples from rats showed weak positive signals for HuNoV GII with real-time RT-PCR (Table 7), but the positive results could not be repeated in these samples. No positive signals for HuNoVs were detected in 88 fecal samples from mice.

5.2.3 HuNoVs in dog samples (IV)

Ninety-two canine fecal samples were analyzed for HuNoV GI, GII, and GIV. Four (4.3%) of the samples tested positive for GII HuNoVs (Table 7). All positive samples were donated from dogs living in households with more than two persons showing symptoms typical of norovirus infection, i.e. vomiting, fever, and diarrhea lasting 1–3 days. Two of the HuNoV-positive dogs also had symptoms; both suffered from nausea and loss of appetite for one day, and one of these dogs also vomited once. All of the HuNoV-positive samples were collected within three days of the disappearance of the owner's symptoms. All households where the dogs gave HuNoV-positive fecal samples contained small children. Therefore, symptomatic children in the household increased the probability of appearance of HuNoV in the feces of the dog significantly ($P = 0.023$).

Based on the genome sequence of the HuNoV in positive samples, the norovirus genotyping tool showed that three fecal samples contained genotype GII.4 and one GII.12 noroviruses. One of the HuNoV strains detected in dog feces was identical to the strain found in the fecal sample of the owner. In this study, other fecal samples from humans were not available.

GenBank accession numbers of HuNoV sequences from dog feces are presented in Table 8.

5.2.4 Phylogenetic analysis of detected HuNoV strains (III, IV)

A phylogenetic tree (Figure 8) was formed using the 223 nt overlapping nucleic acid sequences from capsid region C of the six HuNoV strains found in bird feces (B), four HuNoV GII strains found in dog feces (D), and 27 GII strains in GenBank. Phylogenetic analysis was performed with the neighbor-joining method using Clustal W software. The manual comparison of the genome sequences of bird isolates B7-09 and B10-09 showed 98.7% and 99.1% identity at the nucleic acid level, respectively, and 100% identity at the amino acid level, to the GII.4-2006b variant (EF126965), which was also the result of the norovirus genotyping tool. The genome sequence of bird isolate B3-09 was almost identical to the GII.4-2006a variant (EF126964; 99.6% and 100% identical in nucleic and amino acids) and that of bird isolate B5-11 was highly similar to the GII.4-2009 variant (JN595867; 98.7% and 100% identical in nucleic and amino acids). The bird isolates B1-10 and B9-11 showed 93.6% and 96.9% identity at the nucleic acid level and 97.3% and 95.9% at the amino acid level to GII.3 strains U02030 and EU187437, respectively. The isolate D3-09 (JF746892) in dog feces was 99.6% and 98.6% identical in nucleic and amino acids to the GII.4-2006b variant (EF12966), consistent with the result of the norovirus genotyping tool. The two isolates in dog feces, collected in 2009 and 2010, namely D1C-09 (JF746890) and D261-10 (JF746891), were identical to each other and closely related to the GII.4- 2008 variant (AB445395, Apeldoorn, 98.7% and 100% identical in nucleic and amino acids), but the genotyping tool did not give a specific variant recognition. The gene sequence of the dog isolate D262-10 (JF746893) clustered with GII.12-recombinant strain HM635106 (98.2% and 100% identical in nucleic and amino acids).

Interestingly, the GII.4-2006b genome sequences of isolates B7-09 and B10-09 found in bird feces in 2009 were also closely related to one of the isolates found in dog feces in 2009 (D3-09, JF746892, 98.2% and 98.6% identical in nucleic acids, respectively, and 98.6% in amino acids). The GII.4-2009 genome sequence of B5-11 in bird feces collected in 2011 was 97.3% identical in nucleic acids and 100% in amino acids to two isolates from dog feces (D1C-09, JF746890 and D261-10, JF746891) collected in 2009 and 2010.

Table 7. Fecal samples from animals.

Animal	Total number	HuNoV-positive samples			
		GII	GI	total	% positive
Bird	115	25	6	31	27
Rat	100	2	0	2	2
Mouse	88	0	0	0	0
Dog	92	4	0	4	4

Table 8. HuNoV-positive avian (B) and canine (D) samples.

Positive samples [a]	HuNoV GI / GII	Bird species / Dog breed	Confirmed[b] (yes/no/ Cq value)	HuNoV genotype capsid region C	Accession no. in GenBank
B1-09	GII	Gull (*Larus fuscus*)	Y/ 38.68	-	-
B2-09	GII	Gull (*Larus argentatus*)	Y/ 39.02	-	-
B3-09	GII		Y/ 39.48	GII.4 2006a	MF444290
B4-09	GII		Y/ 39.20	-	-
B5-09	GII	Crow (*Corvus corone cornix*)	Y/ 38.11	-	-
B6-09	GII		Y/ 40.44	-	-
B7-09	GII		Y/ 37.18	GII.4 2006b	MF444291
B8-09	GII		Y/ 39.62	-	-
B9-09	GII	Gull (*Larus hyperboreus*)	Y/ 39.34	-	-
B10-09	GII		Y/ 38.64	GII.4 2006b	MF444292
B11-09	GII		Y/ 39.85	-	-
B1-10	GII	Gull (*Larus fuscus*)	Y/ 39.63	GII.3	-[c]
B2-10	GII	Gull (*Larus hyperboreus*)	N/ 38.49	-	-
B3-10	GII	Gull (*Larus hyperboreus*)	N/ 39.53	-	-
B4-10	GII	Gull (*Larus fuscus*)	N/ 38.34	-	-
B5-10	GI		Y/ 39.18	-	-
B6-10	GI		N/ 39.48	-	-
B7-10	GI	Gull (*Larus hyperboreus*)	Y/ 41.49	-	-
B8-10	GI		N/ 40.63	-	-
B9-10	GI		N/ 40.24	-	-
B1-11	GII		N/ 40.42	-	-
B2-11	GII		Y/ 38.91	-	-
B3-11	GII		N/ 39.47	-	-
B4-11	GII		N/ 38.33	-	-
B5-11	GII		Y/ 38.16	GII.4 2009	MF444293
B6-11	GII		N/ 38.64	-	-
B7-11	GII		N/ 38.36	-	-
B8-11	GII		N/ 40.72	-	-
B9-11	GII	Crow (*Corvus corone cornix*)	Y/ 35.87	GII.3	MF444294
B10-11	GII		Y/ 38.29	-	-
B11-11	GI		N/ 39.71	-	-
D1C-09	GII	Irish setter	Y/ 23.06	GII.4	JF746890
D3-09	GII	Dachshund	Y/ 29.96	GII.4 2006b	JF746892
D261-10	GII	Poodle	Y/ 37.23	GII.4	JF746891
D262-10	GII	Poodle	Y/ 33.67	GII.12	JF746893

a: running number of the sample- year of collection

b: positive real-time RT-PCR result on at least two occasions

c: nucleotide sequence too short for GenBank accession

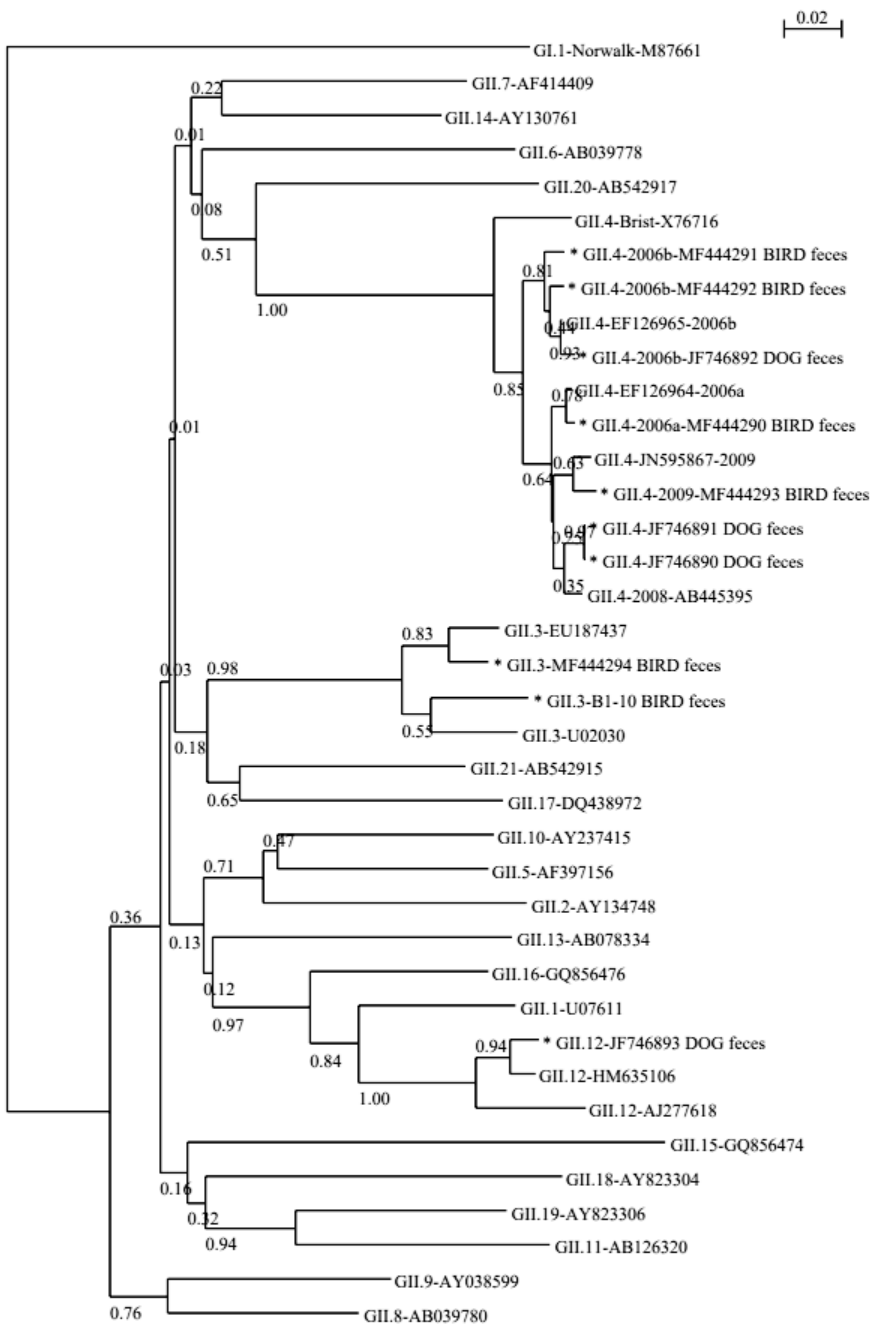

Figure 8. A phylogenetic tree formed of the 223-nt sequence alignments of of a partial capsid gene from HuNoV GII strains found in six avian and four canine fecal samples (indicated “BIRD feces” and “DOG feces”) and 27 HuNoV GII genotypes in GenBank. Norwalk GI.1 was added as an outgroup. Bootstrap values (1000 replicates) are shown on the branches, and the scale of the tree is indicated by the bar.

6 DISCUSSION

6.1 Food-related norovirus infections

Between the years 2005 and 2016, the Finnish Food Safety Authority Evira, currently Finnish Food Authority verified 173 food-borne outbreaks caused by HuNoV in Finland (Table 1, Niskanen et al. 2006, 2007, 2010a, 2010b, 2011, Pihlajasaari et al. 2012, 2016). According to the reports, only rarely (in 35 outbreaks) was suspected food analyzed for HuNoVs in these outbreaks. In these 173 outbreaks, only in seven cases was HuNoV detected in food samples and in one case in surface samples taken from the kitchen. Unfortunately, published data concerning the reported outbreaks in 2011-2013 (and personal communication concerning the years 2014-2016) did not contain detailed information of how many food samples were analyzed for HuNoV during the outbreaks, and thus, the numbers may not be all-inclusive for the years 2011-2016.

Based on this information, in the majority of food borne-norovirus outbreaks the causative agent is identified only in fecal samples of patients and food handlers and only rarely in food samples. The same tendency was observed in the 53 reported outbreaks in other countries in 2000-2010 reviewed by Mathijs et al. (2012), where HuNoV was detected in only 17 food samples.

However, HuNoV has quite often been detected in food samples not related to outbreaks. Baert et al. (2011) gathered the results of screening tests of fresh produce in Belgium, France, and Canada. Of 850 tested samples, 216 were positive for HuNoV using real-time RT-PCR. Only 52 of these gave a positive result when using conventional RT-PCR, and only from 18 samples was a specific sequence obtained. In their conclusions, Baert et al. (2011) stated that the actual risk of HuNoV infection via the food testing positive for the HuNoV genome using real-time RT-PCR is unknown.

So paradoxically, HuNoV has been detected in numerous food samples not related to outbreaks, but only rarely in food samples associated with outbreaks.

6.2 Challenges in detecting HuNoVs in foods

6.2.1 Technical challenges, low contamination levels, and uneven division of the virus

Evaluation of the methods tested in this study revealed some technical challenges. In today's world, all results must be obtained rapidly. Methods that contain long centrifugation times or numerous processing steps do not fulfill this requirement.

One challenge in detecting viruses in foods is that the viruses are often present in food samples only in low levels. Thus, the virus extraction method should be sufficiently

sensitive to capture all of the virus particles in the sample and effective in removing the inhibitors that harm the RT-PCR reaction. Capturing the virus particles includes two main goals: to release all virus particles from the food surface and to not lose them during further processing.

It remains somewhat unclear how the virus particles are attached to the food surface. In the majority of the extraction methods reviewed in Section 2.7.2, the food is washed with an alkaline buffer (pH > 9). Many research groups have concluded that a high pH level is most effective for different food matrices (Park et al. 2010, Cheong et al. 2009, Butot et al. 2007, le Guyader et al. 2004a, Dubois et al. 2002). In new Method 1 in Study II, the substantial drop in pH of pure sparkling water during the washing step harmed the sensitivity of virus extraction (unpublished data). Therefore, the sparkling water was supplemented with a small volume of alkaline TGBE buffer (pH 11) after a short wash to prevent the drop in pH.

Beef extract (BE) as a carrier protein in the elution buffer has been shown to improve release of the viruses from the filter membrane in virus analyses of water (Schwab et al. 1995, 1996) and has therefore been widely used also in food analysis (Dubois et al. 2002, Butot et al. 2007, Kim et al. 2008, Cheong et al. 2009, Park et al. 2010, Scherer et al 2010, Stals et al. 2011a, 2011c). Developing experiments for the new methods in Study II showed that the commonly used 1% level of BE increased the inhibition of the RT-PCR reaction (unpublished data). To minimize the inhibitory effects of BE, it was decided to reduce the level to 0.5% in new Method 1 in Study II.

In Study I, gas bubbles were seen on the surface of raspberries when using carbonate-based buffers for washing the berries (unpublished data). Gas bubbles were also formed on the surface of the food when testing sonication in the washing step according to Jones et al. (2009), who showed that the use of sonication increased virus recoveries when combined with ultrafiltration. Fraisse et al. (2011) also used vacuum-induced bubbles when washing lettuce in inactivation tests for HuNoV surrogates. Based on these findings, the idea arose of a washing fluid with bubbles that might release virus particles from the food surface more efficiently. When testing different washing fluids in Study II, the two bubbling fluids eluted HuNoV GII.4 particles from the berry surfaces more efficiently than the other liquids tested. However, the bubbles seemed also to release more food debris from the food surface relative to the non-bubbling fluids (unpublished data). The release of the food debris was also seen when using an inadequate volume of elution buffer during the washing step, resulting in the need for mechanical handling of the food to avoid incomplete washing (Method 1, Study I). According to experiments of this study, the released food debris needs to be removed from the sample to ensure successful further processing. Usually, centrifugation or washing steps are used for this purpose. In Method 1 in Study I, the lack of a step for removing the remaining solid food before ultrafiltration caused problems in the process because the membrane in the device became clogged.

Most of the virus extraction methods presented in Tables 2 and 3 discard the washing buffer after certain processing steps such as centrifugation (used e.g. to remove solid food debris from the samples and during a concentration step where the volume of the

sample is reduced for nucleic acid extraction) or washing of beads (all methods in Study I, PEGP in Study II). All steps that discard components from the sample entail, however, the risk of virus particle loss. For this reason, the discarding steps were kept to a minimum in the new methods in Study II.

The highest mean virus recovery efficiencies in this study, about 30%, were similar or slightly higher than those published by other research groups (Butot et al. 2007, Kim et al. 2008, Rutjes et al. 2006, Rzezutka et al. 2005, Fraisse et al. 2017, Bartsch et al. 2016, de Abreu Corrêa et al. 2013, El-Senousy et al. 2013, Stals et al. 2011a, 2011b). All methods tested in Study II were sufficiently sensitive to detect viruses at an inoculation level of 1000 virus particles. At the level of 100 virus particles, only a minor part of the samples was positive. None of the methods detected viruses at a level of 10 virus particles. The infectious dose of HuNoV may be as low as 10-100 virus particles, and thus, the sensitivity of the methods may be inadequate for virus detection. The methods, thus, require further development.

The contamination route is an important factor for how the virus spreads on the food surface. Contaminated irrigation water (Wei and Kniel 2010, Lowther et al. 2008, Kokkinos et al. 2012, Maunula et al. 2013) probably spreads the virus widely to the food batch. A contaminated surface or hand (Kokkinos et al. 2012, Boxman et al. 2009) as a point-source, however, likely spreads the virus unevenly as small patches on the food, as also concluded by Baert et al. (2011). A part of the food portion may contain high numbers of viruses, whereas other parts may be totally free of the viruses. This fact poses a great challenge for virus detection; if the sample is taken from the part not containing any viruses, the result will be negative despite the food infecting people. This may also hinder epidemiological research when only some of the people consuming the same unevenly contaminated food show symptoms.

6.2.2 RT-PCR inhibition

The processing of food samples in virus extraction procedures often releases such components as organic compounds, fats, sugars, and Ca^{2+}, which are known to inhibit the RT-PCR reaction (Wilson 1997). To avoid false-negative results caused by strong RT-PCR inhibition, many virus extraction protocols include a step for removing PCR inhibitors. The PEG precipitation methods presented in Table 2 include the two most commonly used steps for inhibitor removal. The first step concerned the berry and fruit analysis; pectinase was added to the elution buffer at the beginning of the protocol. The second step was to use a chloroform–butanol (CB) treatment at the end of the protocol. The CB treatment efficiently removes PCR inhibitors, especially from raspberry samples, which was shown in Studies I and II. However, when developing the two methods presented in Study II, new Method 2 was also tested with CB treatment. When analyzing naturally contaminated raspberries, new Method 2 produced a weak positive HuNoV signal only when performed without this treatment (data with CB treatment not shown), perhaps partly because of loss of virus particles when separating the water phase from the organic phase during CB treatment. Instead, PEG, which is usually used to concentrate the viruses, was used as a supplement because it was found to reduce the effect of PCR inhibitors released from raspberries.

In Study I, a bead-based method with washing steps (Method 2) and the use of Catfloc, a cationic polyelectrolyte (Method 3), proved to be the most efficient ways to remove inhibitors from lettuce and ham. Catfloc has successfully been used also by other research groups (Le Guyader et al., 2004a, 2004b, Rzezutka et al., 2005). Several research groups have also efficiently removed inhibitors by using Plant RNA Isolation Aid (Cheong et al. 2009, Sánchez et al. 2012, Brassard et al. 2012, Müller et al. 2014) after the RNA extraction.

A tenfold dilution of viral RNA from the samples clearly reduced the inhibition of RT-PCR in Studies I and II. However, the analysis of both neat and diluted RNA was found to be necessary for reliable detection of HuNoV in food samples.

Coudray-Meunier et al. (2015) concluded that the use of digital RT-PCR was not influenced by PCR inhibitors as much as was the real-time RT-PCR when analyzing lettuce samples, because the impact of inhibitors was reduced in individual micro-reactions in digital RT-PCR. However, Fraisse et al. (2017) got higher virus recoveries when using an additional OneStep PCR inhibitor removal kit along with RNA extraction kit and digital RT-PCR relative to the results without the additional step when analyzing berry samples.

6.3 Animals transmitting HuNoVs

HuNoV genome was detected in fecal samples of three animal species, showing that the viral genome can pass through the gastrointestinal tract of dogs, birds, and rats. Consequently, these animals may potentially carry HuNoV genomes from one place to another. During the norovirus epidemic seasons of 2009-2011 the HuNoV variants GII.4-2006b, GII.4-2006a, GII.4-2008, and genotype GII.3 circulated commonly in the human community in Finland (Hulkko et al. 2010, 2011, Jaakola et al. 2012) and were also present in canine and avian fecal samples analyzed in Studies III and IV. All ten partial HuNoV genome sequences detected in canine and avian fecal samples were identical or nearly identical to previously published genome sequences from human samples and differed from canine, porcine, or murine noroviruses, and avian caliciviruses (highest similarity around 70%). The possibility of animal transmission of the HuNoV genome is also supported by previous studies, which observed HuNoVs in the feces of cattle, pigs, and a rat and antibodies against HuNoVs in dogs (Caddy et al. 2015, Mattison et al. 2007, Wolf et al. 2013).

6.3.1 Wild animals

Wild birds are strictly controlled in primary food production and food premises because the food industry and legislation regard them as noxious animals that may spread a large variety of diseases (Tsiodras et al. 2008). Birds have been demonstrated to spread salmonella or campylobacter in food and water via their feces (Butterfield et al. 1983, Tsiodras et al. 2008), but the findings of this study show that contamination with HuNoV could also be possible. According to results of Study III, large bird flocks could pose a

risk for norovirus infection to humans, as they may contaminate uncovered food, bathing water, and beaches as well as raw water sources for household water.

All of the avian samples in Study III were collected in late winter from a dump site, where the virus will readily remain infectious in discarded textiles, tissues, and diapers if infected persons have contaminated them with feces or vomit. The virus load in the trash may be very high, especially during the epidemic seasons. HuNoVs infect frequently children of diaper-wearing age (Pringle et al. 2015, Robilotti et al. 2015); especially HuNoV genotypes GII.4 and GII.3 infected children globally in 2004–2012 (67% and 16% respectively) (Hoa Tran et al. 2013). These types of HuNoV genomes were also found in avian samples in Study III, suggesting a child source. In HuNoV-positive bird samples, bird species that could be identified were gulls and crows, which are common transmitters of many human pathogens (Tsiodras et al. 2008). These species were also seen every time when collecting samples from the dump site.

As only the viral genome was detected in the avian samples, the results of Study III do not confirm whether HuNoVs can replicate in the gastrointestinal tract of birds. The number of viral genomes, according to the Cq values of the positive samples, was low in the bird samples, even when compared with positive dog samples. The *FUT2* gene, which codes the HBGA carbohydrates proposed to be needed for HuNoV binding to host cells, has been found in many animal species including mice, rats, and dogs, according to Yamamoto et al. (2014). However, no bird species mentioned in the study (flycatcher, zebra finch, turkey, duck, chicken) have the gene. The lack of the gene and the low number of viral genomes in the fecal samples do not support HuNoV replication in birds.

HuNoV has once been detected in the intestinal content of a rat living in a sewer system (Wolf et al. 2013). The waste-water in the sewer systems of urban areas along with dump sites and waste bins are possible sources of HuNoVs for rats and other rodents. However, only two of the rat samples in Study III were weakly positive for the HuNoV genome, although the majority of the samples were collected in early spring time from the same dump site as the positive bird samples. One explanation for these results could be the low quality of the samples; the rat feces were rather old and dry. The *Apodemus* mice, the source of the HuNoV-negative mouse samples in Study III, were trapped in the autumn, which is not a typical season for HuNoV infections. The normal diet of these mice usually consists of human food or food waste, so they may not even come into contact with HuNoV-contaminated waste, such as diapers or sewage, in human settlement areas. To the best of our knowledge, no research group has detected HuNoVs in mouse samples to date (Leibler et al. 2016, Lõhmus et al. 2013).

6.3.2 Dogs

According to Chomel and Sun (2011), up to 45% of pet dogs sleep in their owner's beds in Europe and the USA. The dogs, however, carry several zoonotic agents, such as pasteurellas, MRSAs, and giardias, and therefore, may serve as an infection source for their owner. The pathogens may also be transmitted when owners kiss or are licked by their pets. If the dog is carrying HuNoV, the virus could also use these transmission routes. Infected persons, who often have uncontrolled vomiting and diarrhea during the

acute phase of infection, secrete high numbers of viruses and easily contaminate surfaces both inside and outside the household. On contaminated surfaces, noroviruses remain infectious for several days if they are not destroyed using strong disinfectants (Weber et al. 2010). Dogs are exposed to HuNoV when they lick or lie on contaminated surfaces or are petted by the contaminated hand of their owner. Dogs may therefore assist in spreading the virus among family members via their fur and muzzle mechanically or via feces. The idea of dogs spreading the HuNoV infection is also supported by a study by Peasey et al. (2004), where a dog living in a household increased the risk for norovirus seropositivity among children under 15 years of age.

It remains unclear whether HuNoVs are able to replicate in the gastrointestinal tract of a dog. Caddy et al. (2015) have shown that HuNoV VLPs bind to canine gastrointestinal samples and that 13% of tested dogs had HuNoV-specific antibodies. HuNoV antibodies in dogs have also been detected by Di Martino et al. (2017). In the HuNoV-positive dog samples in Study IV, the viral load was moderate to low compared with human samples taken during acute infections. All positive samples were collected within three days of the recovery of the owner, indicating that the time-frame for the detection of HuNoVs in dog feces seems to be shorter than in human samples, where HuNoV can be detected even several weeks after the symptoms have disappeared. Nevertheless, two HuNoV-positive dogs showed mild symptoms, supporting the notion that replication is possible, assuming that the symptoms were caused by HuNoV. If virus replication is possible in dogs, genetic recombination between human and canine noroviruses may occur, as Wang et al. (2005) proposed between porcine and human noroviruses. Recombination has been shown already in HuNoV GII.4 variants and GII.12 viruses (Motomura et al. 2010, Vega and Vinje 2011). At the same time, the health-care of dogs is approaching that of humans, with dogs living longer and having access to immunomodulative therapies. Old and immunodeficient dogs may be more susceptible to virus infections, potentiating recombination events and point mutations and probably also allowing the virus to adjust to the new host.

7 CONCLUSIONS

The method evaluation for detecting HuNoV in foods showed that the method based on alkaline elution and PEG precipitation was the most reliable detection method for all food matrices tested. The recovery efficiency of the method with frozen raspberries was on average of 28%. Interestingly, the recovery efficiency of this method stayed at the same level (24%) in Study II, which was performed a few years later, suggesting that the method is stable and reliable. The method was a modification of the ISO standard 15216-1:2017. The standard method was not published during the laboratory work, and therefore, the exact protocol could not be used as a reference method in method comparisons.

The new rapid method presented in Study II, based on direct RNA extraction, yielded the same recovery levels as the PEG precipitation method (32%) in raspberry analyses. The method was slightly more sensitive than the PEG precipitation method, yelding four positive samples (of nine) with virus load of 10^2 gc, whereas PEG precipitation succeeded with only one sample. The method detected HuNoV also in naturally contaminated berry samples. The new method thus is a relevant alternative to the standard method, especially in outbreak situations, when the results of virus analysis are needed quickly.

In the experiments of Study III, the washing fluid used in virus elution from the food surface had an important role in virus recovery. Sparkling water was found to elute viruses most efficiently from frozen raspberries. However, if the frozen food matrix itself releases liquid when thawed, a separate elution fluid may not even be required. Fresh foods and frozen non-juicy food matrices, however, need an elution fluid to wash the contaminated surfaces, and sparkling water was shown to be an efficient choice.

General virus analysis includes the RT-PCR step, which is vulnerable to PCR inhibitors released from the majority of food matrices. PEG as a supplement was shown to decrease the effect of PCR inhibitors. Also a treatment with chloroform-butanol mixture and dilution of samples were efficient ways to remove inhibitors, but the loss of viruses during these processes decreased the sensitivity of the detection method. Therefore, further improvements are needed to increase the sensitivity of genome detection without the need to use steps that lose virus particles. Additional purification steps for extracted nucleic acid and/or digital RT-PCR, which is less influenced by inhibitors, may offer solutions to overcome the current deficiencies in virus analysis.

In the screening study of this work, all retail frozen berries tested negative for HuNoVs GI and GII. The Finnish epidemic data (no reported HuNoV outbreaks related to frozen raspberries in Finland during the sampling periods) support this result. The numerous outbreaks caused by frozen raspberries in the 2000's have also increased the knowledge of the need for hygienic conditions throughout the raspberry supply chain

and most likely led to less contaminations. On the other hand, the conclusions based on these results are limited due to the low number of samples analyzed.

The HuNoV genome was found in the feces of 31 birds, two rats, and four pet dogs. The genotypes found in six bird samples and all of the HuNoV-positive dog samples were the same genotypes present in human samples at the time of sampling. Two of the HuNoV-positive dogs suffered also from mild symptoms. These results strongly support the transmission of HuNoVs via wild birds, pet dogs, and possibly also rats. At the very least, the mechanical transport of the virus genome between the different locations through these animals is possible. The suggestion of animals as a contamination source of HuNoV for food, water, and surfaces is reasonable. The replication capability of HuNoV in these host animals should be investigated. Future studies could, for instance, search for viral antibodies in blood or whole virus particles in feces.

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