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Noroviruses on surfaces: Detection, transfer and inactivation

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ACADEMIC DISSERTATION

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ABSTRACT

Human noroviruses (HuNoVs) are a leading cause of foodborne gastroenteritis worldwide, and are easily spread among humans via the faecal-oral route. They can also be spread via environmental surfaces and ready-to-eat (RTE) foods that are often prepared by infected food handlers. A low infective dose (10–100 virus particles), a high viral load in the vomit and faeces of infected persons (up to 10^9 genomic copies/g), a lack of long-term immunity following previous infection, and a high environmental stability of the viruses all enhance the spreading of HuNoV in the population.

The aim of the research in this thesis is to investigate the prevalence of HuNoVs on environmental surfaces and to observe and measure virus transfer from surface to surface during manual food preparation. A further aim is to investigate the transfer characteristics and prevalence of HuNoV in the environment. A feasible method for the detection of HuNoV on environmental surfaces is optimized and used in the laboratory, and also in field studies, both in a resort and in food preparation premises. Finally, a measure for controlling the disinfection using ultraviolet light irradiation (UV), is tested as a means to inactivate the HuNoVs from environmental surfaces.

The prevalence and transfer of HuNoV and its surrogate murine norovirus (MuNoV) was investigated by swabbing, after which the viruses were eluted from the swabs and their genomes were extracted by a commercial kit. HuNoV and MuNoV genomes were detected using reverse transcription quantitative polymerase chain reaction (RT-QPCR) method using specific primers and probes. The effects of UV on the viruses were investigated both by viability assays (MuNoV) and by RT-QPCR (MuNoV and HuNoV). An enzymatic pre-PCR treatment was conducted before RT-QPCR detection to distinguish infective viruses from non-infective viruses.

Out of the four swab materials tested for swabbing HuNoV on surfaces, the recovery rates of the viruses were highest for swabs made of microfiber and polyester, which had been moistened with glycine buffer solution, pH 9.5. A semi-direct lysis phase, in which the elution and lysis of the viruses happen simultaneously, was found suitable for inclusion in the swabbing protocol. When stored at 4°C, HuNoV persisted well in swabs, whereas at 22°C, viruses persisted better on swabs moistened by phosphate buffered saline (PBS, pH 7.2) than by glycine buffer.

HuNoV and MuNoV transferred easily from the hands to the gloves when gloving. In the sandwich preparation simulation, HuNoV and MuNoV were repeatedly transferred to the first recipient surface (left hand, cucumber, and knife) during the preparation process. It was estimated that when the hands of the food handler contain a 3 $\log_{10}$ or more load of infective HuNoV before gloving, a transfer of at least one infective virus particle from the contaminated hands to the prepared sandwich was likely to occur during the preparation process. Virus-contaminated gloves were estimated to spread HuNoV to the food servings more efficiently than a single contaminated cucumber can during handling.

In the field studies, HuNoV was repeatedly detected on environmental surfaces. In a resort, where a gastroenteritis outbreak had taken place and HuNoV was the suspected cause, virus ribonucleic acid (RNA) was detected on samples taken from several environmental surfaces, including door handles both in common and kitchen areas. HuNoV was detected in 10/36 swabs (27.8%), and further genotyped as a new HuNoV variant, GII.4 Sydney_2012. In the field study that was conducted in

three food-processing companies with no recently reported outbreaks of gastroenteritis, 5/90 swabs (5.6%) in 2010 and 7/82 swabs (8.5%) in 2012 were found to be HuNoV GII-positive. The three positives were detected in a production line and nine positives were obtained from the food handlers' break room and restroom areas. Of the 168 swab samples collected during the one-year HuNoV prevalence study conducted at the same ready-to-eat food manufacturing companies, four (2.4%) were HuNoV GII positive. Positive swabs were collected from fridge door handles and coffee machines in the break rooms of the food industry employees.

UV was observed as a potential inactivation method for HuNoV: a loss of infectivity and a 4 $\log_{10}$ reduction of HuNoV surrogate MuNoV were observed when the virus-containing surfaces were exposed to UV dose of 60 mJ/cm^2 or higher. In the RT-QPCR assay, a distinct difference in RNA levels of HuNoV and MuNoV were not observed until UV doses of 300–1800 mJ/cm^2, when the RNA levels of untreated samples remained at a level of 2–2.3 $\log_{10}$ polymerase chain reaction units (pcr-u) while the RNA levels of enzyme-treated samples declined to less than 1 $\log_{10}$ pcr-u. Methods based on genome detection seemed to overestimate HuNoV persistence even when samples were pre-treated before the genome detection was conducted.

In order to get more detailed picture of the epidemiology and transmission routes of HuNoVs, sensitive and feasible methods for their detection, such as the one presented in this thesis, are essential. As seen in the studies included in the thesis, HuNoV is transmitted very easily from human hands to food and environmental surfaces. Proper hand hygiene combined with effective measures to inactivate HuNoV from surfaces, such as UV, is needed to manage the transmission of this very persistent enteric virus. Due to the rapid onset of HuNoV outbreaks, the origin of the virus is often hard to define, but it would be necessary to be able to target the control measures efficiently. Therefore, adequate and regular monitoring of the environment for virus contamination in potential fountainheads of gastroenteritis outbreaks, such as in hospitals and restaurants serving RTE foods, could prevent or restrict HuNoV outbreaks.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referenced in the text by their Roman numerals:

I Rönnqvist, M., Rättö, M., Tuominen, P., Salo, S., Maunula, L., Swabs as a tool for monitoring the presence of norovirus on environmental surfaces in the food industry, Journal of Food Protection, 2013, 76:8, 1421–8

II Rönnqvist, M., Aho, E., Mikkelä, A., Ranta, J., Tuominen, P., Rättö, M., Boxman, I., Maunula, L. Norovirus transmission between hands, gloves, utensils, and fresh produce during simulated food handling. Applied and Environmental Microbiology, 2014, 80:17, 5403-5410

III Polkowska, A., Rönnqvist, M., Lepistö, O., Roivainen, M., Maunula, L., Huusko, S., Toikkanen, S., Rimhanen-Finne, R., Outbreak of norovirus GII.4 Sydney variant after a wedding reception at a course centre in Finland, 2012. Epidemiology and Infection, 2014, 142:9, 1877–83

IV Rönnqvist, M., Mikkelä, A., Tuominen, P., Salo, S., Maunula L., Ultraviolet light inactivation of Murine Norovirus in comparison with human Norovirus GII: PCR may overestimate the persistence of Norovirus even when combined with pre-PCR treatment. Food and Environmental Virology, 2014, 6:1, 48–57

The author's contributions to the articles included in this thesis:

I Participated in designing, performed the laboratory work and statistical analysis. Main responsibility for interpretation of the results and writing the paper.

II Main responsibility for designing. Performed the laboratory work together with the second author. Performed the statistical analysis together with the third author. Main responsibility for interpretation of the results and writing the manuscript.

III Participated in the laboratory work, interpretation of the results and writing the paper.

IV Main responsibility in designing. Performed the laboratory work. Main responsibility of interpretation of the results and writing the paper.

ABBREVIATIONS

PBS	phosphate buffered saline
C_q	quantification cycle
pcr-u	polymerase chain reaction unit(s)
RT-QPCR	reverse transcription quantitative polymerase chain reaction
RT-PCR	reverse transcription polymerase chain reaction
RT-LAMP	real-time reverse transcription loop-mediated isothermal amplification
NASBA	nucleic acid sequence based amplification
RT-HDA	reverse transcriptase helicase dependent isothermal amplification
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT	room temperature
UV	ultraviolet light irradiation
HuNoV	human norovirus
MuNoV	murine norovirus
FCV	feline calicivirus
MS2	bacteriophage MS2
CrFK	feline kidney cells
RAW 264.7	mouse leukaemic monocyte macrophage cell line
pfu	plaque forming unit
DMEM	dulbecco's modified eagle medium
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
$TCID_{50}$	50% tissue culture infective dose
RTE	ready-to-eat
GI, GII	genogroup I, genogroup II
SD	standard deviation
CI	credible interval
ProMED-mail	the Program for Monitoring Emerging Diseases
ORF1, ORF2	open reading frame 1, open reading frame 2
MPa	megapascal
EA	ethoxylated alcohols
QA	quaternary ammoniums

1 INTRODUCTION

Human noroviruses (HuNoVs) are the most common cause of non-bacterial gastroenteritis outbreaks, and sporadic infectious intestinal disease in the community (1-3). The virus was first found in 1972 in a faecal sample by using immunoelectron microscopy (4). It remained little studied until 1990, when its genome was cloned and characterized (5). Nowadays, noroviruses are cathegorized as belonging to the *Caliciviridae* family, and are further classified into five genogroups, three of which (GI, GII, and GIV) are found in humans (6). The genogroups are further subdivided into genetic clusters called genotypes, of which GII.4 is currently the most common genotype that causes infections in humans (7).

HuNoVs are transmitted from person to person either directly or indirectly via water, food, or contaminated surfaces (7, 8). The route of infection is the ingestion of HuNoV contaminated faeces or vomit. HuNoVs are shed in large numbers, up to 10^9 genomic copies/g, in the faeces of infected individuals (9), whereas the infective dose is believed to be as low as 10–100 virus particles (10). The virus can be shed in the faeces of a normal infected person for over a month and for several months in the faeces of a person whose immune status has been compromised (11, 12). Symptomatic HuNoV infections, present most commonly with vomiting and diarrhoea, are usually relatively mild and self-limiting in otherwise healthy adults. Nevertheless, they may be more severe among young children, the elderly and the immunocompromised persons (12, 13), causing human suffering and also economical losses due to hospitalization and absentees from work.

The role of food handlers in the transmission of HuNoV is well documented (14-16). It is not surprising that the most common food items that cause foodborne outbreaks are those that are prepared by hand and eaten without further cooking, such as sandwiches and salads (14). Modern lifestyle has lead to an increase in the consumption of these food products and thus their significance as virus vehicles. HuNoVs have been shown to transfer easily between foods, food preparation surfaces, hands, and the environment in laboratory studies (17), but more information on the transfer of HuNoVs in actual food preparing situations is needed in order to prevent the facilitation of viral transfer caused by infected food handlers in HuNoV outbreaks.

In addition to being highly contagious, HuNoV is very resistant towards environmental hazards (18, 19). HuNoV is extremely difficult to culture in cell lines, thus most of the persistence studies have been performed using surrogate viruses, or the reduction of the virus has been measured by changes in ribonucleic acid (RNA) levels (17). Results obtained in those studies indicate that HuNoV persists on different surfaces for days at room temperature (RT) (17) and for hours on human hands (20). HuNoV seems to tolerate well relatively high or low temperatures, variable pH levels, and several disinfectants. Promising methods to inactivate HuNoV, such as ultraviolet light irradiation (UV), still need more investigation before they can be recommended for disinfection of the environment, including environmental surfaces (21).

This doctoral thesis was conducted to supplement the existing knowledge on the methods used for detecting HuNoV on surfaces, the incidence of HuNoV on and transfer between environmental and food surfaces, in addition to the means to inactivate HuNoVs from these surfaces. A treatment that would ensure that the polymerase chain reaction (PCR) is only copying genomes of infectious viruses in the sample was also validated in this research.

2. REVIEW OF THE LITERATURE

2.1 The history of HuNoVs

HuNoV is the causative agent for gastroenteritis disease of non-bacterial origin and it remained unknown in spite of intensive volunteer studies until 1972. In 1972, Kapikian and his co-workers isolated and identified it from a faecal sample by immune electron microscopy with covalescent sera in patients of the same outbreak (4). The virus was named Norwalk agent, or later Norwalk virus, after the location in which the virus had caused an outbreak of gastroenteritis among children at an Elementary School in 1968. At that time, HuNoVs were only detected by using specialized equipment and methods, such as immune electron microscopy. HuNoVs were described as being small, round, structured viruses according to what was seen under the microscope (Fig 1).

It was not until 1990, that the genome of Norwalk virus was cloned and characterized (5). Sequencing of the genome showed that these viruses have a genomic organization consistent with viruses belonging to the order *Picornavirales*, and the family *Caliciviridae*. The characterization of Norwalk virus, later shortened to norovirus, led to more intensive studying and better an understanding of the molecular virology, epidemiology and worldwide incidence of these viruses.

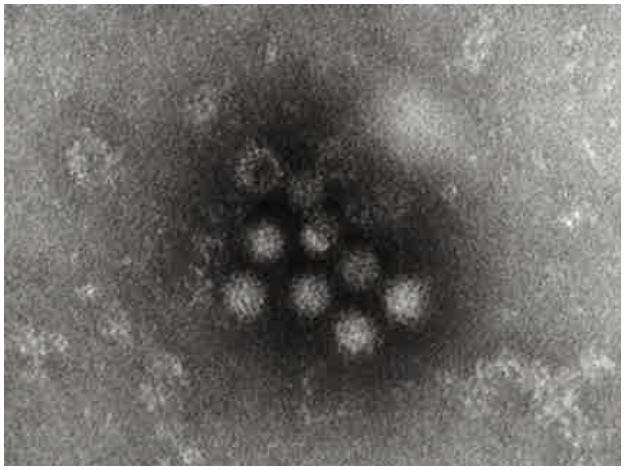

Fig 1. Transmission electron micrograph of HuNoV (photo was taken by Anssi Mörttinen, Department of Virology, University of Helsinki). *Caliciviruses*, including HuNoV, have obtained their name from the Latin word for chalice, calix, according to the cup-shaped structures on the surface of intact capsids.

2.2 Structure and genome of HuNoVs

HuNoV virions are non-enveloped and small: they consist of a capsid and a nucleic acid that measure about 27 to 30 nm in diameter (6, 22-25). The nucleocapsid, as seen by using an electron microscopy, is rounded and exhibits an icosahedral symmetry. The capsid is mainly constructed of a single protein, which is organized into 180 similar protein units according to icosahedral T-3-symmetry.

The virus genome consists of a linear molecule of single-strand RNA, as seen in Fig 2 (5). It has a positive polarity and it can serve as messenger RNA and infect target cells directly. Its complete genome contains approximately 7.5 kb and consists of 45%–56% of cytosine + guanine (C + G). The genome 3’ end has a poly A tail, which is characteristic of the messenger RNA of eukaryotic cells, whereas the 5‘ end presents the VPg protein, needed in virus infectivity and initial translation. The

genome includes three open reading frames: the first ORF encodes a 194-kDa polyprotein, the second a structural 60-kDa capsid protein (VP1), and the third the 23-kDa basic protein (VP2), which interacts with the genome RNA when the virion formation occurs (6, 22-25). VP1 protein is believed to participate in the identification of the target cells receptors. It consists of a capsid which has P1, P2 and S domains. Amino acid changes in the P2 domain, which contains antigen-presenting sites and carbohydrate-receptor binding regions, may facilitate infection of target cells (26).

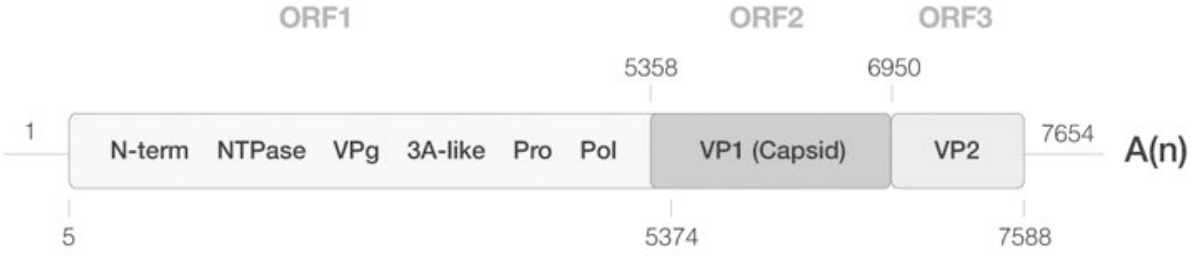

Fig 2. HuNoV genome structure from 5' end to 3' end (27). N-term: amino-terminus, NTPase: nucleotide triphosphatase, VPg: viral protein genome-linked, 3A-like: region of the nonstructural polyprotein, Pro: protease, Pol: polymerase, VP1: capsid protein, VP2: basic protein, A(n): poly A tail

2.3 Classification and taxonomy of noroviruses

The *Caliciviridae* family, in addition to Genus Norovirus, include the following genera: Lagoviruses, Neboviruses, Sapoviruses, and Vesiviruses. Additional genera have been proposed to be included in the family: Recovirus, Valovirus and a number of other unclassified caliciviruses including the chicken calicivirus (28). At the moment, the classification of noroviruses is based on ORF2 phylogenetic analyses of 164 norovirus sequences into five genogroups, from GI to GV (2, 22-24). Three of the genogroups, GI, GII, and GIV, are found in humans. The genogroups are reported to subdivide further into genetic clusters called genotypes and numerous subgroups (29, 30). In 2014, GI was known to have consisted of nine, GII of 22 and GIV of at least two genotypes. The prototype strain, Norwalk virus, has been classified as a GI.1.

Recent studies indicate that there are differencies in the evolution potential between the HuNoV genogroups (31). It seems that some HuNoVs belonging to the II genogroup, especially GII.4, are more prone to evolve than some HuNoVs belonging to GI, which may have only limited potential for evolution. The differences between HuNoV strains may be one of the major reasons why during recent decades, HuNoV GII.4 has been the most common genotype to have caused viral gastrointestinal infections in humans worldwide (7). New epidemic variants of GII.4 have emerged every two to three years during this time. Rapid evolution, population immunity, and antigenic variation of the genotype have led to emergence of these new virus variants, associated with several global epidemics (32). Lindesmith and co-workers suggested in 2008 that the carbohydrate ligand binding domain in the HuNoV GII.4 capsid is subject to heavy immune selection and probably evolves by antigenic drift (33). This evolution of the HuNoV GII surface structures would facilitate escape from the protective herd immunity and facilitate virus persistence in the community. These evolved GII.4 variants have been commonly named. The pandemic variants of GII.4 include US 1995/96, Farmington Hills_2002, Hunter_2004, Yerseke_2006a, Den Haag_2006b, New Orleans_2009, and most recently, Sydney_2012. Other GII.4 variants that have not exhibited pandemic characteristics

have been described, including Henry_2001, Japan_2001, Asia_2003, Osaka_2007, and Apeldoorn_2008 (32).

2.3.1 HuNoV GII.4 Sydney variant

In 2012, several countries, including the United Kingdom (UK), the Netherlands, and Japan, announced an increase in HuNoV outbreaks (34). The first molecular data, reported in Australia in March 2012, indicated that the increase was associated with the emergence of a new variant of GII.4, named Sydney_2012. This variant has been shown to have a common ancestor with two previously dominant GII.4 variants, Apeldoorn_2007 and New Orleans_2009. In the winter of 2012/2013 November-March, the Sydney_2012 was the most frequently detected variant during HuNoV outbreaks all over the world, except in the Netherlands (35). The peak of these outbreaks was reached approximately two months earlier, in November 2012, compared to previous HuNoV seasons.

2.4 HuNoV surrogates

Since the first detection of HuNoV, several attempts have been made to cultivate it in the laboratory (36, 37). Duizer and co-workers tested several cell lines, able to support growth of other enteric viruses, to cultivate HuNoVs (37). Although HuNoVs could be detected in some of the cell lines for several passages, no cytopathic effect caused by the viruses could be detected in these cells. Later, adult human duodenal tissues were infected successfully by HuNoVs but when foetal ileum tissue was infected, only limited virus replication was detected (38). Most recently, HuNoVs were cultivated in a three dimensional cell culture model consisting of Int-407 and Caco-2 cells (39). Despite detecting changes in the cells, no significant increase in the viral titer of HuNoV was observed.

Since HuNoVs cannot feasibly be cultured *in vitro*, most studies on the persistence and inactivation of HuNoVs cannot directly examine virus survival under different conditions, except in volunteer studies. Indirect data on the infectivity of these viruses can be collected by measuring the decreasing levels of viral RNA during challenge studies, or by using surrogate viruses instead of HuNoVs in infectivity studies. Surrogate viruses are viruses that are related to the pathogens they have been chosen to represent. The selection of a surrogate for HuNoV has been based on the ability of the surrogate to be propagated in culture, and its genetic, physical, or chemical relatedness to the HuNoV pathogen.

Bacteriophage MS2 (MS2), Feline calicivirus (FCV), Murine norovirus (MuNoV) and more recently Tulane virus have been used as surrogates for HuNoV (40). MS2 has been used to indicate faecal contamination, and possibly HuNoV contamination, in the environment. The advantage of MS2 is that it can be cultured in *Echerichia coli* cells. Its suitability to be used as a surrogate is, however, questionable due to its distant relatedness to HuNoVs (41). FCV is a respiratory virus that belongs to the genus *Vesivirus*. It can be easily cultured in feline kidney cells (CrFK) and it has been widely used as a surrogate for HuNoV (42). MuNoV-1, norovirus of genogroup V and the first norovirus to be grown in a cell culture, is more closely related to HuNoVs than FCV (43). The virus has been shown to replicate in both macrophages and dendritic cells *in vitro* (44), of which the RAW 264.7 cell line is the most commonly used. It is transmitted from mouse to mouse via the faecal-oral route, but it does not cause gastroenteritis symptoms in healthy mice but rather a wasting syndrome in immune deficient individuals. Tulane virus was recently discovered in a rhesus monkey and it represents a

new genus in the family of *Caliciviridae*, Recovirus (45). Although readily cultivable *in vitro*, it has not been shown to cause gastroenteritis in monkeys like the HuNoVs in humans.

2.5 HuNoV infection

HuNoVs infect people of all ages (46). The disease occurs all year round, but the outbreaks caused by HuNoV tend to peak in cold weather, which in the Northern hemisphere is from November to March (22). Therefore, HuNoV disease has been historically called 'winter vomiting disease' and the name is still widely used. Clinical HuNoV infection has an incubation period of between 10 to 51 h, typically 24–48 h (46). HuNoV gastroenteritis patients often present with nausea, vomiting, abdominal pain, and watery diarrhoea. Other symptoms include anorexia, malaise, fever, and occasionaly, bloody diarrhoea. Recently, GII.4 HuNoVs have been observed to result in more severe symptoms of gastroenteritis and a higher frequency of painful symptoms compared to infection caused by other HuNoVs (11, 47). The disease can lead to hospitalization due to dehydration especially when the patients are very young, very old, or immunocompromised. A correlation between HuNoV gastroenteritis outbreaks and deaths in the elderly population has been observed (13, 48). Asymptomatic infections are also common: they have been estimated to occur in approximately one third of infected persons (49). Clinical symptoms usually pass within two to three days, although immunocompromised persons can suffer from them for several more days.

Transmission of HuNoVs occurs from person to person mainly through the faecal-oral route: a person ingests infective virus particles, which were previously shed in the faeces by infected persons (22). Virus shedding can also occur during vomiting and lead to the transmission of the pathogen through air droplets (50). Transmission of HuNoVs also occurs either directly or indirectly via contaminated water, food, or environmental surfaces. The low infective dose of HuNoVs is approximately 10 to 100 virus particles and this, facilitates the transmission of the virus (10). HuNoVs are shed in large numbers, up to 10^9 genomic copies per g, in faeces of infected individuals, especially one to three days after the onset of the illness (9, 51, 52). These numbers suggest that each gram of faeces during peak shedding from an infected person might contain approximately 5 billion infectious doses of HuNoV (21). Shedding can continue for up to eight weeks in previously healthy persons and for more than several months in patients who are immunocompromised (9). Even longer shedding time has been observed for the prototype strain, Norwalk virus GI.1 (11). Phillips and co-workers (2010) reported that the highest risk for getting HuNoV infection occurs upon contact with a person with HuNoV gastroenteritis symptoms (53). It is common that small children in a family fall ill first and then transmit the disease to other family members.

Immunity in volunteers after HuNoV infection has been studied to investigate the development and duration of immunity. These studies have suggested that patients develop a short-term immunity after infection, but that infections can occur by encountering with other strains or with the same strain sometime later in life (54). IgG antibodies against HuNoV GII.4 are commonly found in children (31). In Finland the antibody prevalence was measured at 91.2% in children older than 5 years (55). Genetic susceptibility to HuNoV infections is related to the expression of histo-blood group antigen carbohydrates on the mucosal surface of the intestinal epithelial cells (23). Host genetic susceptibility and histo-blood group binding patterns appear to be HuNoV strain-specific. For instance, individuals who carry a gene encoding a functional alpha-1,2-fucosyltransferase (secretors), needed to express histo-blood group antigens, are more susceptible than average to get the HuNoV GI.1 infection (33).

Individuals with defects in the alpha-1,2-fucosyltransferase gene (non-secretors) do not express the appropriate histo-blood antigens and are thus resistant to the HuNoV GI.1 infection.

2.5.1 HuNoV vaccine

Studies of the epidemiology of HuNoVs increasingly show that the incidence and severity of the disease caused by these viruses is such, that immunization against the agent would be highly beneficial (56). Unfortunately, since HuNoVs are very species-specific, there are no robust small animal models of human infection and disease, which makes the development of a vaccine difficult. The development of HuNoV like particles (VLPs), which are morphologically and antigenically similar to native HuNoVs but empty of RNA, has offered a promising route toward effective vaccine candidates designed to protect against multiple circulating HuNoV strains (57, 58). Vaccine development is mostly concentrated to be efficient against HuNoV GII.4 and GI.1 strains, but discussion has been going on whether they should protect also against other HuNoV strains, such as GII.3 (31).

In Finland, an injectable rotavirus-HuNoV combination vaccine was developed to prevent infection or gastroenteritis induced by these viruses (59). The vaccine has been shown to produce antibody responses that exceed six months towards HuNoV GII.4 in mice (60). Recently, in the Unites States, an intramuscular bivalent HuNoV vaccine, which was designed to protect against GI.1 and GII.4 strains of HuNoV, provide a protection against the symptoms of HuNoV infection in a small volunteer study (61). Both of these rotavirus-HuNoV and HuNoV GI.1/GII.4 vaccines were planned to be studied in clinical trials in 2014.

2.6 HuNoV epidemiology and outbreaks

HuNoV has been estimated to cause approximately 21 million cases of acute gastroenteritis each year in the United States alone (1). The incidence of HuNoV-associated gastroenteritis has been calculated to be 4.5 cases per 100 person-years in England (3). It has also been assumed to be similar in other countries, although it has not been intensively studied (1). Due to the often mild course of the disease people rarely seek medical aid for treating the disease and thus, only a fraction of HuNoV cases are reported to the official registers around the world. Outbreaks of HuNoV gastroenteritis are reported more extensively, although the reports are sometimes not commonly available. The investigation of HuNoV outbreaks is also difficult, as secondary infections among the population are common. It has been estimated that HuNoV is responsible for approximately 50% (range: 36%–59%) of all reported gastroenteritis outbreaks in the United States and in Europe (2). Periodic increases in HuNoV outbreaks tend to occur in association with the emergence of new GII.4 strains that evade population immunity (32). The economic impact of HuNoV infections, including the costs of hospital visits, is likely to be substantial, amounting to billions of euros (1, 62).

The program for Monitoring Emerging Diseases (ProMED-mail) is a worldwide outbreak reporting system, which has been used to report HuNoV outbreaks with extensive coverage (63). In the United States, outbreaks are also reported to a national surveillance system called ‘CaliciNet’. From the inception of CaliciNet in March 2009 through to May 2010, 552 outbreaks had been uploaded to this national database (64). In Europe, ‘Food-Borne Viruses in Europe Network’ has collected reports of 7 636 HuNoV outbreaks and sporadic cases in Denmark, Finland, France, Germany, England and Wales, Hungary, Ireland, Italy, the Netherlands, Norway, Sweden, Slovenia, and Spain in 2001–2006 (65). During this time, HuNoV activity was the most pronounced in the epidemic seasons 2002–2003 and

2004–2005. The main HuNoV strain responsible for the outbreaks during these two seasons was a new variant of GII.4 (66). At the same time, GI strains of HuNoV were mostly associated with foodborne outbreaks, especially those that had originated from bivalve molluscs (67).

In Finland, a PCR method for the detection of HuNoVs was developed in the Department of Virology, University of Helsinki at the end of 90's, which boosted the diagnostics of outbreak investigations of HuNoV (68). The recently mounted method allowed further characterisation HuNoV strains, as well as more accurate study of the epidemiological issues that are related to the HuNoV outbreaks (69). Cases of HuNoV infection have been registered by the National Institute for Health and Welfare since 1998 (70). The number of reported cases have increased considerably in the most recent decade, being at the moment around 1 500 to 3 000 cases per year (Fig 3).

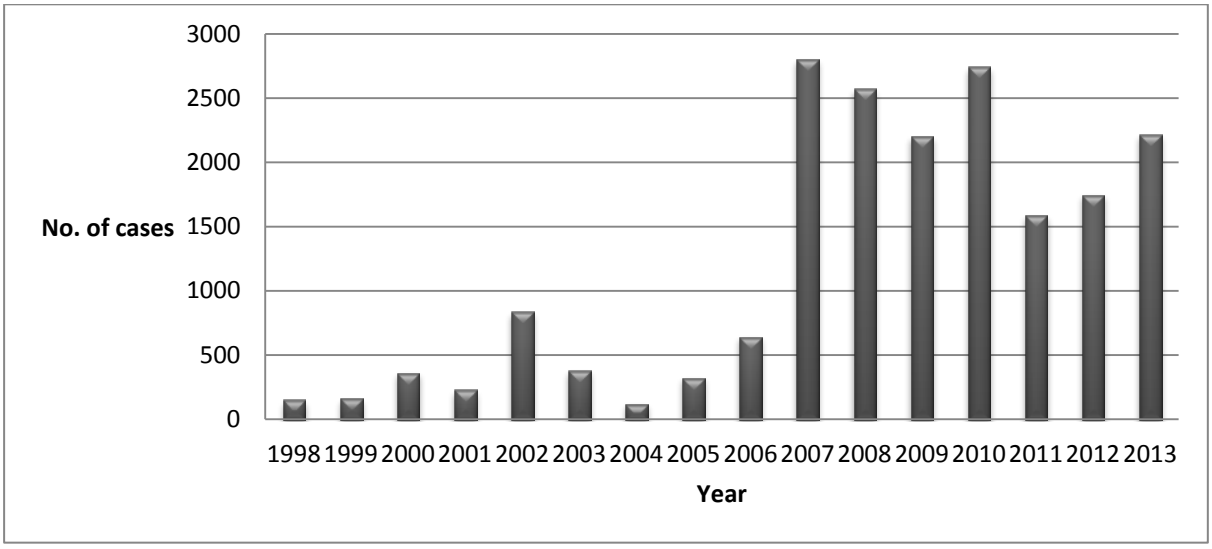

Fig 3. Registered cases of HuNoV infection in Finland from 1998 to 2013.

Outbreaks of HuNoV gastroenteritis commonly occur in closed settings, such as hospitals, hotels, cruise ships, and day-care centres (71). According to data collected by Matthews and his co-workers (2012), which consisted of 902 HuNoV outbreak descriptions reported in articles published from 1993 to 2011, the majority of the outbreaks occurred in healthcare facilities and foodservice settings (7). In over 50% of the outbreaks the transmission route was reported to be associated with food. In less than one third of the outbreaks, HuNoV was directly transmitted from person to person, while in only 60 of the 902 outbreaks HuNoV was transmitted via the environment. Foodborne outbreaks have greater attack rates compared to person-to-person and environmental outbreaks. This may be partly due to ingestion of higher infectious doses of HuNoV. An overview of the HuNoV transmission routes, which often lead to outbreaks of gastroenteritis, is shown in Fig 4.

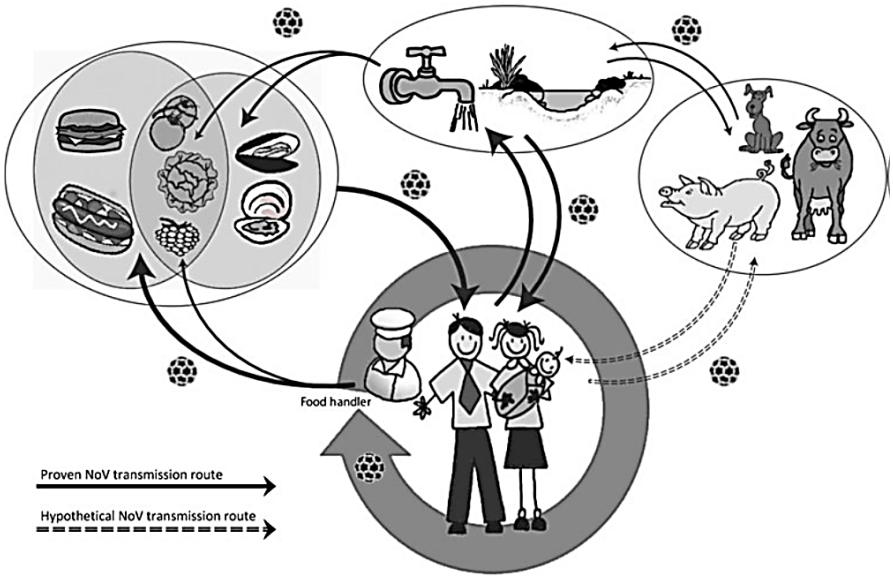

Fig 4. Overview of the proven and hypothetical transmission routes of HuNoVs, as presented by Mathijs and colleaques (16). The thickest arrows present the most common transmission routes for HuNoV, while the thin arrows indicate less frequent transmission routes. Dashed lines represent hypothetical transmission routes for HuNoV. NoV=HuNoV

HuNoVs are rarely detected in samples obtained from food items in food-borne gastroenteritis outbreak investigations for several reasons. First, food items may simply not be available for analysis. Second, the levels of HuNoVs in food may be low, especially when the food has become contaminated by a food handler infected with the virus (16). Third, the portion of foods analysed may not have contained HuNoVs due to uneven distribution of these viruses in the foods that were sampled. Fourth, foods may contain such substances that inhibit the detection of HuNoVs. In the absence of methods for HuNoV detection in food samples, four epidemiological features have been used to classify outbreaks, for which HuNoV is the suspected cause (72). These Kaplan criteria include vomiting which is a reported symptom in over half of the ill persons, the incubation period of the disease ranges between 24–48 hours, the symptoms pass within 12 to 60 hours, and no bacterial pathogens are present in the faecal samples of the ill patients.

2.6.1 Outbreaks caused by RTE food products

Ready-to-eat (RTE) food is food that is ordinarily consumed in the same state as that in which it was sold or distributed. RTE food meant to be consumed without heating or other further handling. Typically, it consists of different food components, such as vegetables and meat products. The most common food items that cause HuNoV outbreaks are bivalve molluscs including oysters, soft fruit including raspberry, and leafy greens (16, 30). RTE food products can be contaminated with HuNoV by contact with faecal material or vomit, which can occur during any stage of the food production. At a pre-harvest level, contamination usually happens by irrigation or washing the fresh produce with virus contaminated water or by use of contaminated manure or contaminated pesticides (16, 73). At post-harvest level, contamination can occur by manual harvesting, processing, or preparation of foods. The manual picking of food ingredients, such as berries, is sometimes also regarded as pre-harvest level contamination. In most outbreaks involving RTE foods, the route of contamination is hard to define. In the following outbreak cases, presented in Table 1, the source contamination was, however, identified.

Table 1. Examples of HuNoV outbreaks in 2003–2010, for which contamination of food happened at pre-harvest.

Food involved	Attack rate	Confirmed HuNoV		Place/event of the outbreak	Country	Reference
		Patient stool	Food			
Oysters	14 cases	2/4 GI.1	5/6 GI.1	Bought from two stores, eaten home	France	(74)
Mussels	103/139	24/24 GI and GII	6/11 GI and GII	Easter/restaurants and picnics	Italy	(75)
Oysters	305 cases	4/5 GII	6/11 GII	Hotels, clubs and restaurants	Singapore	(76)
Oysters	202 cases	29/53 GI.4/6, GII.4/8/b	3/3 GI.4, GII.4, GII.8	International outbreak	Italy, France	(77)
Raspberries*	~200 cases	2/2 GI.4	3/5 GI.4	Catering setting, daycare center	Finland	(78)
Lettuce	260/480	2/25 GI 12/25 GII	1/2 GII	Sandwich lunch	Denmark	(79)

Modified from (16)
*either pre-harvest or post-harvest contamination

2.6.2 Outbreaks caused by an infected food handler

Food handlers have been confirmed to play a major role in HuNoV transmission which led to food-borne outbreaks (16). Data collected by Todd and his co-workers (80), indicate direct, bare hand contact between the food handler hands and food was the most common factor associated with outbreaks involving a food handler. Often food handlers have been asymptomatic virus shedders, or they have returned to work after the gastroenteritis symptoms had passed but the shedding of virus was still continuing (81), as shown by the faecal samples taken during an outbreak investigation. In some outbreaks, however, the food handler had come to work still suffering from the gastroenteritis symptoms. In one of the most extreme cases, a food handler had vomited inside the kitchen area due to the sudden onset of the disease thereby spreading the infective HuNoV particles to the surroundings and food (81). Most common food items associated with HuNoV outbreaks where the food handler was involved have been RTE foods, especially delicatessen sandwiches, hereafter referred to in this text as deli sandwiches, as seen in Table 2.

Table 2. Examples of HuNoV outbreaks in 2001–2012, in which food handler involvement has been confirmed.

Food involved	Attack rate	Confirmed HuNoV		Place/event of the outbreak	Country	Reference
		Non-food handlers	Food handlers			
Deli sandwich	140/231	15/16	- (ill 5/8)	Buffet lunch in a dance theatre	The Netherlands	(82)
Deli sandwich	38/57	12/14	2/4	Hotel	Spain	(83)
Wedding cake	332/2700	2/2	2/2	46 weddings	The United States of America	(84)
Deli sandwich	34/427	12/14 GI.3	1/1 GI.3	Hospital cafeteria	Spain	(85)
Deli sandwiches	87/142	21/21	1/1	Luncheon in a restaurant	The United States	(86)
Deli sandwich	231/505	24/27 GII	1/1 GII	Buffet lunch	The Netherlands	(81)
Salad vegetables	182/325	5/6 GII.7	4/5 GII.7	Telephone company canteen	Austria	(87)
Salad vegetables in pastry	more than 23 persons	22/23 GII.4	3/3 GII.4	School lunch	Japan	(88)
Salad vegetables	60/106	6/13 GII.6	1/1 GII.6	Barbeque	England	(89)
Ham rolls	21/63	3/21 GII	1/1 GII	Pre-Christmas celebration	Austria	(90)
Chips, spare ribs and bread	14/31	13/13 GI	4/6	Restaurant	The Netherlands	(91)
Mushroom dish,wedding cake	26/103	2/26 GII	6/14 GII.4	Wedding	Austria	(92)

Modified from (16)

2.6.3 Outbreaks caused by the environmental transmission of HuNoV

Environmental transmission of HuNoV is a less important mode of spread than direct human-to-human transmission or food or food handler transmission in HuNoV outbreaks as a whole. Nevertheless, environmental transmission has played a major role in a number of outbreaks (1). The outbreaks where groups in a common setting, but who have had no known direct contact and have been sequentially affected, are the most easily identified to involve environmental HuNoV transmission. In the two following examples of outbreaks, environmental transmission was confirmed.

In the autumn of 2009, an airline medical team was informed that several flight attendants who had worked in different crews had fallen ill with gastroenteritis over a time period of six days (93). All

these crews had worked on the same aeroplane over successive flight sectors over the six day period. The outbreak had started, when one of the passengers on a flight had vomited and soiled the carpet next to his seat. A total of 27 out of 77 flight attendants who had worked on that aeroplane developed gastroenteritis symptoms. HuNoV GI.6 was identified in two stool samples that were collected for analysis from the flight attendants.

In the winter of 1999, the Environment and Public Protection Division in Wales was notified of outbreaks of gastroenteritis at two primary schools, which affected 163 out of 315 (57.7%) children (94). The children had attended the same lunchtime concert at a large concert hall. The environmental surfaces of the concert hall had been contaminated by HuNoV-containing vomit the day before the children attended. Concert hall staff had cleaned up the vomit and the carpeted areas of the concert hall but no hypochlorite-based product was used. After the lunchtime concert, gastroenteritis was also reported by 37 other attendees of the same concert. Ten of them attended an event on the same day as the index case, nine attended on the same venue three days after the index case, and 12 on the day after that. Six persons fallen ill attended to the venue that took place five days after the initial vomiting episode.

2.6.4 Outbreaks in Finland

In Finland, HuNoV has been the most common cause of food-borne gastroenteritis outbreaks in recent years (95). The virus caused 31% of the food-borne outbreaks in Finland in 2010, two of the outbreaks being the largest gastroenteritis outbreaks in that year. In these outbreaks HuNoV was only rarely detected directly from food items. Therefore most of the outbreaks have been classified as caused by HuNoV based on epidemiological investigation, which was supported by data showing the same HuNoV genotype in the faecal samples of the victims and the food handlers. More recently, swabs from food handling areas which tested positive for HuNoV have sometimes strengthened the evidence for classification of the outbreaks.

In a five-year study (1998–2002) in Finland, HuNoV was shown to have caused 60.6% of the investigated 416 viral gastroenteritis outbreaks during the study period (69). Most of the outbreaks were caused by HuNoV GII, whereas GI was detected in only 35 of the 416 (8%) outbreaks. The outbreaks had most often occurred in hospitals (30.6%) followed by restaurants and canteens (14.3%). In one of the outbreaks, which occurred between December 1999 and January 2000, a prolonged HuNoV outbreak took place in a rehabilitation centre (96). An environmental contamination of HuNoV was confirmed, for the virus was found in swab samples taken from rooms of people that had fallen ill and also from common areas, including the handle of an ultrasound physiotherapy instrument. Six years later, in the winter of 2006–2007, HuNoV variant GII.4.-2006b caused an outbreak in a large tertiary care hospital (97). The outbreak affected both the staff of the hospital (205 infected) and the patients (240 infected), contributed to nine deaths among the patients.

In 2009, the virology laboratory in the Department of Food and Environmental Hygiene in the University of Helsinki received an increasing amount of frozen raspberry samples, related to gastroenteritis outbreaks with HuNoV as the suspected cause (78). In total 21 notifications of these HuNoV outbreaks, which affected about 200 people, were sent to the National Institute for Health and Welfare (98). HuNoV GI.4 was detected both in samples from people who had fallen ill in the outbreaks and from raspberries that were suspected as the source of the outbreak. A pre-harvest

contamination by the raspberries was suspected, because the berries had been imported from Poland and distributed by a single wholesaler, and they were the only common factor in the described outbreaks (78). Although heating of raspberries of foreign origin before consuming had been recommended by the Finnish Food Agency, currently Finnish Food Safety Authority Evira since 2000, the berries had been used in desserts and cakes without heating.

During the spring of 2012 in Southern Finland, environmental swabs were used in an epidemiological investigation of restaurants and school canteens where a gastroenteritis outbreak was suspected (99). In half of the outbreaks, swabs were positive for HuNoV contamination on the environmental surfaces of the food handling premises or the toilet areas that were dedicated for food handlers. The faecal samples of the people who had fallen ill also revealed HuNoV. These outbreaks could be classified as foodborne HuNoV outbreaks, even though food items were not available for analysis.

2.7 Detection methods for HuNoVs on surfaces

It has been known for several decades that HuNoVs cause large outbreaks of gastroenteritis. The development of molecular techniques for the detection of HuNoVs that was based on the sequence of the virus published in 1990 enabled direct diagnostics of HuNoV since it enabled a very specific identification (5). Prior to this innovation, HuNoV had been only detected in human clinical samples: by (immune) transmission electron microscopy in faecal samples or by methods that detect a specific antigen in serum samples. Nowadays it is possible to use the sequence-based detection methods of HuNoV, which are more sensitive methods compared to electron microscopy. They are used in food, water and environmental samples, and they facilitate outbreak investigations and research on virus transmission and transfer routes.

Samples obtained from environmental surfaces can be used for the evaluations of hygiene standards in food processing environments and in semi-closed communities, such as in day-care centres, elderly homes and hospitals. In these communities, the residents often use the same areas for eating and the same restrooms, and the immune status of the residents is often low. Swabs can be used to monitor the incidence of harmful viruses to circumvent large outbreaks (100).

Environmental samples can also be used as a tool for outbreak investigations for foodborne outbreaks and also for monitoring the direct spread of HuNoV from human to human (101). Environmental samples do not give direct information on the extent of contamination in the foods or the people. Environmental samples do show that the pathogen is present, which helps the investigators to narrow down the list of possible causes. This is especially the case in outbreaks where human stool samples or food items related to the outbreak could not be collected or the bacterial analyses of food items did not indicate a cause for the outbreak.

HuNoVs hitherto have not been grown in cell culture. Therefore, the current methods for detection of HuNoV focus on detecting viral RNA or HuNoV antigen (8). Nowadays, the detection of viral RNA is often used when detecting HuNoV in water, food, and environmental samples. The levels of the virus are usually very low in these specimens, which leads to the need of concentrating and/or extracting the virus from the sample material. Validated methods for these techniques are now available for bottled water, bivalve mollusc shellfish, soft fruit and leafy vegetables, and food surfaces (102). According to the ISO standard, the recommended method for HuNoV sampling for food surfaces is done by swabbing with a moistened swab, followed by RNA extraction and reverse transcription quantitative polymerase chain reaction (RT-QPCR).

2.7.1 Swab sampling

Swab sampling or swabbing describes a protocol, whereby environmental or food surfaces are wiped by a swabbing tool, such as a cotton wool stick or cotton cloth, the viruses are subsequently detached from the tool, and the virus or its genome is detected by a variety of different methods (103). In the case of HuNoVs, the detection method is almost always either RT-PCR or RT-QPCR.

The effectiveness of a swabbing method is often measured by calculating the percentage of viruses that were recovered from a certain surface to the known amount of viruses that were applied to the surface before swabbing. This fraction of viruses is called the viral yield or viral recovery rate. Previous studies implicate that the swab material, the surface material, and the solution used to remove the viruses from the surface and later, from the swab, have an impact on the recovery rates of viruses. In a review conducted in 2011 by Julian and co-workers, a polyester swab moistened in saline solution was to give the highest recovery rates of the phage MS2, which ranged from 45% to over 100% (103). In a study by Scherer and co-workers, the recovery rates when swabbing surfaces with a cotton wool stick moistened by phosphate buffered saline (PBS) solution were from 10% to 58%, which found the highest rates for ceramic surfaces and the lowest for wood surfaces (104). Gibson and co-workers tested the removal of HuNoV surrogates MuNoV and FCV from solid surfaces by wiping the surfaces with microfiber cloths, and reported a removal of 3.5 $\log_{10}$-units (PFU) of both viruses from these surfaces (105).

2.7.2 Other methods for surface sampling

In addition to swabbing, HuNoVs can be collected from surfaces by back and forth pipetting, as described by D'Souza and co-workers (2006) (106). The viruses do not need to be detached, or eluted, from the swabbing material when using this method. Scraping the sampled surfaces with a cell scraper while pipetting, was suggested to aid in sampling and indeed it has been reported to enhance the virus recovery from surfaces (107). In the third method for surface sampling, disks of surface materials are placed on a petri dish or plate and covered with an elution solution (103). It is feasible for only very small disks of surface materials and it has therefore been used mainly in experimental studies in the laboratory. Viruses are then eluted from these surfaces by shaking the petri dish to generate a flushing effect. In 2013, Stals and co-workers reported viral recoveries for HuNoV that ranged from 5% for lettuce to 40% for nitrile glove using this method (108).

2.7.3 Elution and RNA extraction

When surface samples are taken by a swab, the next step in the preparation for analysis is virus elution. For HuNoV samples, which are usually analysed by PCR assays, the most common elution agent has been saline solution, such as PBS that is adjusted at neutral pH (103). Viruses are usually eluted from the swab material in a separate elution step, by using lysis buffer directly, or by a combination of the two approaches. The elute pH seems to affect the viral recoveries. Taku and co-workers (2002) reported higher recoveries, 36–50%, when glycine solution at pH 6.5 was used than when the viruses were eluted with a glycine solution at pH 9.5, at which time the recovery was 20–35% (107).

Surface samples may contain substances, such as the organic layer of soils that can inhibit the enzymatic activity of the reverse transcriptase and DNA polymerase enzymes used in RT-PCR assays (109). Thus, purification of HuNoV nucleic acids is needed before the RT-PCR assay. Nowadays, the RNA extraction is usually performed using commercial kits, such as those that involve the filtration or

treatment of the sample with chemical substances. Of the chemicals that are used in RNA extraction, Freon 113 (1,1,2-trichloro-1,2,2-trifluoroethane) and later, a more environmentally friendly substitute, Vertrel® XF (1,1,1,2,3,4,4,5,5,5-decafluoropentane), have been utilized to purify viruses from *inter alia* food matrices, for they can separate lipid matrices from polar molecules, such as virus RNA (110). Also phenol-clorophorm solution and later, a commercial guanidinium thiocyanate-phenol-chlorophorm extraction Trizol®, have been used in HuNoV RNA extraction. The use of a mixture of guanidinium thiocyanate together with the nucleic acid-binding properties of silica particles, followed by chloroform extraction and precipitation of nucleic acids in alcohol, was first described by Boom and co-workers in 1990 (111). Commercial systems are available, such as the the MiniMag System which have silica on the magnetic beads, and the QIAamp Viral RNA Mini kit system, in which the silica is built on a membrane in a column (112).

2.7.4 RT-PCR methods

Nowadays the most common method for detection of HuNoVs in several matrices is the reverse transcription polymerase chain reaction (RT-PCR) method (109). It was developed in the 1990s to identify HuNoV in faecal samples. Later, in 2003, Kageyama and co-workers compared the sequenced HuNoV genomes and found that the most conserved region of the genomes is the open reading frame 1 (ORF1)-ORF2 junction region (113). They used the most conserved sequences from that region to react with diverse HuNoVs to design primers and TaqMan probes that are suitable for detection of HuNoV GI and GII by RT-PCR or RT-QPCR. Both RT-PCR and RT-QPCR methods have been successfully used to identify HuNoV in clinical specimens in addition to food, water, and environmental surface samples. Being highly sensitive, they are especially useful for swab and other surface samples, for which the concentration of HuNoVs collected from surfaces are usually low, perhaps only a few viral particles per a square centimeter (103).

In RT-PCR, the viral RNA template is first converted into a complementary DNA by using a reverse transcriptase enzyme (114). The complementary DNA is then used as a template for the exponential amplification using PCR. These two phases can be performed separately, or in the same test tube, which is referred to as one-step RT-PCR. After the amplification phase, the lengths of DNA, known as PCR products, are made visible by dyeing them using various fluorescent dyes and then separating the labeled products according to their electrical charge and molecular size by gel electrophoresis using electric current. A modification of RT-PCR method is RT-QPCR. When a target DNA sequence is amplified using RT-QPCR the amplification can be monitored during the PCR run in real time, and thus no later visualization of the DNA lengths is needed. The detection of the DNA is usually based either on TaqMan chemistry, which uses a labelled fragment of DNA called probe to detect the target DNA, or the use of a fluorescent dye, such as SYBR Green I (114). An example of TaqMan chemistry, modified from the work of Koch and co-workers (115), is given in Fig 5.

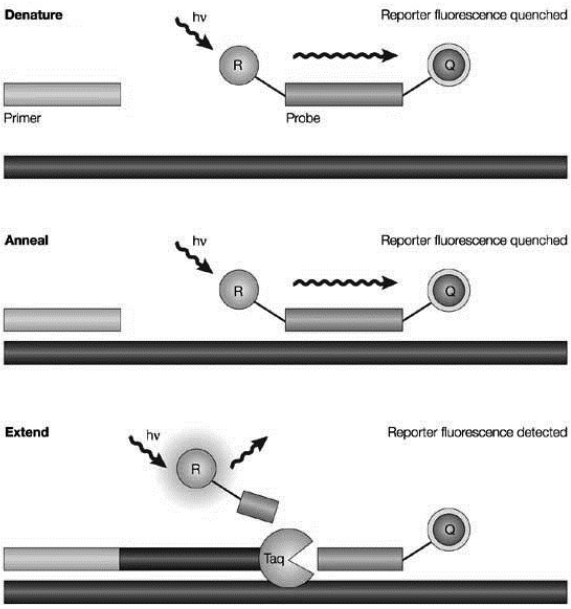

Fig 5. TaqMan hydrolysis probe principles (115). The 5'-nuclease activity of thermostable polymerases used in the polymerase chain reaction (PCR) cleaves hydrolysis probes during the amplicon extension step, which separates the detectable reporter fluorophore (R) from a quencher (Q). Fluorescence is emitted when excited by an external light source (hv) at each PCR cycle which is directly proportional to the amount of product formed.

In RT-QPCR, the concentration of amplified DNA can be designated by absolute or relative quantification (114). In theory, absolute quantification measures the undisputed number of DNA length copies in the sample, when the strength of the signal from the target DNA is compared to the strength of the signal of several reference samples with known concentrations of DNA. One RT-QPCR detectable polymerase chain reaction unit (pcr-u) is usually defined as the highest 10-fold dilution of the reference samples with a positive signal. It is used to describe the relative copy number of DNA lengths in the tested samples. One pcr-u is not a universal copy number of the target DNA lengths, because it is dependent on the RT-QPCR conditions and the specific virus strain. Relative quantification measures the concentration of target DNA lengths compared to a known reference sample, whose concentration is at a certain level in certain conditions. Relative quantification is used *inter alia* for studying the differences in gene expression of bacteria under different conditions by comparing the expression levels of a studied gene to the expression level of a housekeeping gene in the same conditions. Quantification cycle (C_q) value indicates the number of amplification rounds before the fluorescence signal exceeds the threshold value of background fluorescence (114). A low C_q value indicates a high concentration of DNA in the sample, whereas high C_q value is an indicator of a low concentration of DNA.

2.7.5 Other methods for molecular HuNoV detection

Although RT-PCR is the most commonly used method for HuNoV detection nowadays, other options for molecular HuNoV detection are available. These alternative methods include reverse transcription loop-mediated isothermal amplification (RT-LAMP), in which amplification is carried out at a single temperature (116), reverse transcription helicase dependent amplification (RT-HDA), in which helicase enzyme is used to denature the DNA before amplification (117), and nucleic acid

sequence-based amplification (NASBA) (118). NASBA seems particularly useful for the detection of HuNoVs. It is an RNA amplification method that allows the use of the initial single stranded RNA genome as the template in the reaction. However, a drawback with this method is a low incubation temperature in the NASBA reaction that is thought to increase non-specific amplification during the reaction (119).

2.7.6 Other methods for norovirus detection from surface samples

The detection of HuNoVs in stool specimens using direct electron microscopy or immune electron microscopy requires high virus concentrations, which are usually observed only in faecal samples (109, 120). Therefore, this method is not feasible for the detection of HuNoVs from swabs. Similarly, Enzyme Immunoassay and Immunochromatography are too insensitive, although they are widely used for detetcting HuNoV in faecal samples.

Although a cultivation technique for HuNoV is still currently not available, its surrogates, such as MuNoV, have been detected in cell cultures of experimentally contaminated environmental surfaces in the laboratory (40). These surrogate viruses, when analyzed in cell culture, are usually eluted from the surfaces by either a neutral buffer solution (pH 7.0), or cell culture media, as demonstrated by Tuladhar and co-workers (121). The eluted viruses are then analyzed in infectivity assays, such as a plaque assay, which determine the number of plaque forming units (pfu) in a virus sample. The pfu/ml value is obtained by observing lysed infected cells in the culture, and represents the number of infective particles within the sample. This determination is based on the assumption that each plaque formed is indicative of one infective virus particle.

2.8 Persistence of noroviruses on surfaces

Descriptions of outbreaks of gastroenteritis, where the cause of the outbreak has been confirmed to be HuNoV and the mode of spread was found to be via contact with environmental surfaces, suggest that HuNoV can stay infective on environmental surfaces for long periods of time. Human volunteer studies that concentrate on the survival of infective HuNoV on environmental surfaces have not been, however, published. In contrast, the viability of HuNoVs in groundwater has been shown to exceed two months (19). In another volunteer study, pressures of up to 600 megapascals (MPa) for 5 min at RT were required to eliminate a 4 $\log_{10}$ load of HuNoV in oysters totally (18).

Non-enveloped viruses, including HuNoVs, are generally more persistent in the environment than enveloped viruses, such as influenza viruses (17). The faecal-oral transmission route of HuNoVs and their transmission via the environment requires a viral capsid that is capable of withstanding the extremely low pH 3 of the stomach, in addition to the conditions outside of the human host. Survival in the environment is facilitated by protection of organic debris of faeces or vomit in which the virus is shed and virus aggregate formation occurs that offer protection from environmental stresses encountered on en route to new human hosts. Even relatively high temperatures do not inactivate the virus: HuNoV like particles have been shown to be highly stable at temperatures up to 55 °C (122).

2.8.1 Persistence of noroviruses on environmental surfaces

Various factors, including surface type, temperature, inoculum matrixes, and relative humidity, are reported to have an impact on how HuNoV remains viable on environmental surfaces (17). In most of the persistence studies conducted on HuNoV and its surrogates, viral RNA of these viruses was still observed even at the last measurement point of the trials, the longest interval being 56 days after

starting the experiment, as seen in Table 3. The infectivity of HuNoV surrogates on environmental surfaces decreased more rapidly than the persistence of the virus genome. The environmental surface, on which the HuNoVs and its surrogates had been inoculated, had little effect on the persistence of the viruses (20, 106). Keeping the surface samples at 4^{o}C or 7^{o}C rather that at RT, however, significantly facilitated the persistence of HuNoV on these surfaces as measured by genome detection (123, 124), and it also reduced the loss of infectivity of HuNoV surrogates (124). The effect of relative humidity on the infectivity of HuNoV surrogates on environmental surfaces was not evaluated, but 86% relative humidity was reported to facilitate HuNoV persistence compared to 30% humidity (123).

2.8.2 Persistence of noroviruses on human hands

The ability to remain infective on human hands is crucial for HuNoVs. The faecal-oral transmission route requires the ingestion of HuNoV particles in order for the new host to get the infection. However, the presence of HuNoV on hands does not enable droplet infection, except in the relatively rare situations when viruses become airborne in droplets that were created by vomiting (50). Therefore, in most cases, HuNoV is transmitted from faecal matter of infected persons to their hands and via those hands to environmental surfaces and food.

HuNoV persistence on hands has mainly been studied by using surrogate viruses or viruses resembling HuNoV in their environmental persistence, such as hepatitis A virus (125). In these studies, enteric viruses have been shown to survive on human hands for several hours. HuNoV RNA has been found in swabs taken from the hands of an ill food handler during outbreak investigation, which suggests prolonged stability of the virus on the hands (91). Recently, Liu and co-workers (2009) demonstrated that HuNoV RNA remained quite stable up to the end of their two hours trial (20). It thus seems that HuNoV survival resembles that of the other enteric viruses, although the decrease in infectivity could not be measured in their study.

2.8.3 Persistence of norovirus on foods

Viruses, unlike bacteria, are strict intracellular parasites and cannot replicate in foods. Therefore, viral contamination of food will not increase during processing, transport, or storage, and the contamination of food products is not detected by the senses. Nevertheless, due to the small infective dose of HuNoV, survival of even a fraction of these viruses on foods can lead to infection of the host when the contaminated foods are consumed.

Previous studies on HuNoV persistence in foods suggest that temperature, food type, and virus strain are important variables when considering HuNoV survival on foods. Freezing facilitates HuNoV survival, as seen in many challenge studies, reviewed in Table 4. The virus persists much longer than the leafy green plants themselves at refrigeration temperatures. Differences in survival of HuNoV surrogates on different foods has not been extensively studied, but the reduction of infectivity of MuNoV on lettuce, strawberries and raspberries has been similar at 4^{o}C (126-128). Moreover, HuNoV persistence studies on environmental surfaces indicate that the viral genome can be detected in foods after much longer time periods compared to detection of the infectivity of HuNoV surrogates. In the following two Tables, 3 and 4, the persistence of HuNoV has been reviewed in more detail.

Table 3. Overview of persistence studies of HuNoV and its surrogates on environmental surfaces

Reference	Virus[a]	Inoculum	Inoculum matrix	Surface	Time points, days	Temp-erature	Relative humidity	Method for detection	Persistence, days
(106)	FCV	10^9 PFU[b]	Cell culture media	Formica	0, 0.02, 0.042, 0.083, 0.17, 0.33, 1, 2, 7	22±2 °C	75–88%	Plaque assay	7
	HuNoV GI	10^4 pcr-u[c]	Faecal suspension	Stainless steel				RT-PCR	7
				Ceramic					
(124)	FCV	5 * 10^5 PFU	Artificial faecal suspension	Stainless steel	1, 2, 3, 4, 5, 6, 7	4°C	54 %	Plaque assay	7, reduction less than 1 $\log_{10}$
						22°C	75–85%		7, reduction 2.5 $\log_{10}$
	MuNoV					4°C	54 %		7, reduction 1.5 $\log_{10}$
						22°C	75–85%		5
(129)	MuNoV	10^8 PFU	Cell culture media	Gauze Nappy		-20, 4,18,30°C		Plaque assay	-20 °C: 40 4 °C: 40, reduction 5.5 $\log_{10}$ PFU 18, 30 °C: <2
(130)	HuNoV GII	10^2 NASBA particle units	Faecal suspension	Stainless steel	0.02, 4, 7, 14, 21, 35, 49, 56	7°C	86%	NASBA	49
							30%		not tested
						20°C	86%		28
							30%		7
				Plastic (PVC)		7°C	86%		56
							30%		not tested
						20°C	86%		28
							30%		7
(20)	HuNoV GI	10^7-10^9 genome copies	Faecal suspension	Ceramic Formica	42	ambient temp-erature	ambient relative humidity	RT-QPCR	42, 0.4-1.2 $\log_{10}$ RNA reduction
	HuNoV GII			Stainless steel	28				28, 1.5-2.9 $\log_{10}$ RNA reduction

Table 3 continued

(127)	MuNoV	10^5-10^6 PFU	Cell culture media	Stainless steel	42	ambient temperature	ambient relative humidity	RT-PCR Plaque assay	42 42, 1.6 $\log_{10}$ RNA reduction
(126)	HuNoV GI	10^4 pcr-u	Faecal suspension	Formica Stainless steel Ceramic	0-42			RT-QPCR	42, 1.5-2.3 $\log_{10}$ RNA reduction
	HuNoV GII								
	MuNoV	$10^{6.5}$ PFU	Artificial faecal suspension	Formica Stainless steel Ceramic				RT-QPCR Plaque assay	42, no RNA reduction 21, 1.5 $\log_{10}$ PFU reduction
			Cell culture media	Formica Stainless steel Ceramic				RT-QPCR Plaque assay	42, no RNA reduction 14, 3 $\log_{10}$ PFU reduction

Modified from (17).

[a] HuNoV = human norovirus, MuNoV = murine norovirus, FCV= feline calicivirus, MS2= coliphage MS2

[b] Plaque forming units

[c] One pcr-u has been defined as the last dilution of the sample from which virus RNA could be amplified with RT-PCR

Table 4. The persistence of HuNoV and its surrogates in foods

Reference	Virus	Inoculum	Inoculum matrix	Food type	Duration, temperature	Method for detection	Persistence, days
(123)	HuNoV GII	10^6 NASBA particle units	Faecal suspension	Lettuce	10 days at 7°C	Real-time NASBA	10, 1 $\log_{10}$ reduction in RNA levels
				Turkey meat			10, small reduction in RNA levels
(131)	MuNoV	6.3 log PFU 2.5 log PFU	Cell culture media	Onion Spinach	6 months at -21 °C	Plaque assay	180, very small reduction in infectivity
(132)	HuNoV GI HuNoV GII	10^5-10^6 pcr-u	Faecal suspension	Berries	90 days at -20 °C	RT-PCR	90, less than 1 $\log_{10}$ reduction in RNA levels for both viruses
				Parsley		Plaque assay	90, 0.3-2.7 $\log_{10}$ reduction in infectivity, most prominent in strawberries
	FCV			Basil			
(127)	MuNoV	10^5-10^6 PFU	Cell culture media	Lettuce	15 days at ambient temperature	Plaque assay	15, 1 $\log_{10}$ reduction in infectivity after 4 days
						RT-PCR	15, very low reduction in RNA levels
(126)	HuNoV GI HuNoV GII	10^4 pcr-u	Faecal suspension	Lettuce	14 days at 21°C or 4 °C	RT-PCR	14, 1 $\log_{10}$ reduction in HuNoV GI RNA, 1.2-1.8 in HuNoV GII RNA, very low reduction in MuNoV RNA
	MuNoV	$10^{6.5}$ PFU	Cell culture media			RT-PCR and plaque assay	14, 1.5 $\log_{10}$ reduction at 4 °C, 3 $\log_{10}$ reduction at 21°C
(128)	HuNoV GII HuNoV GI	10^6-10^7 genome copies	Faecal suspension	Raspberry Strawberry	7 days at 4 °C and 10 °C	RT-PCR and plaque assay	7, very low reduction in RNA levels at 4 °C, less than 1 $\log_{10}$ reduction in RNA levels at 10 °C
	MuNoV		Cell culture media		3 days at 21 °C		3, reduction in infectivity: raspberries 1.1 $\log_{10}$, strawberries1.4 $\log_{10}$

Modified from (17).

[a] HuNoV = human norovirus, MuNoV = murine norovirus, FCV= feline calicivirus, MS2= coliphage MS2

[b] Plaque forming units

[c] One reverse transcription PCR unit (pcr-u) has been defined as the last dilution of the sample from which virus RNA could be amplified by using RT-PCR

2.9 Transfer of noroviruses between surfaces

The transfer of HuNoVs from infected persons to foods or to environmental surfaces has been identified as the source of several large outbreaks of gastroenteritis in recent decades (16). Although the role of human hands and other fomites as transmission vehicles of HuNoVs has been suspected for a long time, laboratory evidence of the transfer has become available only recently (17). In these experiments the drying time of the inoculum and the pressure applied during the transfer have been seen to have a major influence on the transfer rates, calculated as the number of viruses transferred from donor to recipient surface divided by the total number of the viruses present on the donor surface before transfer. For instance in 2012, Sharps and co-workers reported that the proportions of HuNoV that transferred from gloved fingertips to fruit, gloves, or stainless steel were much higher when the virus did not have time to dry on the donor surface compared to after HuNoV had dried for 30 minutes (133). In the same year, Escudero and co-workers showed that a pressure increase from 100 to 1000 g/cm^2 during MuNoV transfer from formica, stainless steel or ceramic to lettuce surfaces, increased the transfer rates from 0–4% to 8–20%, respectively (126).

2.9.1 Transfer of noroviruses between human hands and food

As described before, fresh food including berries and vegetables can become contaminated with HuNoV during harvesting, if the pickers working in the fields are infected and the level of hand hygiene is insufficient in the farms (8). At post-harvest phase, almost any type of food can become contaminated via a food handler, as a result of handling food without gloves while infected with HuNoV (80, 134).

The transfer of HuNoVs from hands to food has been studied mainly using HuNoV surrogates. For instance in 2004, Bidawid and co-workers studied FCV transfer from fingerpads to food (ham and lettuce) and vice versa. These authors found mean transfer percentages as high as 46 ± 20.3% when contaminated fingers touched these surfaces, but only up to 14 ± 3.5% when clean fingers touched contaminated surfaces (135).

A mean of 3.86 $\log_{10}$ genomic equivalent copies of HuNoV has been detected on the hands of infected persons who were experiencing gastroenteritis symptoms (136). Therefore, even low transfer rates from hands to foods can be important when considering the risk of the food picker or food handler contaminating foods with HuNoV while handling food items.

2.9.2 Transfer of noroviruses between human hands and environmental surfaces

The transfer of HuNoV from contaminated hands to environmental surfaces plays an important role in HuNoV transmission in hospitals and other facilities (100). HuNoVs that were detected by swabbing as a part of epidemiological studies, have often been detected on those environmental surfaces that are often touched by hands (100, 101). These environmental surfaces include handles of refrigerator and microwave ovens, soap dispensers and television consoles.

Experimental studies have shown that HuNoV is easily transferred from hands to environmental surfaces. In 2004, Barker and his co-workers reported that fingers contaminated with HuNoV after contact with soiled toilet paper were capable of sequentially contaminating seven clean melamine surfaces upon touch (137). Bidawid and co-workers reported in their study that the transfer efficiency of FCV from fingers to stainless steel surface was 13 ± 3.6%, and from stainless steel to fingers 7 ± 1.9% (135). One potential transfer route for HuNoV via environmental surfaces could be

paper money and coins, which have been found to contain other pathogens that spread in the population via the faecal-oral route, such as *Escherichia coli (138)*.

2.9.3 Transfer of noroviruses between environmental surfaces and food

Transfer of HuNoVs from environmental surfaces to food is considered a minor route of contamination compared to the transfer of the viruses from human hands to foods (16). Nevertheless, the transmission route is widely studied in the laboratory, using both HuNoVs and their surrogates in the experiments.

In 2006, D'Souza and co-workers studied the transfer of HuNoV GI and FCV from stainless steel to lettuce surfaces (106). They found eight out of nine lettuce samples to be positive for HuNoV after coming into contact with the surfaces. The transfer efficiencies of FCV varied from 4.3% to 6.8% after 60 min of drying when the lettuce on which the virus had been inoculated was wet and 0.2% to 4.9% after 60 min of drying when the lettuce had been dry. Sequential transfer of MuNoV to produce items was demonstrated by Wang and co-workers (135). In their study, contaminated produce was first prepared using knives and/or graters, and then the contaminated kitchen utensils were sequentially used to prepare seven produce items (cucumbers, strawberries, tomatoes, cantaloupes, carrots, or honeydew melons). In nearly all cases, the virus was detected on the seventh item prepared with the contaminated utensil (139).

The transfer efficiencies from environmental surface to food and vice versa have also been studied. In 2012, Escudero and co-workers measured the transfer of a HuNoV GII to foods from environmental surfaces, and found transfer percentages of viruses to lettuce, 0–26%, to be lower than their transfer, 55–95%, to sliced turkey deli meats (126).

Transfer of HuNoV from gloves to food has been only recently experimentally studied by Stals and co-workers (2013) (104). Those authors measured HuNoV and MuNoV transfer from contaminated nitrile gloves to sandwich components: meat, lettuce, and bun. They found different rates of transfer to each food type, ranging from 2.2%, obtained from boiled ham to 8%, obtained from a sandwich bun. Sharps and co-workers reported that the transfer rates of a norovirus cocktail, including HuNoV GII, HuNoV GI and MuNoV, to raspberries, blueberries and grapes after handling by operators wearing contaminated latex gloves varied on average from 25 to 108% when the inoculum did not have time to dry and from 5 to 65% when the inoculum was dry before transfers occurred (133).

2.10 Inactivation methods for noroviruses on surfaces

The environmental stability of HuNoVs has caused problems both in food producing chains and healthcare environments, probably for as long as these viruses have existed (1). Traditionally, food hygiene measures in food processing have been designed to control the growth of harmful bacteria in food, for instance by continuous chill chain (40). HuNoVs do not multiply outside human hosts and persist for long periods in the environment, thus cool and moist conditions enhance their survival instead of inactivating them. Consenquently, food hygiene solutions tailored to inactivate enteric viruses in addition to controlling bacteria are needed in order to prevent foodborne outbreaks. In healthcare environments, strict hygiene measures are regarded as a feasible way of controlling the transmission of HuNoVs (140). However, more accurate knowledge on the effects of inactivation methods against HuNoV is needed. For example the effect of several chemicals, such as ethanol based hand sanitizers, against HuNoVs are still unclear.

Inactivation, by definition, refers to the elimination or reduction in infectivity of a virus. According to European disinfection standards (141, 142), inactivation of viral infectiousness in the quantitative suspension test and environmental surface test by at least 4 $\log_{10}$ stages (= 99.99% titre reduction) must be achieved in all test batches at 20°C over a maximum of 60 minutes. Both chemical and physical inactivation methods, including disinfection with sodium hypochlorite, heating, and UV; have been tested against HuNoVs and other enteric viruses (8). As stated before, HuNoV infectivity cannot be measured in the laboratory except in volunteer studies, thus inactivation experiments have been performed using HuNoV surrogates, or the resistance of HuNoV particles including RNA, have been measured by genome detection methods (143).

2.10.1 Inactivation using chemicals

Chemical disinfection is the most common approach to interrupt the chain of HuNoV transmission via food contact and other environmental surfaces (21). The United States Environmental Protection Agency (EPA) maintains a list of approved products for HuNoV disinfection, which was last updated in 2009 (144). The list is based on the efficacy of the products against FCV, although this respiratory virus exhibits different physiochemical properties to those of HuNoV. At the moment, chemical agents are often tested on several surrogate viruses to assess the efficacy of these agents, as seen in Table 5.

Centres for Disease Control and Prevention (2011) recommend that environmental surfaces potentially contaminated by HuNoV should be disinfected using a sodium hypochlorite solution (21). The recommended concentration of chlorine bleach solution for treating hard, nonporous, environmental surfaces is in the range of 1 000–5 000 ppm. Although effective towards HuNoV surrogates (145-148), sodium hypochlorite apparently does not destroy HuNoV RNA completely, which leads to very little reduction of HuNoV levels detected by RT-QPCR (137, 149). Hydrogen peroxide, which is available as a slowly evaporating commercial product Oxivir®, and commercial product Virkon S®, have also been approved for HuNoV disinfection (144).

There is some contradiction in the reports on the effects of alcohols against HuNoV. Only one commercial product containing ethanol has been registered with the EPA as being effective against HuNoV (144). Magulski and co-workers reported that 55–60% ethanol inactivated 6 $\log_{10}$ MuNoV over 5 min (150). Girard and co-workers reported that a disinfectant consisting of 2-(1-Butoxy) propanol and ethoxylated alcohols had no effect on either HuNoV or on MuNoV (146).

Table 5. Inactivation of HuNoV and its surrogates on environmental and other surfaces by chemicals.

Reference	Virus[1]	Inoculum	Surface	Chemical	Concentration	Affecting factors[2]	Time, min	Inactivation
(151)	FCV	9.5 $\log_{10}$ $TCID_{50}$	Fabric: Cotton Polyester Carpets: Olefin Nylon Polyester	2.6% glutaraldehyde Phenol compound Quaternary ammonium compound 70% isopropanol	undiluted 1:128 1:32 undiluted		1, 5, 10	Fabrics: Glutaldehyde 10 min: 100% Phenol compound 10 min: 95-99% Q. ammonium 10 min: 92-97% Isopropanol: 91-99% Carpets: Glutaldehyde 10 min: 99-100% Phenol compound 10 min: 60-96% Q. ammonium 10 min: 17-95% Isopropanol: 68-97%
(152)	FCV	6.8 $\log_{10}$ MPN	Petri dish	Calcium carbonate	100 and 1 000 ppm	20°C ± 2°C, 5%	10	100 ppm: 3.2 $\log_{10}$ MPN 1 000 ppm: 6.6 $\log_{10}$ MPN
(145)	HuNoV MuNoV MS2	4-5 $\log_{10}$ pcr-u 5.3 $\log_{10}$ PFU 7-8 $\log_{10}$ PFU	Stainless steel Ceramic	Sterilox hypochlorous acid	50-190 ppm		1, 5, 10	188 ppm 1 min: MS2 3 $\log_{10}$ PFU both surfaces HuNoV 3 $\log_{10}$ pcr-u both surfaces 38 ppm 5 min: MS2 3 $\log_{10}$ PFU both surfaces HuNoV 3 $\log_{10}$ pcr-u ceramic

Table 5 continues

(150)	MuNoV	not reported	Stainless steel	Peracetic acid Glutaraldehyde Ethanol 1-propanol 2-propanol	50, 200, 500, 1 000, 1500, 2000, 2500 ppm 20-60 %		5	Peracetic acid 1 000 ppm: 4 log_{10} Glutaraldehyde 2 500 ppm: >4 log_{10} Ethanol 55% and 60%: 6 log_{10} 1-propanol 40%, 50%, 60%: 6 log_{10} 2-propanol 60%: 3 log_{10}
(146)	HuNoV MuNoV	10^4 pcr-u 10^5 PFU	Stainless steel	3% Sodium hypochlorite Ethoxylated alcohols (EA) Quaternary ammoniums (QA)	1:1, 20 µl	pH 12.3 pH 9.8 pH 10.7	5, 10	Sodium hypochlorite 5 min: MuNoV 5 log_{10} PFU, HuNoV 2 log_{10} pcr-u 10 min: MuNoV 5 log_{10} PFU HuNoV 3.5 log_{10} pcr-u EA 10 min: MuNoV, HuNoV not affected QA 10 min: MuNoV 1.5 log_{10} PFU HuNoV not affected
(153)	HuNoV MuNoV FCV MS2	4.0 log_{10} pcr-u 4.8 log_{10} PFU 4.1 log_{10}	Stainless steel	Sodium hypochlorite	250, 500, 1000, 2500, and 5 000 ppm	pH 7.0	2, 4, and 10	5 000 ppm 2 min: HuNoV < 1 log_{10} pcr-u MuNoV 1.3 log_{10} PFU FCV 3 log_{10} PFU MS2 1.2 log_{10} PFU

		PFU 5.7 log_{10} PFU all faecally soiled						
(154)	MuNoV	3.8 g/liter 50µl	Stainless steel	Hydrogen peroxide, liquid and vapour	1%, 2%		5,10	1% 5 min: 2 log_{10} PFU 1% 10 min: 3 log_{10} PFU 2% 5 min: 2.9 log_{10} PFU 2% 10 min: 3.1 log_{10} PFU
(147)	FCV MuNoV	$10^{8–9}$ $TCID_{50}$/ml	Stainless steel	Chlorine	0-5 000 ppm	22°C ± 2°C	0 to 5	5 000 ppm 5 min: FCV, MNV 5.20 $TCID_{50}$
(148)	MS2 MuNoV	6 log_{10} PFU	Stainless steel PVC plastic	Chlorine-based electrochemical oxidants (ECO)	500-2 500 ppm	± 80 mg/liter humic acid	30 s	1 000 ppm: MS2 6.9 log_{10} PFU 2 500 ppm: MS2 7.3 log_{10} PFU MuNoV 1.7 log10 PFU 5 000 ppm: MuNoV 2.4 log10 PFU

Data collected by the author.

[1]HuNoV = human norovirus, MuNoV = murine norovirus, FCV= Feline calicivirus, MS2= coliphage MS2

[2]Temperature and pH if reported

2.10.2 Inactivation using chemicals on human hands

Appropriate hand hygiene is regarded as the single most important method to prevent HuNoV infection and control virus transmission nowadays (21). Ethanol hand rubs are commonly available and acknowledged to inactivate bacteria and respiratory viruses. However, they have been suggested to be ineffective against HuNoV. Therefore, the old hand hygiene method of washing hands with soap and water, in addition to certain types of hand sanitizers, have been tested alongside ethanol to find suitable methods for effective hand hygiene against HuNoVs. Standard test methods for the evaluation of hygienic hand wash and hand rub formulations have been developed to evaluate the effects of these sanitizers (155, 156).

According to the U.S. Food and Drug Administration 2010 Food Safety Survey, washing hands with soap and water before food preparation at home results in a reduction in the self-reported incidence of foodborne illness (157). Indeed, hand washing with soap and water has been shown to reduce the number of enteric microbes on the hands via the mechanical removal of these micro-organisms (158). The procedure was observed to remove nearly 2 $\log_{10}$ PFU/ml of the HuNoV surrogate MS2 viruses from the hands. Liu and co-workers reported that the removal of HuNoV by soap and water was somewhat less effective, 0.67 to 1.20 $\log_{10}$ reduction in genomic copies (159). The inactivation of HuNoV by ethanol and other alcohols is shown in Table 6.

Table 6. Inactivation of HuNoV and its surrogates by chemicals on human hands.

Reference	Virus[1]	Inoculum	Chemical	Concentration	Time	Inactivation/Reduction
(160)	FCV	10 µl 1:1	Ethanol 1-Propanol 2-Propanol	70%, 90%	30 s	0.83, 0.64 $\log_{10}$ ID_{50} 0.92, 0.33 $\log_{10}$ ID_{50} 0.50, 0.19 $\log_{10}$ ID_{50}
(161)	MuNoV	10 µl 1:1	Ethanol+ polyquaternium-37+ citric acid	70%	30 s	2.48 $\log_{10}$ PFU
			Ethanol	75%		0.91 $\log_{10}$ PFU
(159)	HuNoV	6.7 $\log_{10}$ genomic copies	ethyl alcohol	62%	20 s	0.27 $\log_{10}$ genome copies
(162)	FCV MuNoV	10 µl 1:1	Ethanol	62% 75%	20 s 30 s	20 s 62-75%: FCV <1 $\log_{10}$, MuNoV 3 $\log_{10}$ 30 s 62%: FCV 2 $\log_{10}$, MuNoV 3.5 $\log_{10}$ 30 s 75%: FCV 2.2 $\log_{10}$, MuNoV 2.7 $\log_{10}$

Data collected by the author.

[1] HuNoV = human norovirus, MuNoV = murine norovirus, FCV= Feline calicivirus

2.10.3 Inactivation by physical inactivation methods

Physical inactivation methods are suitable for HuNoV inactivation on those environmental surfaces that cannot, or are difficult to treat effectively with chemicals (21). Physical inactivation methods

include heating, ultrasound techniques, pressure treatment, non-ionizing and ionizing irradiation. These inactivation methods against HuNoV on surfaces have not been widely studied, because researchers have mainly focused on studying the inactivation of the virus in suspension (124, 163-165). One study that did focus on inactivation of HuNoV surrogates on surfaces is reported by Schultz and co-workers, who in 2012 observed that a method combining heating and ultrasound inactivated MS2 almost completely, whereas the inactivation levels of two other surrogates, FCV and MuNoV, where also high but not complete, over 4 $\log_{10}$ PFU (166). Further details on the studies of inactivation of HuNoV on surfaces by physical inactivation methods are shown in Table 7.

2.10.4 Inactivation by ultraviolet light irradiation

UV is electromagnetic radiation with wavelengths shorter than visible light. UV can be separated into various frequency bands that range from 10 to 400 nm, including UVC between 280 to 100 nm. This narrow spectral range UV (more specifically 254 nm) is considered harmful to micro-organisms, including viruses for it disrupst the double-bond stability of adjacent carbon atoms in molecules including pyrimidines, purines and flavin (167). UV inactivation of micro-organisms results from the formation of dimers in RNA (uracil and cytosine) and DNA (thymine and cytosine). UV has been used for a long time to disinfect drinking water, natural water, and waste water (168). More recently, UV has been used in hospitals and other health care facilities to sanitize air and environmental surfaces (140, 169). Most publications that describe the sanitation effects of UV on HuNoV have been carried out as suspension tests (129, 149, 170-172). According to de Roda Husman and co-workers, UV at a fluence of 120 J/m^2 (12 mJ/cm^2) was able to cause a 3 $\log_{10}$ reduction of FCV in suspension (173).

Jean and co-workers studied the effects of UV on MuNoV on surfaces in 2011, and reported that UV was effective against this HuNoV surrogate, as long as the surfaces were free of soil (Table 7)(174). They also reported that the reduction of MuNoV on stainless steel and polyvinyl chloride (PVC) surfaces at a fluence of 60 mJ/cm^2 was 5 $\log_{10}$.

Table 7. Inactivation of HuNoV and its surrogates on surfaces by physical inactivation methods.

Reference	Virus[1]	Inoculum	Surface	Method	Time	Inactivation
(154)	MuNoV	3.8 g/l 50 µl	Stainless steel	UV	5 min	2.9 $\log_{10}$ PFU
(174)	MuNoV	5 $\log_{10}$ PFU	Stainless steel Polyvinyl chloride (PVC)	UV, 60 mJ/cm^2 Presence/ absence of foetal bovine serum (FBS), pulsed UV	2, 3 s	Stainless steel: Clean: 5 $\log_{10}$ FBS: 3.6 $\log_{10}$ PVC: Clean: 5 $\log_{10}$ FBS: 2.3 $\log_{10}$
(166)	MNV FCV MS2	6.43 $\log_{10}$ PFU 6.5 $\log_{10}$ PFU 9.8 $\log_{10}$ PFU	Petri dish	Steam-ultrasound, 160 dB, 130 °C	0–3.0 s	3 s: MNV 4 $\log_{10}$ PFU FCV 4.5 $\log_{10}$ PFU MS2 9 $\log_{10}$ PFU

Data collected by the author.

[1]HuNoV = human norovirus, MuNoV = murine norovirus, FCV= feline calicivirus, MS2= coliphage MS2

2.11 Discrimination between infectious and non-infectious HuNoV

Currently, the infectivity of HuNoVs can only be determined by human volunteer studies (40). These studies are very expensive and although the results that have been achieved by them are definitive, they are ethically problematic to carry out because there is no curative treatment to be given to those volunteers who develop gastroenteritis symptoms after being exposed to the virus and the volunteers suffer. The permission process to carry out volunteer studies can also be difficult. These foregoing reasons have entailed the following alternative approaches to be developed to study HuNoVs: determining the physical presence of HuNoV RNA and using cultivable surrogate viruses. Surrogate viruses have been reviewed in 2.4, whereas this chapter focuses on discrimination between infectious and non-infectious HuNoVs using RT-PCR or RT-QPCR.

PCR techniques can indicate the presence and quantity of HuNoV RNA in a sample by copying and indicating a part of a strand of nucleic acid that was transcribed from RNA to DNA. This strand of RNA must be intact for the copying to take place, but the rest of the genome, and also the viral capsid, can be damaged. A variable part of a virus batch used in the experiments consists of damaged viruses, therefore non-infective viruses are almost always present before inactivation studies are initiated. Thus it is not surprising that considerable difference in HuNoV surrogate persistence is reported between infectivity assays and RT-PCR assays (164, 175). Different pre-treatments will already have been included in the PCR assays to exclude the non-infective HuNoVs that were present in the sample prior the RT-PCR assay. Four approaches have been applied: PCR has been modified to target longer regions than usual to increase the probability of copying only intact genomes, infectious virus particles will have been captured exclusively so that only they are analysed in the PCR assay, non-infectious viruses will have been degraded by enzymatic activity, or they will have been bound so that the enzymes in PCR assay do not recognize them or they will have already been discarded before the assay (40).

Analysis of a long target region of the viral genome during amplification has been suggested to help in distinguishing non-infective and infective viruses, as the damage done to the viral genome is more likely to fall on multiple sites (176). Unfortunately, this approach has been reported to reduce the sensitivity of PCR detection, which can lead to a possible underestimation of the concentration of infective viruses in the samples.

An immunocapture technique has been suggested as an effective means to collect only infectious enteric viruses from environmental samples and thus distinguish them from non-infective virus particles (176). In this method, viruses are attached to an antibody, present on a surface of a paramagnetic bead, and then separated from the sample matrix by a magnet. The target virus can then be released from the antibody and subsequently detected by PCR methods. Although it is able to distinguish between infective and non-infective polioviruses, the method has been shown not to differentiate similarly another enteric virus, hepatitis A virus (177). The method is also highly dependant on the nature of the capturing antibody. Thus, the applicability of the technique for determining and quantifying HuNoVs remains unclear and requires further research.

In a widely used approach to degrading the virus samples by enzymatic activity, proteinase enzyme breaks up the protein coat of damaged HuNoVs and RNAase digests the viral RNA (171, 178), as seen in Fig 6. Protocols also exist were RNAase is used alone (179). In 2002, Nuanualsuwan and Cliver used

proteinase K enzyme to break down the damaged virus particles, which resulted in negative signals in traditional RT-PCR from inactivated FCV samples (171).

Although enzymatic pre-PCR treatment has been a more widely used approach to discriminate between infectious and non-infectious HuNoV, other treatments have also been described. One such approach is based on the binding properties of infective viruses to porcine gastric mucins, which results in the unbound virus particles being discarded and thus not copied in the PCR assays (168). Ganglioside GD1a (attachment receptor of MuNoV on RAW 264.7 cells) has also been suggested to apply as receptors in a similar manner (180). In another approach to binding-based RT-PCR detection of infectious HuNoVs, propidium or ethidium monoazide was allowed to penetrate through damaged or compromised virus capsids and bind covalently to viral RNA upon exposure to visible light, which made this bound RNA unavailable for amplification (181, 182). An alternative approach that is based on the detection of carbonyl groups produced by oxidation on viral capsid protein has also been described (183).

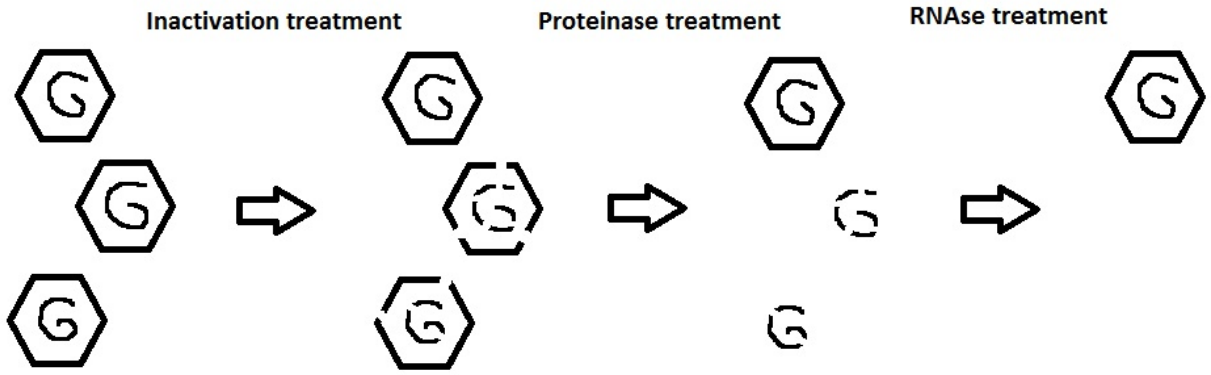

Fig 6. The principle of enzymatic pre-PCR treatment using proteinase and RNAse enzymes. Illustration by the author.

3 AIMS OF THE STUDY

The objectives of this work were to develop a feasible protocol for HuNoV detection in samples obtained from environmental surfaces and to apply this protocol in a laboratory setting for investigation of HuNoV transfer, in addition to field studies on the incidence of HuNoV on environmental surfaces. Further objectives of this work were to demonstrate the effectiveness of UV against HuNoV on environmental surfaces and to determine, whether infectious and non-infectious HuNoV particles could be differentiated by an enzymatic pre-PCR treatment.

The specific aims were to:

1. Optimize an environmental surface sampling protocol for two components of the methodology, namelly: the swab materials and the buffer solutions, then collect optimal recoveries of HuNoVs from different surfaces (I)
2. Determine the transfer of HuNoV from hands to gloves during gloving, and from contaminated hands of the food handler or from contaminated food ingredient to food servings during a simulated preparation of a cucumber sandwich (II)
3. Use the developed swab sampling protocol to investigate HuNoV incidence in a setting where a HuNoV outbreak was suspected and also in an environment where no outbreak had been reported (I,III, unpublished data)
4. Examine the persistence of HuNoV and its surrogate MuNoV to UV: by measuring the decrease of infectivity (MuNoV) and the decrease of viral RNA levels (both viruses) (IV).
5. Analyse whether the enzymatic treatment for virus particles could differentiate infectious and inactivated viruses from one another thus making the results of the PCR method closer to that of the true infectivity level (IV)

4 MATERIALS AND METHODS

An overview of the laboratory methods used in this thesis is given in Fig 7.

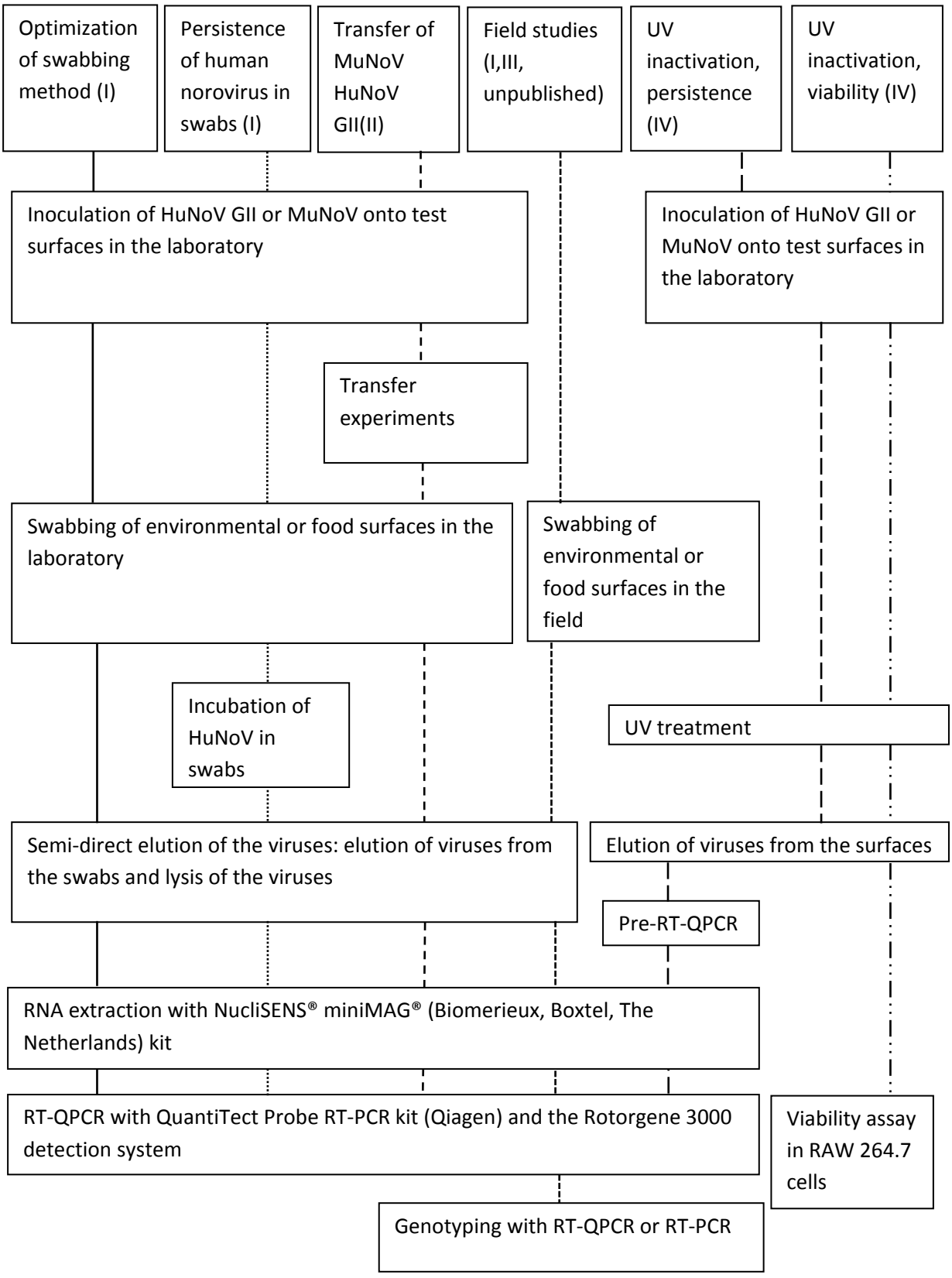

Fig 7. An overview of the laboratory methods used in the thesis.

4.1 Viruses (I-IV)

Three viruses, HuNoV GI, HuNoV GII, and MuNoV (MNV-1) were used in this research, as described in Table 8.

Table 8. An overview of the use of the viruses in the research

	Inoculation dose for each protocol		
Protocol	**HuNoV G II.4**	**HuNoV G I.6**	**MuNoV MNV-1**
Stock RT-QPCR	10 $\log_{10}$ pcr-u/ml	8 $\log_{10}$ pcr-u/ml	10 $\log_{10}$ pcr-u/ml
Stock infectivity assay	-	-	7 $\log_{10}$ PFU/ml
Optimization of swabbing	4 $\log_{10}$ pcr-u or 2 $\log_{10}$ pcr-u in 100 µl		
Persistence of HuNoV in swabs	2 $\log_{10}$ pcr-u in 100 µl		
Transfer of MuNoV and HuNoV while putting on latex gloves	6 $\log_{10}$ pcr-u in 100 µl		6 $\log_{10}$ pcr-u in 100 µl
MuNoV and HuNoV transfer during the manual preparation of deli sandwich	3.5 $\log_{10}$ pcr-u in 100 µl		3.5 $\log_{10}$ pcr-u in 100 µl
UV inactivation	8 $\log_{10}$ pcr-u in 100 µl		8 $\log_{10}$ pcr-u or 5 $\log_{10}$ PFU in 100 µl
Process control			4 $\log_{10}$ pcr-u in 5 µl
PCR positive control	3 $\log_{10}$ pcr-u in 5 µl	3 $\log_{10}$ pcr-u in 5 µl	3 $\log_{10}$ pcr-u in 5 µl

4.1.1 Human noroviruses (I-IV)

Stool samples that contained either HuNoV GII.4 or GI.6 were diluted into 10% suspensions in PBS (pH 7.2). The suspensions were cooled to 5 °C for 2 h and then frozen at -70 °C in aliquots. The concentrations of viral RNA in both of the virus stocks were estimated by RT-QPCR endpoint dilution (Table 8). A standard curve was plotted for sequential 10-fold dilutions of RNA, and one RT-QPCR detectable pcr-u was defined as the highest 10-fold dilution of the sample, which shows a positive result with $C_q < 40$.

4.1.2 Murine norovirus (I-IV)

MuNoV was obtained from Dr. Herbert W. Virgin at the Washington University School of Medicine (St. Louis) and was cultured in RAW 264.7 cells (ATCC-CRL-2278) in high-glucose Dulbecco's Modified Eagle's Medium (DMEM), which contained 10% heat-inactivated foetal bovine serum (FBS, Gibco), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 1% glutamine-penicillin-streptomycin. The cells were grown under standard conditions at 37 °C with 5% carbon dioxide (CO_2) and maintained according to animal cell culture protocols (184). Cells from passage 2 to passage 20 were used for the experiments.

MuNoV stock was produced by cultivating the viruses on confluent RAW 264.7 cell monolayers for 2 to 3 days, until the cytopathic effect was observed. The virus stock was prepared according to Park and co-workers (2011) by ultrafiltration of the cell lysate (Amicon Ultra-15; Millipore, Billerica, MA, USA) at 4500 x g for 10 min at 4 °C, and filtration through a 0.2 µm syringe filter prehandled with Polysorbate 20 solution (Sigma, Saint Louis, MO, USA) (149). The resulting MuNoV stock was stored

in aliquots at -70 °C. The concentration of viral RNA was estimated to be similar to those of the HuNoVs by RT-QPCR endpoint dilution (Table 8).

4.2 Swabbing method (I-III)

The swabbing method was used in the studies I, II, and III, and in the one-year HuNoV prevalence study (unpublished data).

4.2.1 Swabs (I-III)

Four swabs: polyester sticks (175KS01, Mekalasi Oy, Finland), flocked nylon sticks (500C50, Mekalasi Oy, Finland), cotton wool sticks (Selefa Trade, Sweden), and microfiber cloths (Taski Microcare, Novakari Oy, Finland) were all compared to obtain an optimum swabbing method for HuNoV on environmental surfaces (I). The microfiber cloths were previously tested by Suvi Laukkanen in her licenciate thesis (185). Two lots of these microfiber cloths (1 manufactured in 2010 and 2 manufactured in 2011), both similarly made of 85% of polyester and 15% of polyamide, were cut into 1.5 cm x 5 cm pieces and riveted to a holder to handle them with tweezers. Microfiber 2 cloths and cotton wool swabs were used in the field study, which surveyed the presence of HuNoV on environmental surfaces in companies manufacturing RTE food products (I and unpublished data). Polyester swabs, consisting of a polyester tip and nylon handler, were used in transfer studies (II). 3M™ EnviroSwabs (ENVSWB25, 3M, Finland) were used in HuNoV outbreak investigation (III).

4.2.2 Test surfaces in the laboratory (I,II)

Cucumber and three environmental surfaces, low-density polyethylene or PELD plastic, stainless steel, and latex surfaces, were used in the optimization of the swabbing method (I). For transfer studies, cucumber and latex glove surfaces were also used, in addition to sterile stainless steel knives and plastic pipette tip box covers, which are referred henceforth in this thesis as ‘breads’ (II). Hands of the researcher, who participated in the study as study subject, were washed with soap and water and left to dry before inoculating MuNoV on them or putting latex gloves on for the inoculation of HuNoV (II).

4.2.3 Inoculation of the surfaces before swabbing in the laboratory (I,II)

HuNoV GII and MuNoV stock solutions, which were used for the inoculation of all test surfaces, were diluted in sterilized water to the planned concentrations according to Table 8. The 5 cm squares on the test surfaces in the optimization study and the persistence study (I) were inoculated evenly with one 100-µl portion of either virus. In the transfer studies (II), the cucumbers were inoculated in similar a manner to that used in the optimization study. The palms of the bare hands and the palms of the latex gloves on the gloved hands of the palm area were evenly inoculated. Drying times at room temperature (RT) in a fume hood before sampling were as follows: overnight for environmental surfaces inoculated with HuNoV in study I, 60 mins for cucumber surface inoculated with HuNoV in study I, 60 mins for environmental surfaces in the persistence evaluation study (I), and 60 mins for all samples in study II.

4.2.4 Buffer solutions and swabbing method (I-III)

Swabs were moistened in their assigned flasks, which contained 2 ml of either PBS (pH 7.2) or 50 mM glycine buffer pH 9.5 (subsequently referred to as glycine buffer). The exception was 3M™ EnviroSwabs, which were moistened with 5 ml of PBS. Swabbing was done by wiping the surface rapidly in an up and down motion while steadily moving across the surface. After careful wiping for

one minute, swabs were placed back in their flasks, which contained either one of the two buffer solutions mentioned above.

4.3 Persistence study setting (I)

On four consecutive days in two subsequent weeks, PELD plastic surfaces were inoculated with HuNoV GII, then left to dry for 60 min, and swabbed with PBS or glycine buffer moistened microfiber 2 cloths, as described under heading 4.2, on each day of the experiment. After swabbing, the cloths were returned to their tubes and were placed on a horizontal plane at either 4°C or 22°C until the end of the experiment.

4.4 Swabbing in field studies (I,III, unpublished data)

In the field studies, swab samples were taken from those surfaces that were frequently touched by bare hands. In the companies producing RTE foods, sampling focused on the production lines, the break rooms and the restrooms of the companies. In the resort, sampling took place in the common areas, the quest rooms, and the kitchen. A summary of the field studies is shown in Table 9.

Table 9. Swabbing in field studies

Study	Year	Swab, elution buffer	Person responsible for swabbing	Examples of swabbing sites	Number of samples	Delivery of the samples to the laboratory
HuNoV prevalence (I)	2010 2012	Microfiber 2 cloth, cotton wool swab, glycine buffer	Author	Production line: control panels Break rooms: coffee machine Restrooms: toilet seat	172	Transport in cooled styrofoam box within 12 h
HuNoV prevalence, one-year study (unpublished)	2011	Microfiber 2 cloth, PBS	Food industry employee	Break rooms: coffee machine, microwave oven, refrigerator Restrooms: toilet seat	168	Transport by mail at variable temperatures within 3 d
Outbreak investigation (III)	2012	3M™ EnviroSwabs, PBS	Outbreak investigator	Resort centre: Kitchen: freezer door handle Common rooms: door handles The quest rooms: toilet seat	36	Transport in styrofoam box at 4°C within 12 h

4.4.1 One-year study on the prevalence of norovirus, questionnaire

A questionnaire study for food industry employees was conducted during 2011 with swab sampling in the one-year study (Table 9). The questionnaire was in two parts: the first period exclusively covered to the first seven months of the year (January—July, 2011) and the second, the remaining year (August—December, 2011). The respondents were asked how many times and when they had experienced gastroenteritis symptoms during the two follow up periods, as well as if they had had contact with symptomatic family members. The respondents were also asked if they had worked while symptomatic or if they had returned to work immediately after they had recovered.

4.5 Virus transfer trials (II)

The transfer study consisted of two parts. First, the transfer of HuNoV and MuNoV was monitored in samples obtained from transfer from virus contaminated hands to latex gloves during gloving. Second, the transfer from virus contaminated donor surfaces to recipient surfaces was observed after simulated preparation of a cucumber sandwich. Inoculation of the viruses on all the test surfaces was carried out as described under heading 4.2.3, doses according to Table 8. Swabbing was performed as also described under heading 4.2.4, using polyester swabs and the glycine buffer solution.

4.5.1 Transfer of MuNoV and HuNoV while putting on latex gloves

The transfer of MuNoV from bare hands to gloves was observed, as was the transfer of HuNoV from worn latex gloves to a clean pair of gloves. Gloving was performed the same way in every trial. The test person took the gloves from a container with the left hand and then put a glove on the right hand first and then the left hand. HuNoV transfer during gloving was not only tested after a drying period of 60 min post inoculation, but also without drying.

4.5.2 Transfer of MuNoV and HuNoV during the manual preparation of a sandwich

To test virus transfer between surfaces in the process of manually preparing a cucumber sandwich, MuNoV or HuNoV was seeded onto the test person's latex gloved right or left hand or on half of a cucumber before the manual preparation began. The preparation of the cucumber sandwich was performed as follows: (1) the right-handed test person took a hold on the cucumber with the left hand, (2) took the knife into the dominant right hand, (3) cut six slices off the cucumber, (4) and placed the slices on top of the bread with the right hand. Swab samples were taken from the following surfaces: (1) the palm and fingers of the glove of the right hand, and then (2) from the left hand, (3) the whole knife, (4) the outer surface of the cucumber, (5) the surfaces of cucumber slices, and (6) the top and sides of the bread.

4.6 Ultraviolet light inactivation (IV)

UV was generated by an ozone-free low-pressure mercury-vapour discharge lamp (Sylvania G15T8, London, UK) with the peak wavelength set at 253.7 nm and an output of 0.48 ± 0.02 mW/cm^2. The lamp was switched on for 10 minutes at room temperature (RT) to reach its maximum output before starting the experiments. Subsequently, the UV intensity was measured by a digital UVX radiometer (IL Metronic Sensortechnik GmbH, Germany).

Before the UV treatments commenced, HuNoV and MuNoV suspensions (Table 8) were inoculated on a circular thin layer of ⌀ 100 mm on a glass slide and dried in a flow hood for 1–2 hours at RT. The UV doses used in the viability assays were 0, 7.5, 15, 30, 60, 90, 120 and 150 mJ/cm^2, whereas doses in the PCR assay were 0, 30, 60, 90, 120, 150, 300, 450, 600, 750, 900 and 1800 mJ/cm^2.

4.7 Virus elution from swabs (I-III)

A semi-direct lysis method was used for virus elution from all swabs. First, swabs were shaken in an orbital shaker (IKAKS 2060 basic, Patterson Scientific, UK) at 250 rpm for 10 min at RT. Then, 4 ml (10 ml in case of 3M™ EnviroSwabs, study III) of NucliSENS® miniMAG® lysis buffer (bioMerieux, Boxtel, The Netherlands) was added to each flask and shaking was repeated to ensure the maximum elution of viruses from the swabs. RNA extraction was continued using the fluid content of the flasks. A total

of 6 ml of fluid was obtained. Out of 15 ml fluid content of the flasks used with 3M™ EnviroSwabs, 9 ml was stored at -21^{o}C.

4.8 Virus elution from glass slides (IV)

MuNoVs that were used for the infectivity assay were eluted from the slides with 500 µl of DMEM containing 2% foetal bovine serum 100µl at a time with back-and-forth pipetting and the infectivity of the virus samples was determined by a viability assay. HuNoVs and MuNoVs that were being prepared for RT-QPCR detection were eluted from the slides with 500 µl of sterile H_2O.

4.9 Viability assay (IV)

The viability assay for MuNoV is described in the paper authored by Verhaelen and co-workers (128). In short, 100 µl aliquots of each dilution of concentrated MuNoV, prepared in DMEM with 2% instead of 10% FBS, were seeded into a 96-well plate, that contained 2×10^4 RAW 264.7 cells/well (Nunc A/S, Roskilde, Denmark). Each dilution was added to six parallel wells on the plate and the plates were incubated in the standard conditions and checked daily for the presence of a cytopathic effect. The tissue culture infectious dose ($TCID_{50}$/ml) and PFU/ml were calculated using the protocol described by H. Morales (186).

4.10 Pre-RT-QPCR treatment (IV)

The protocol for the pre-RT-QPCR was modified from that described earlier (123, 171). The protocol was optimized by testing three doses of pronase, 3 mg, 6 mg, and 9 mg and two doses of RNAse, 0.02 mg and 0.04 mg before carrying out the pre-RT-QPCR treatment. The following protocol was selected for the study: 6 mg of pronase enzyme was dissolved in sterile H_2O (200 mg/ml, Roche) then mixed with the virus sample and the whole was shaken at 37 °C for 10 min. The reaction was stopped by adding 2 µl of 200 µM phenylmethane sulfonyl fluoride (Aldrich Sigma Canada Ltd., Oakville, Ontario, Canada) to the suspension, and the suspensions were left at RT for 15 min. RNAse (0.04 mg, Roche Diagnostics, Indianapolis, IN, USA) was subsequently added and the mixtures were incubated at 37 °C for 40 min, after which 80 U of RNAse inhibitor solution (Promega US, Madison, WI, USA) was added. The protocol was then continued with the RNA extraction phase.

4.11 RNA extraction (I-IV)

RNA extraction was performed according to the manufacturers instructions given in the NucliSENS® miniMAG® (Biomerieux, Boxtel, The Netherlands) kit, apart from the following steps: 60 µl of magnetic beads were added to the sample tube instead of 50 µl in studies I, II and III, and the samples were shaken in an orbital shaker at RT at 150 rpm for 10 minutes instead of incubating them without shaking.

4.12 RT-QPCR (I-IV)

Detection of the viruses was performed using a QuantiTect Probe RT-PCR kit (Qiagen, Hilden, Germany) with primers and a probe, showed in Table 10, specific to each virus. Each reaction used a 20 µl volume that contained 10 µl of QuantiTect Probe RT-PCR Master Mix, 0.2 µl of QuantiTect RT mix, 0.4 µl of RNAse-free water, 1 µM each of both primers, 0.2 µM of probe, and 5 µl of the sample RNA solution. Amplification was performed with the Rotorgene 3000 detection system (Corbett Life Science, Sydney, Australia) under the following conditions: initial activation for 25 min at 50°C, second activation for 15 min at 95°C, followed by 45 cycles, during which two phases, one cycle at 95°C for 15 sec and another at 60°C for 60 sec, rotated. Measurements of fluorescence were

performed after the annealing step. In the data analysis, the threshold of the PCR was set at 0.02 with a cut-off of 40.

Table 10. Primers and probes used for RT-QPCR (I-IV)

Virus	Primer +	Primer -	Probe	References
HuNoV GII	QNIF2d (+)	COG2R (-)	QNIFS (+)	(187)
HuNoV GI	QNIF4(+)	QNIF3 (-)	JJV1P	(112)
MuNoV	MNVfor	MNVrev	MNV	(188)

4.13 Genotyping/Sequencing (I,III)

HuNoV positive samples from study I and from the one-year study of HuNoV prevalence (unpublished data) were screened for GII.4 genotype using a specific set of primers (F1 5'-act ctc tgt gca ctc tcc gaa gt-3' and R2 5'-gct ttg ctg tca act tct ctg g-3') published in 2012 by Maunula and co-workers (189). The samples were subjected to RT-QPCR using a SYBR green I PCR kit (Qiagen, Venlo, the Neatherlands) and Rotorgene RG-3000 PCR cycler. A melting curve analysis was performed, and samples with a melting temperature of 79 ± 0.5 ^{o}C were regarded as positive.

Sequencing of the HuNoV isolates in study III was done as follows: HuNoV RNA was amplified using a one-step RT–PCR kit (Qiagen, Hilden, Germany) according to the method described by Vinjé and co-workers with a degenerate primer set from the capsid protein (VP1) region (190). After this, nucleic acid sequences of the amplicons were determined at the DNA Sequencing Service, in the Institute of Biotechnology, University of Helsinki. A genotyping tool (www.rivm.nl) and BLAST search in Genbank were used for genotype determination.

4.14 Controls

The controls in the studies are presented in Table 11.

Table 11. Controls used in the studies (I-IV)

Study	Control							
	Sample handling			RNA extraction	RT-QPCR, RT-PCR			
	Parallel samples	Process control	Negative sample	Negative sample	RNA duplicates	Positive samples[1]:	Negative sample	Non-template control
Optimization of swabbing (I)	+ (2)		+	+	+	+	+	+
Persistence of HuNoV in swabs (I)	+ (3)		+	+	+	+	+	+
Transfer while putting on latex gloves (II)	+ (3)		+	+	+	+	+	+
Transfer during the manual preparation of a deli sandwich (II)	+ (3)		+	+	+	+	+	+
HuNoV prevalence (I)		+		+	+	+	+	+
One-year study (unpublished)		+		+	+	+	+	+
Outbreak investigation (III)		+		+	+	+	+	+
UV inactivation (IV)	+ (4 * 3)		+	+	+	+	+	+

[1] HuNoV GI, HuNoV GII and/or MuNoV depending on the expected viruses in the samples

4.15 Virus recovery rate and transfer rate calculations (I,II,IV)

The recovery rates were calculated as the pcr-u count expressed as a proportion of the viruses recovered from surfaces divided by the observed original pcr-u count of the virus inoculation dose multiplied by 100% (I, II, IV). The transfer rates were similarly calculated by expressing the pcr-u count of the acceptor surface as a percentage of the inoculation dose (II). The calculated transfer rates in study II were converted from the transfer rates by the following pattern: transfer rate x (100/observed virus recovery rate of the acceptor surface from study I). The inactivation levels were measured by comparing the titres of the viruses after the inactivation treatment to the titres of the viruses before the treatment (IV).

4.16 Statistical testing (I,II)

Statistical analysis for study I was performed using one-way analysis of variance (ANOVA), used to compare means of two or more sample groups, followed by the Bonferroni post-test with a cut-off of 0.05 to counteract the problem of multiple comparisons. Set as statistical significance the analysis

was performed using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com). HuNoV and MuNoV transfer rates in study II were analyzed statistically with the student's t-test in SPSS software (SPSS Statistics, International Business Machiness, New York, USA) to determine if two sets of transfer data were significantly different. Significance was determined at the 0.05 level.

4.17 Statistical models (II,IV)

Two Bayesian statistical models, constructed by Antti Mikkelä, Evira, were used in the thesis. The first was used for analysing HuNoV and MuNoV transfer studies. The second used the data and the information from the publication by Qian and co-workers (191). Computations of the models were performed with WinBUGS 1.4.3 or OpenBUGS software (192). The number of MCMC iterations was 10000.

4.17.1 Statistical model used for the transfer studies (II)

The data consisted of the calculated MuNoV and HuNoV transfer efficiencies converted from the raw pcr-u transfer data, based on the findings that the two viruses transferred in similar quantities during the simulation study.

The aim of the modelling was to evaluate the extent of HuNoV contamination in the prepared cucumber sandwiches and their associated contact surfaces, when the virus contamination originated from either the hands of the food handler or from a single food ingredient. It was assumed that the observed transfer rates from hand to glove during the glove changing $\mathrm{Tc}^{\mathrm{hg}}$, from glove to food ingredient during contact $\mathrm{Tc}^{\mathrm{gf}}$, and from food ingredient to glove during contact $\mathrm{Tc}^{\mathrm{fg}}$, follow a Beta distribution:

$$\begin{aligned} \mathrm{Tc}_{\mathrm{i}}^{\mathrm{hg}} &\sim \mathrm{Beta}(\alpha_1, \beta_1), \ \ \mathrm{i}=1,...,11 \\ \mathrm{Tc}_{\mathrm{j}}^{\mathrm{gf}} &\sim \mathrm{Beta}(\alpha_2, \beta_2), \ \ \mathrm{j}=1,...,6 \\ \mathrm{Tc}_{\mathrm{k}}^{\mathrm{fg}} &\sim \mathrm{Beta}(\alpha_3, \beta_3), \ \ \mathrm{k}=1,...,6 \end{aligned} \qquad \text{(1, Antti Mikkelä)}$$

where i, j, and k denote the number of trials. A conventional uninformative Exponential (0.01) distribution was used as a prior for both of the parameters of the Beta distributions.

A predicted transfer rate from a food handler to food was $\mathrm{Tc}_{\mathrm{pred}}^{\mathrm{hg}} \cdot \mathrm{Tc}_{\mathrm{pred}}^{\mathrm{gf}}$, where the values of $\mathrm{Tc}_{\mathrm{pred}}^{\mathrm{hg}}$ and $\mathrm{Tc}_{\mathrm{pred}}^{\mathrm{gf}}$ were simulated from their posterior predictive distributions. The predicted transfer rate from food ingredient to food was $\mathrm{Tc}_{\mathrm{pred}}^{\mathrm{fg}} \cdot \mathrm{Tc}_{\mathrm{pred}}^{\mathrm{gf}}$, where the values of $\mathrm{Tc}_{\mathrm{pred}}^{\mathrm{fg}}$ and $\mathrm{Tc}_{\mathrm{pred}}^{\mathrm{gf}}$ were similarly simulated from their posterior predictive distributions.

The predicted amount of HuNoV contaminated food servings after repeatedly preparing cucumber sandwiches was modelled for two scenarios. In the first scenario, the hands of the food handler were assumed to contain initially loads from 1 to 4 $\log_{10}$ of virus particles before gloving and preparing a series of sandwich servings. In the second scenario, the first single food ingredient (cucumber) that the food handler touched before preparing a series of sandwiches was also assumed to contain a load that ranged from 1 to 4 $\log_{10}$ virus particles. The amount of HuNoV particles on gloves was

assumed to decrease during every contact so that the expected amount of virus particles remaining on the gloves after t preparations with the same gloves, $E(x_t)$:

$$E(x_t) = n^{gl} \cdot e^{(t \cdot \log(1 - Tc_{pred}^{gf}))} \quad \text{(2, Antti Mikkelä)}$$

where n^{gl} is the initial expected amount of HuNoV on the gloves, transferred from either hands or from the initial single food ingredient, $n_0^h \cdot Tc_{pred}^{hg}$ or, $n_0^f \cdot Tc_{pred}^{fg}$ according to chosen scenario 1 or 2. The expected amount of viruses in the next food serving y_{t+1} was then

$$E(y_{t+1}) = E(x_t) \cdot Tc_{pred}^{gf} \quad \text{(3, Antti Mikkelä)}$$

4.17.2 Statistical model for the UV inactivation studies (IV)

The aim of the modelling was to determine the relationship between UV doses and the reduction of MuNoV and HuNoV particles on dry surfaces. A Deviance Information Criterion was used to compare different regression models (192, 193).

The slope parameter (β_{uv}) and the precision parameter (τ) were estimated from the data. Both parameters were given practically uninformative prior distributions:

$$\beta_{uv} \sim \text{Norm}(0, 100^2)$$
$$\tau \sim \text{Gamma}(0.01, 0.01) \quad \text{(1, Antti Mikkelä)}$$

After testing several competing regression models, the following model fitted best to the data:

$$Y_{i,j} \sim N(\mu_{i,j}, \tau)$$
$$\mu_{i,j} = \alpha + \beta_{uv} \cdot \log(X_{i,j} + 1) \quad \text{(2, Antti Mikkelä)}$$

The response variable ($Y_{i,j}$) that used is the $\log_{10}$ inactivation of the MuNoV or the HuNoV. The values of the response variable (inactivation in the i^{th} UV dose level in the j^{th} trial) were analytically calculated from the data. The intercept parameter (α) of the model was fixed at zero, so that no inactivation should take place when the UV dose was zero.

5 RESULTS

5.1 Optimization of the swabbing method (I)

A previously existing protocol (104) was optimized for swab material and elution buffer for two inoculation doses of HuNoV in order to find a feasible swabbing method for detecting HuNoV on environmental surfaces. A semi-direct lysis technique, in which both the elution buffer and lysis buffer are used to detach viruses from swabs, was introduced as part of the swabbing protocol.

When 2 $\log_{10}$ pcr-u of HuNoV had been inoculated onto latex, plastic, and stainless steel surfaces and the virus-inoculated surfaces had been swabbed by one of the following: polyester, flocked nylon, microfiber, or cotton wool swabs, the virus was detected in all tested swab materials in every trial. The recovery rates were calculated as the mean recovery rate for the three environmental surfaces, and they varied from 27.8 ± 15.2% to 78 ± 15.1%, as seen in Fig 8. The difference in recovery rates between the swab materials was statistically significant ($p<0.05$) only when comparing the combination of microfiber 1 cloth and glycine buffer elution to the other materials and elution buffer combinations. HuNoV recovery rates for polyester swabs were slightly higher than the corresponding rates for cotton wool and flocked swabs, but this could not be confirmed statistically due to the high variance in recovery rates of single trials. Glycine buffer at pH 9.5 was beneficial for the recovery of HuNoV when the viruses were eluted from swabs of microfiber 1 cloth, flocked and polyester swabs, whereas eluting the viruses from swabs using PBS as the solution produced higher recovery rates for cotton wool swabs and microfiber 2 cloths. The lowest recovery rate was obtained from a latex surface was 11.1 ± 5.2% (flocked swab, PBS), whereas the two highest rates were 41.2 ± 9.0% (polyester swab, glycine buffer) and 66.2 ± 18.0% (microfiber 1 cloth, glycine buffer). The recovery rates from plastic surfaces varied from 25.1 ± 9.5% (flocked swab, PBS) to 88.7 ± 1.7 % (microfiber 1, glycine buffer), and the recoveries from stainless steel varied from 26.1 ± 12.6% (flocked swab, PBS) to 79.0 ± 10.2% (microfiber 1, glycine buffer).

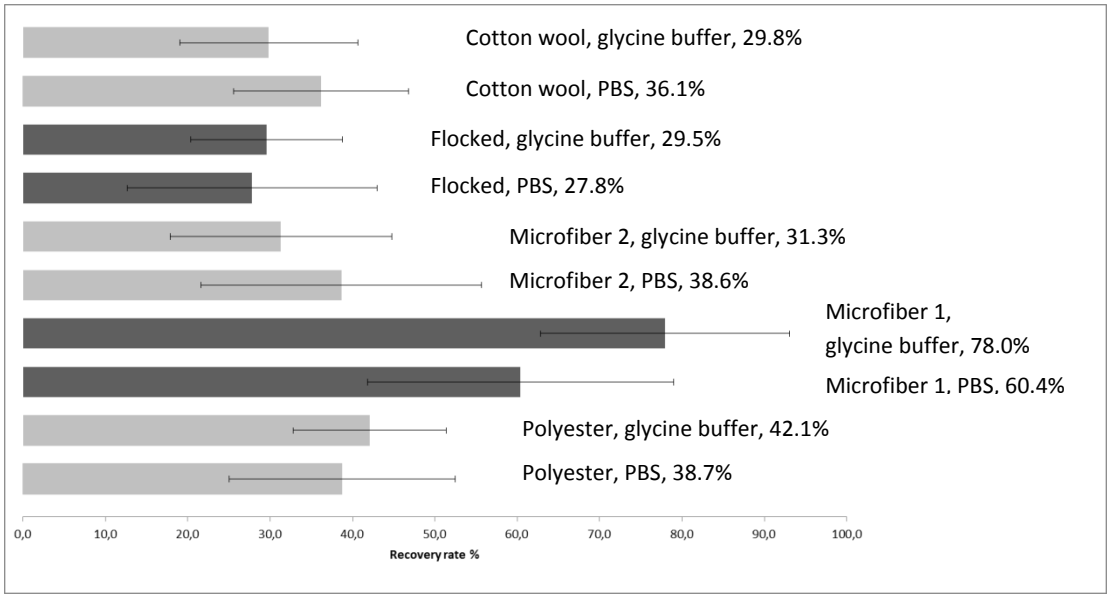

Fig 8. HuNoV recovery rates (%; mean ± SD) using the surface swab sampling protocol. Results of three environmental surfaces in study I, PELD plastic, stainless steel, and latex surfaces, have been combined. Inoculation dose was 2 $\log_{10}$ pcr-u in 100 µl (Table 8).

When the experiments were repeated with a 4 $\log_{10}$ pcr-u inoculation load of HuNoV, a dramatic decrease in recovery rates was observed, which affected the virus recovery rates obtained for all swab materials that were available and for all environmental surfaces. Only microfiber 2 could be acquired for this trial from batches of microfiber cloths. The recovery rates, calculated as an average of all tested environmental surfaces as described before, varied from 12.6 ± 0.9% (flocked swab, PBS) to 40.9 ± 10.8% (polyester swab, glycine buffer). The advantage of using either one of the two elution buffers was less clear on the results obtained with the higher of the two inoculation doses, except for the recovery rates of HuNoV obtained from latex surfaces: the rates were higher when glycine buffer was used in the elution of the viruses.

The mean recovery rates of HuNoV, which were calculated from the combined results of all swab materials were the lowest (7.6 ± 2.3%, PBS and 16.8 ± 3.6%, glycine buffer) when detecting viruses on latex surfaces, and the highest, (40.4 ± 6.2%, PBS and 43.1 ± 4.7%, glycine buffer) when detecting viruses on stainless-steel surfaces. When swabbing the outer surface of the cucumber, the lowest virus recoveries (15.8 ± 2.7%) were obtained for the polyester swab using the PBS buffer for the elution, and the highest (45.2 ± 5.2%) for the microfiber 2 cloth using PBS for the elution.

The stability of HuNoV in microfiber 2 cloths was evaluated as changes in ct-values at 4^{o}C and 22^{o}C on days 0, 1, 2, and 3, as seen in Fig 9. All samples remained positive for HuNoV over the incubation time regardless of the temperature or buffer solution used. The reduction in viral RNA was less than 1.4 $\log_{10}$ pcr-u during this time. The reduction curves at 4^{o}C between days 0 and 1, were less steep than at 22^{o}C. There was, however, no difference in overall reduction rates of HuNoVs between the two temperatures at the end of the experiment.

The HuNoV levels in microfiber 2 cloths at 4^{o}C that had been moistened with either PBS or glycine buffer, showed similar reduction curves with no statistical differences (Fig 9A). The reduction of HuNoV on day 1 was small, but the largest drop in virus levels was seen between days 1 and 2. At 22^{o}C, the reduction curves of the viruses on PBS- or glycine buffer - moistened cloths differed statistically ($p<0.05$) on day 1 (Fig 9B). HuNoV levels on PBS-moistened cloths were reduced only 1.5 $\log_{10}$ in 24 h, whereas the levels of the virus on glycine buffer moistened cloths was reduced by as much as 3.8 $\log_{10}$. On day 2, the difference between the two sample types was still pronounced, but on day 3 there was no longer difference between them.

Based on the stability results presented in Fig 9, buffer solution for the prevalence studies, shown in Table 9, was chosen case-by-case, taking into consideration the time from the swabbing to the start of laboratory analysis.

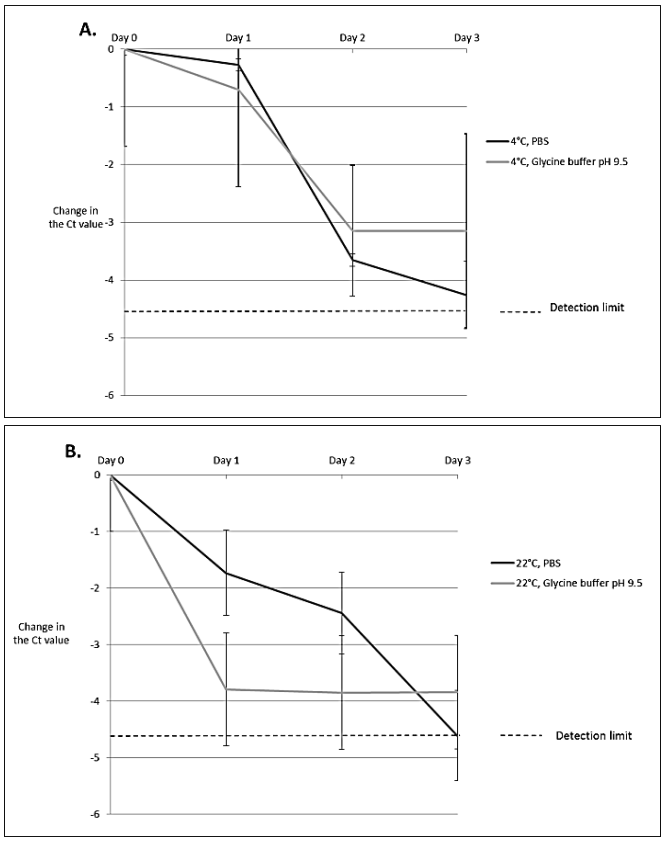

Fig 9. Reduction of HuNoV RNA levels in microfiber cloths that were moistened with either PBS or glycine buffer (pH 9.5) and incubated at 4°C (panel A) or 22°C (panel B).

5.2 Transfer of noroviruses (II)

Transfer studies on HuNoV and its surrogate MuNoV were conducted in two parts to enable the estimation for the extent of the virus transfer from bare hands of a food handler to the sandwich servings. These were the gloving experiment and the experiment on HuNoV or MuNoV transfer during the preparation of cucumber sandwiches. The two parts of the transfer study were then combined to mathematically estimate the expected transfer rates of HuNoV and the expected amounts of sandwich servings that could become contaminated by the virus, when the virus was present on the food handlers' hands prior to gloving and sandwich preparation.

5.2.1 Gloving experiment

MuNoV was used as a surrogate for HuNoV in the transfer experiments. Transfer from either hand to the latex gloves was observed repeatedly, overall 10/12 times (83.3%), as seen in Table 12. The transfer rates of MuNoV RNA to gloves varied from 0.1% to 7.0% when the left hand was contaminated by the virus and from 0.0% to 0.2% when the right hand was contaminated. The calculated transfer rates of virus particles revealed a difference in the rates between left or right contaminated hands, though this could not be verified statistically. The mean calculated transfer rate, converted from virus recovery rate of 33% for the latex surface, from the left contaminated hand to both recipient gloves was 6.1 ± 5.6%, while the corresponding value for the right hand was a mean rate of only 0.2 ± 0.1%.

When HuNoV was used in the experiments, the virus was inoculated onto the gloved hands before donning a clean pair of latex gloves. HuNoV RNA was transferred from the gloved hands to the clean donned gloves 10/12 times (Table 12). The transfer rates of HuNoV varied from 0.0% to 8.7% regardless of the inoculation site (left or right hand). No statistical difference in transfer rates was observed between the HuNoV and MuNoV results in these trials.

The transfer of HuNoV was also investigated by donning gloves immediately after inoculation of HuNoV. Virus transfer was then observed 11 out of 12 (91.7%) times. The mean concentration of viruses on the swabbed gloves (6.1 $\log_{10}$ pcr-u per hand) was higher ($P < 0.05$) when the virus inoculation remained wet than when it was left to dry (5.0 $\log_{10}$ pcr-u per hand).

5.2.2 Transfer of MuNoV or HuNoV during the preparation of cucumber sandwiches

MuNoV transfers between a donor surface (left-hand glove, right-hand glove, or outer surface of a cucumber) and acceptor surfaces (left-hand glove, right-hand glove, outer surface of a cucumber, cucumber slices, knife and bread) were investigated in the process of simulating a manual preparation of a cucumber sandwich (Table 12). The transfer of MuNoV from cucumber or individual gloves to the first acceptor surface was detected in every trial. The highest virus transfer rates in the trials were observed from a glove to cucumber (0.5–1.6%), followed by the rates from cucumber to a glove (0.4–1.3%). The smallest virus transfer rates were observed from a cucumber to a knife (<0.1%), from a glove to a bread (<0.1%), and from a glove to a knife (0.1-0.5%). MuNoV was more easily transferred from gloves than from cucumber to acceptor surfaces: the remnant mean recovery rate from artificially virus-contaminated glove surfaces was 5.8±5.7% and 6.6±6.1% for the left and right hand, respectively, whereas the remnant recovery rate from cucumber surfaces was 18.4±26.4%.

HuNoV was transferred from the donor surfaces to the acceptor surfaces in quantities similar to those of MuNoV. However, in one experiment, HuNoV was transferred from the cucumber surface to acceptor surfaces more extensively than MuNoV. HuNoV was also more widely transferred to the gloved left hand and a knife to the gloved right hand and to a bread. As in the MuNoV trials, more virus particles were transferred from the glove to a cucumber contact (0.2–4.3%) than vice versa (0.2–0.9%), although this could not be confirmed statistically.

Table 12. Virus recoveries, transfer rates, and calculated transfer rates of MuNoV and HuNoV between surfaces after gloving, and in the manual preparation of cucumber sandwiches (II).

Virus	Inoculation site	Inoculation dose	Surface	Virus concentration log_{10} pcr-u/ml	Recovery % (pos/total)	Transfer rate % (pos/total)	Calculated transfer rate % [1]
MuNoV	Left hand	6 log_{10} pcr-u					
			left hand	5.6±5.2		1.5±0.5 (3/3)	4.4±1.5
			right hand	6.0±5.2		2.6±3.2 (3/3)	7.8±9.6
	Right hand	6 log_{10} pcr-u					
			left hand	0.0±3.0		0.0±0.0 (1/3)	0.0±0.0
			right hand	4.5±4.4		0.1±0.1 (3/3)	0.3±0.2
	Cucumber	3.5 log_{10} pcr-u					
			cucumber	4.7±4.8	18.4±26.4 (3/3)		
			right hand	<1[2]			
			left hand	3.3±3.1		0.7±0.5 (3/3)	2.1±1.6
			knife	1.7±1.9		0.0±0.0 (1/3)	0.3±0.5
			cucumber slices	2.9±2.4	0.3±0.1 (3/3)		
			'bread'	<1			
	Left hand	3.5 log_{10} pcr-u					
			cucumber	3.6±3.1		1.2±0.6 (3/3)	5.4±3.1
			right hand	nc[3]			
			left hand	4.2±4.1	5.8±5.7 (3/3)		
			knife	<1			
			cucumber slices	<1			
			'bread'	<1			
	Right hand	3.5 log_{10} pcr-u					
			cucumber	nc			
			right hand	4.3±4.2	6.6±6.1 (3/3)		
			left hand	nc			
			knife	2.9±2.7		0.2±0.2 (3/3)	0.4±0.3
			cucumber slices	<1			
			'bread'	0.0±1.6		0.0±0.0 (2/3)	0.1±0.1

Table 12 continues

HuNoV	Left hand	6 $\log_{10}$ pcr-u					
			left hand	4.0±2.9		0.1±0.0 (3/3)	0.3±0.0
			right hand	4.2±4.0		0.1±0.1 (2/3)	0.4±0.3
	Right hand	6 $\log_{10}$ pcr-u					
			left hand	5.7±5.5		3.6±3.6 (3/3)	11.0±10.9
			right hand	4.4±4.5		0.2±0.3 (2/3)	0.7±1.0
	Cucumber	3.5 $\log_{10}$ pcr-u					
			cucumber	4.2±4.2	7.9±7.1 (3/3)		
			right hand	1.9±2.1		0.0±0.1 (1/3)	0.1±0.2
			left hand	2.9±2.7		0.4±0.2 (3/3)	1.2±0.7
			knife	3.3±0.3		0.4±0.1 (1/3)	0.1±0.1
			cucumber slices	2.3±2.5	0.0±0.1 (3/3)		
			'bread'	1.9±2.1		0.0±0.1 (1/3)	0.2±0.2
	Left hand	3.5 $\log_{10}$ pcr-u					
			cucumber	3.7±3.8		2.2±2.9 (3/3)	10.1±16.0
			right hand	nc			
			left hand	4.3±3.7	8.2±1.2 (3/3)		
			knife	<1			
			cucumber slices	<1			
			'bread'	<1			
	Right hand	3.5 $\log_{10}$ pcr-u					
			cucumber	nc			
			right hand	4.3±3.7	7.0±0.7 (3/3)		
			left hand	nc			
			knife	2.7±2.8		0.2±0.3 (2/3)	0.4±0.5
			cucumber slices	<1			
			'bread'	<1			

[1] When converting the calculated transfer rates the following recovery rates were used: outer surface of cucumber 22%, surface of plastic 27%, surface of stainless-steel 62%, and surface of latex gloves 33%

[2] Under the detection limit 1 $\log_{10}$ pcr-u

[3] No contact with virus

5.2.3 Expected transfer of HuNoV

Using the Bayesian analysis, which utilized the data from both gloving experiment and sandwich preparation simulation as prior information, estimations were formed of the probabilities for HuNoV transfer from hands to food servings during manual preparation of cucumber sandwiches. As a result of this analysis, HuNoV-contaminated hands of a food handler were calculated to transfer the virus during the preparation to more sandwich servings than a sporadic HuNoV contamination of a single food ingredient. It was calculated that if 3 $\log_{10}$ HuNoV particles were present on the hands of the food handler before gloving and food preparation, there would still be 50 % chance that the 8th cucumber sandwich would be contaminated with at least one infective HuNoV. If, however, the same amount of particles, i.e. 3 $\log_{10}$, were present on the surface of a cucumber, the probability of transfer to even the first serving would be less than 5%.

The expected number of HuNoVs on the first sandwich, when the hands of the food handler were contaminated with 3 $\log_{10}$ infective virus particles, would be less than 2 as has been illustrated in Fig 10 A. However, if the hands were contaminated by 4 $\log_{10}$ or more infective virus particles, the expected load of HuNoVs on the first serving would already be more than 10 virus particles, as seen in Fig 10 B.

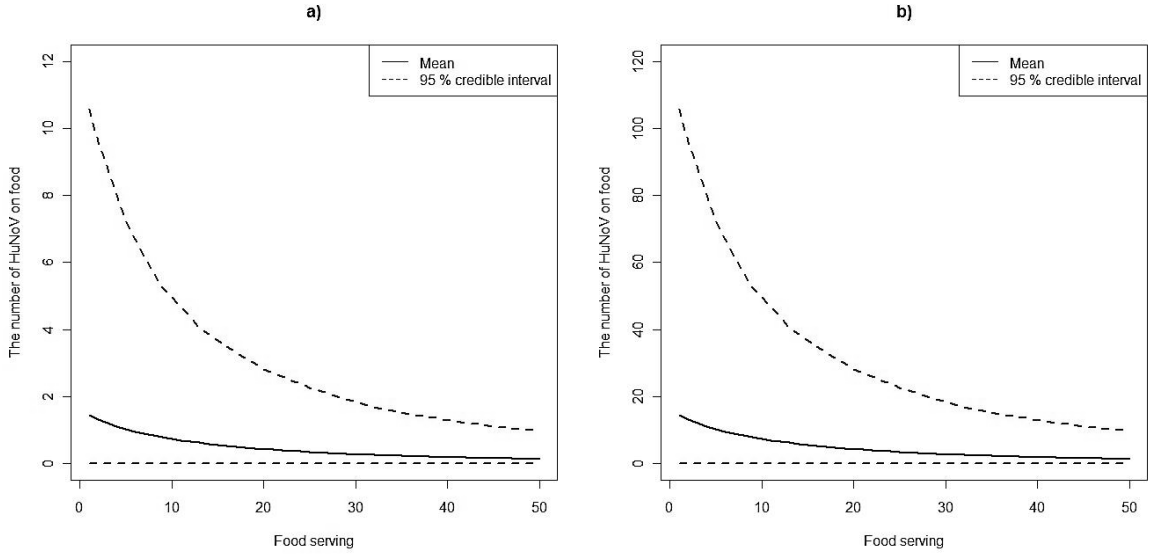

Fig 10. The expected number of HuNoV particles transferred on food after preparing a series (0-50) of food servings. The hands of the food handler had either 3 $\log_{10}$ (panel a) or 4 $\log_{10}$ (panel b) load of HuNoV particles before gloving and manual preparation of food servings.

5.3 Prevalence of noroviruses on environmental surfaces (I,III, unpublished data)

Environmental swabs were collected from food industry premises with no reported gastroenteritis outbreaks, an also from an activity centre where a suspected HuNoV outbreak had taken place. Although analysed for the presence of both HuNoV GI and GII RNA, only HuNoV GII was detected in the swab samples from both sites, as seen in Table 13.

In HuNoV prevalence studies conducted during spring in 2010, HuNoV was detected in five swabs, three of which were detected on the production lines that produced RTE foods and the rest on surfaces in break rooms. All positive findings from the production line and two of the findings from the break rooms were from a single company. In 2012, HuNoV was detected in three swabs collected from break rooms, in four swabs collected from restrooms in two separate companies that produced RTE foods. In a one-year prevalence study conducted in 2011, four positive samples were taken from break rooms in January, May and November from a single company. During the HuNoV prevalence studies in 2010-2012, 12 out of 16 of the HuNoV positive samples were typed as HuNoV GII.4 by screening primers.

In the resort centre where a HuNoV outbreak was suspected, HuNoV RNA was detected in 10 swab samples. HuNoVs could be sequenced from three of the swabs and revealed the genotype GII.4 Sydney_2012. After the virus had been detected in the resort, the resort was cleaned and more swab samples were taken to verify its success in eradicating the virus from the resort. The cleaning was not a complete success, for HuNoV was still detected in one room in the resort. After second round of thorough cleaning, HuNoV was no longer detected.

Table 13. Prevalence of HuNoV in field experiments in RTE companies and one resort

Study	Swab, elution buffer	Positive samples, genotype	HuNoV positive swabbing sites and surfaces
HuNoV prevalence (I)	Microfiber 2 cloth, cotton wool swab	12/172, 7% 10/12 GII.4	Production line: a handle of a knife, a cover of a box containing raw produce, gloves of an employee Break rooms: coffee machine, microwave oven Restrooms: toilet flushing knobs, door handles
HuNoV prevalence, one-year study (unpublished)	Microfiber 2 cloth, PBS	4/168, 2.4% 2/4 GII.4	Break rooms: coffee machine, microwave oven, refrigerator
Outbreak investigation (III)	3M™ EnviroSwabs, PBS	10/36, 27.8% 3/10 GII.4 Sydney_2012	Kitchen: freezer door handle Common rooms: door handles Hotel rooms: mattresses, toilet seats, tap handles, soap devices

A questionnaire was given to food industry employees to inquire about their gastroenteritis episodes. Data from the questionnaire and the study were related to the one-year study of HuNoV prevalence on the environmental surfaces of the food industry premises. The total number of respondents in the study was 190, which gave a response rate of 63%. According to respondents,

38.9% of the food industry employees had suffered gastroenteritis symptoms at least once during the first period of the study (January—July 2011) and 20.3% during the second. The peak of illness was in March and the symptoms had lasted for a mean of 2–3 days. About 50% of the respondents admitted working at least once while suffering from gastroenteritis symptoms.

5.4 UV Inactivation of noroviruses (IV)

UV inactivation of noroviruses was investigated by two means: MuNoV inactivation was measured using a viability assay and the reduction in viral RNA levels for both MuNoV and HuNoV was measured by using RT-QPCR. For each UV dose given two parallel sample groups were detected using RT-QPCR: one group was enzymatically pre-PCR treated with pronase and RNAse enzymes, and the other was not treated enzymatically. The results are shown in Figure 11.

5.4.1 Inactivation of MuNoV with UV irradiation

The levels of viable MuNoVs, measured in the RAW 264.7 cells, were observed to decrease in two phases: the virus titre decreased rapidly from 5 $\log_{10}$ PFU to 2.7 $\log_{10}$ PFU when the UV fluence was increased from 0 mJ/cm^2 to 7.5 mJ/cm^2, after which the decrease was more moderate, from 2.7 $\log_{10}$ PFU to 1 $\log_{10}$ PFU when the UV fluence was increased from 7.5 mJ/cm^2 to 60 mJ/cm^2. A total loss of infectivity was achieved at a fluence of 90 mJ/cm^2 at a 3 min time point.

5.4.2 Decrease in MuNoV and HuNoV levels measured using RT-QPCR

The decrease in virus levels detected by RT-QPCR, when the virus-contaminated surfaces were treated by UV, was UV dose-dependent and data between MuNoV and HuNoV were comparable, as seen in Figures 11 A and C, respectively. When the UV fluence was increased gradually from 0mJ/cm^2 to 300 mJ/cm^2, MuNoV levels decreased from 4.5 $\log_{10}$ pcr-u to less than 3.5 $\log_{10}$ pcr-u (Fig. 11 A). Positive PCR signals were, however, detected even when the UV fluence was as high as 1800 mJ/cm^2. The RNA reduction curve of HuNoV was less steep than that of MuNoV as the levels of HuNoV decreased from 4.4 $\log_{10}$ pcr-u to 3.8 $\log_{10}$ pcr-u when UV fluence was increased from 0 mJ/cm^2 to 300 mJ/cm^2 (Fig. 11 C).

Enzymatic pre-PCR treatment using pronase, RNAse, or both was validated before inactivation and virus stability experiments in study IV by using the same protocol on either MuNoV samples that did not receive UV treatment or samples that received maximum UV, 1800 mJ/cm^2. The combination of pronase and RNAse enzymes was chosen, for it was the only pretreatment option that did not show a positive signal for MuNoVs in RT-QPCR after treatment.

The addition of enzymatic pre-PCR treatment to the UV protocol caused a total decrease of 4–5 $\log_{10}$ in MuNoV levels when UV fluence was 1800 mJ/cm^2, as can be seen in Figure 11 B. The total decrease in pre-PCR treated HuNoV levels was somewhat smaller, 2.5–3 $\log_{10}$, with the same UV fluence, demonstrated in Figure 11 D.

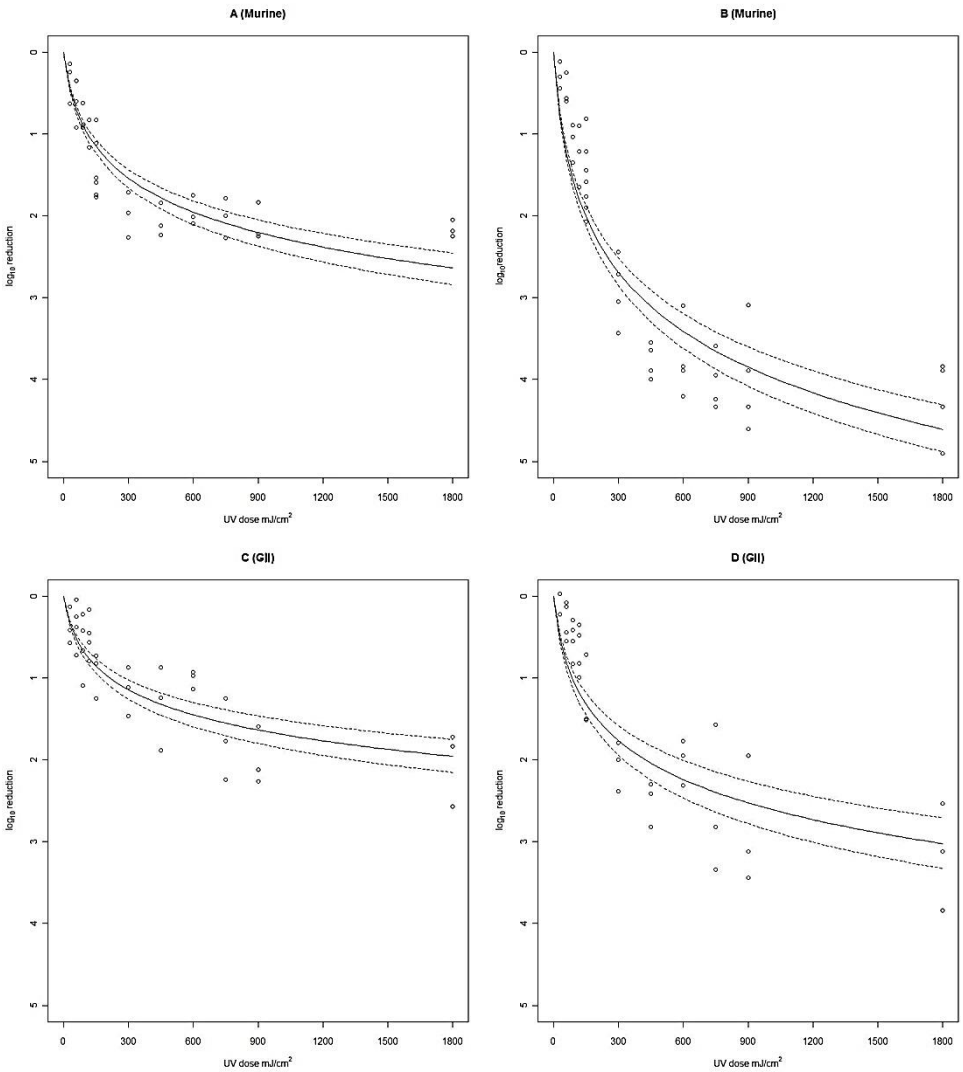

Fig. 11. Observed reduction rates (circles) and prediction distributions (dashed lines) for the expected reduction rates of MuNoV and HuNoV GII, either with no enzymatic pre-PCR treatment (A, C) or with enzymatic pre-PCR treatment (B, D) (IV).

6 DISCUSSION

6.1 Swabs as a tool in epidemiological investigations and prevalence studies (I,III)

Outbreaks of gastroenteritis that are caused by HuNoV have become a major public health concern, especially during the last decade (1). The modern lifestyle, which includes travelling and eating food that is intensively handled, facilitate HuNoV circulation in the population. Furthermore, the population is comprised of larger groups of immonocompromized persons, such as those suffering from cancer, to which the HuNoV infection could be serious, even fatal. Effective control and prevention of the disease depends, in part, on the identification of the source of contamination. Reports of HuNoV outbreaks indicate that in addition to the transmission of these viruses via food and water, contamination of environmental surfaces significantly contributes to the spread of this virus during outbreaks (16). The distribution of HuNoVs in the environment can be studied by detecting HuNoVs on environmental surfaces by swabbing (103). The swabbing protocol was fast and easy to follow. It has been successfully used in large follow-up studies, in continuous monitoring of environmental surfaces in food processing companies, catering companies, hospitals, and in elderly homes (100).

At present, researchers use multiple techniques to recover and quantify viruses, including HuNoV, on surfaces (103). The recovery rate of viruses, detected in swabs taken from environmental surfaces, depends on two features: the effectiveness of the swab to collect the viruses from environmental surfaces and the effectiveness of elution of viruses from the swab. The first is the interaction between the features of the virus, the swabbed surface, and the characteristics of the swab, whereas the second is a result of interaction between the swab, the viruses, and the elution buffer. Swabs rarely have any biological substances on their surfaces before swabbing and thus can not provide receptors for viruses. Therefore, the interactions between the virus and the swabs are purely non-biological.

Physical features of environmental and food surfaces, such as porosity seem to affect the fraction of HuNoVs that can be collected from these surfaces. In general, the easiest collection of HuNoVs is from smooth and hard surfaces such as ceramic and stainless steel (104, 107), as also seen in study I. Most HuNoV findings in epidemiological studies have as well been obtained from hard and non-porous surfaces, such as toilet seats and toilet handles and taps (100). Gloves of food handlers have been rarely examined for HuNoVs in field studies, but in the laboratory, HuNoVs have been recovered from various glove materials. Stals and co-workers (2013) reported that the recovery rate for HuNoVs in swabs of vinyl gloves was approximately 40% (108), whereas the recovery rates for latex gloves in our study were lower, 30% with a 2 $\log_{10}$ inoculation dose or a mean of 21% for inoculation doses 2 $\log_{10}$ and 4 $\log_{10}$. In contrast, Suriyanarayanan and co-workers reported higher recovery rates from latex than from vinyl gloves (194), by using the glove juice technique where buffer solution is poured inside the gloves on hands and virus is recovered from the buffer (195). Recovery rates obtained from different surfaces varied in our study despite our efforts to achieve a controlled test environment, as seen also in other studies (104, 108). The high variability may be due to the heterogeneous structures of test surfaces, variable attachment of the virus to these surfaces, virus aggregation and the low number of repeated experiments.

Virus removal from environmental surfaces can be interpreted as virus recovery of the swab. Microfiber cloths have been shown to remove 3.5 $\log_{10}$ PFU of HuNoV surrogates MuNoV and FCV

from solid surfaces by wiping, which indicated these were potential swabbing materials for HuNoV sampling (105). In a recent review of virus surface sampling methods, the authors observed that several papers reported that viruses have been detected from surfaces most reliably with antistatic sampling materials, such as microfiber cloths, followed by polyester, cotton and rayon swabs. Similarly, the highest mean recovery rates of MuNoV and HuNoV in this study were achieved when microfiber cloths were used for sampling. However, even small differences in microfiber cloths, such as electrical charge, seem to affect virus recovery rates, as was seen in our study with microfiber 1 and microfiber 2 cloths, whose material composition and the weaving were reported to be identical, but their performance differed. A feasible explanation might be that the production process of microfiber cloths may have changed between manufacturing the two batches of cloths used in the study, which would influence virus attachment or release.

Glycine buffer at pH 9.5 was seen slightly more effective than PBS at pH 7.2 in eluting viruses from microfiber cloths and polyester swabs in our study. The electrochemical adhesive interactions between the glycine buffer moistened swabs and the viruses may have been stronger than when swab had been moistened in neutral buffer. The persistence of HuNoV particles and RNA in swabs was, nevertheless, hindered if the swabs were moistened by glycine buffer instead of PBS before sampling and the persistence of the viruses was measured at RT. Biophysical methods have shown that decreased persistence of HuNoV-like particles can result from changes in the secondary structure of HuNoVs at pH levels over 8 (122).

In the three field studies included in this present research, the swab samples were collected by different persons who used different swab materials and elution buffers. Nevertheless, HuNoV could be detected in swabs in all three studies (Table 13). In Finland, the HuNoV incidence according to the register by National Institute for Health and Welfare during seasons 2010, 2011 and 2012 was 2 700, 1600 and 1800 cases respectively (Fig 3). It thus seems that strains of HuNoV, including Sydney_2012 variant at the time of the outbreak investigation study (III) in 2012, were circulating actively in the Finnish population during the time of the field studies. Not surprisingly, the highest fraction of HuNoV positive samples was found during the outbreak investigation (27.8%), although HuNoV has been detected even more often in swabs taken during outbreak investigation in hospitals (196).

Prevalence study in food manufacturing companies with no known HuNoV outbreak showed that the fraction of positive findings was somewhat higher than the prevalence of 1.7% reported in 2011 by Boxman and co-workers for environmental swabs taken in catering companies (101). One reason for this may be that Boxman and her group collected the swabs from restaurants throughout a single year, whereas in the current study the prevalence was investigated at the same time in the springs of 2010 and 2012, when HuNoV prevalence in the population is usually at its annual peak (22). The prevalence of HuNoV in our one-year study was only 2.4%. One reason for this may be that swabs were transported to the laboratory by mail under varying temperature conditions, which may have decreased the level of HuNoVs in the swabs under the detection limit of the sampling method. In further studies, alternative means for transport could increase the amount of HuNoV findings from swab samples. According to the questionnaire, responses of the employees of the RTE companies, conducted during the follow-up year, the employees had mostly suffered from gastroenteritis in the early spring. Two of our positive HuNoV findings were also from that same time period.

6.2 The role of food handlers in norovirus transmission (I,II,III)

Food workers have been one of the known sources of foodborne HuNoV outbreaks for as long as HuNoV outbreaks have been recorded, and it seems the trend is continuing (16). Multi-ingredient foods have been most frequently noted to be involved in foodborne HuNoV outbreaks, perhaps because of the intensive handling during preparation, which may have increased the chance for contamination (14). In HuNoV outbreaks where a food handler connection has been confirmed, a deli sandwich has often been a vehicle of transmission (Table 2).

The use of protective gloves is generally considered to prevent microbial contamination of food effectively by the food handler, as long as the gloves are intact and properly used (197). Indeed, data collected by Todd and co-workers (2007) found that, bare hand contact of the food handler with food was responsible for 105 out of 376 investigated foodborne HuNoV outbreaks, whereas gloved hand contact with the food had been responsible for only one of the outbreaks (80). Several laboratory studies show, however, that when they are contaminated, gloves can transfer HuNoVs to food quite easily (108, 133). Contamination of protective gloves while gloving repeatedly happened in study II. According to a volunteer study by Liu and co-workers (2009), the levels of HuNoVs on the hands of infected individuals could lead to the contamination of the gloves when gloving (20). The HuNoV surrogate, MS2, has also been observed to transfer from protective gloves to hands while removing the gloves (195). Proper hand hygiene, including proper hand washing, not just before gloving but also when changing the gloves should be strictly followed, but it is not always performed in real life (198). Education and regular remainding of hand hygiene would benefit the efforts to prevent HuNoV outbreaks originating from food handlers.

Transfer of HuNoV between gloves, environmental surfaces, and food has been documented in laboratory conditions (106, 108, 126, 133, 135, 137, 139). It seems that only few previous reports on HuNoV transfer during simulated preparation of sandwiches have been published before study II. Our finding that HuNoV and its surrogate are transferred more easily from hands to food ingredients than from hands to stainless steel is supported by the observations of Bidawid and co-workers in 2004 (135) and Stals and co-workers in 2013 (108). It was observed in study II that gloves are an efficient donor surface compared to food or smooth and hard environmental surfaces. This result is in agreement to that found by Bidawid and co-workers, who observed that the transfer rates of FCV in the direction from bare fingers to food and environmental surfaces were much higher than opposite direction (135).

The transfer of HuNoV and MuNoV in study II was detected by RT-QPCR, as has been reported in previous reports (106, 108, 126, 133, 137). Although RT-QPCR cannot discriminate between infectious and non-infectious particles transferred between the hands, gloves, and food products during sandwich preparation, the determination does give direct information on the risk of contamination by infective viruses in preparing RTE foods. Sandwiches are not usually heated or otherwise sanitized after their preparation. Therefore, if the contaminated sandwiches are consumed soon after their preparation, the infectious HuNoVs transferred from the gloves of the food handler to the food would still be infectious.

It was estimated in study II that 4 $\log_{10}$ pcr-u or higher loads of HuNoV on contaminated hands would lead to contamination of approximately all the sandwich servings prepared after gloving on the same working shift despite covering of the hands with gloves. The quantitative exposure model of

Mokhtari and co-workers (199) and the recent HuNoV transfer model described by Verhaelen and co-workers (128) support our findings that hands are a significant vehicle in HuNoV transmission during the processing of RTE foods. In our model, the transfer of only one infective virus particle was considered sufficient to cause contamination of gloves, utensils and food servings. However, in reality, the collecting of HuNoV particles, on aggregates containing from two to hundreds of infectious viruses, could impact upon the transfer rates of HuNoVs from surfaces to surfaces and result in higher or lower transfer rates in food preparation situations (200).

Food handler involvement in the HuNoV outbreak in the resort, described in study III, was suspected, as HuNoV was detected on the freezer door handle and the kitchen tap handle, which indicated that contaminated hands had been touching them. As is often the case (16), no stool samples from the staff and no samples of the food that had been served was available for analysis. The general hygiene of the centre's kitchen was visually good and none of the kitchen staff had been symptomatic at work about the time of the outbreak. After the outbreak it was impossible to tell if the food handlers had fallen ill after the initial case of HuNoV had already contaminated the resorts surfaces, or if one of the food handlers was the initial case, and had transmitted the disease to served food and to environmental surfaces.

In order to exclude food handler involvement in the future, a regular sampling to detect HuNoVs in the kitchens preparing RTE foods should routinely occur. Swabbing could be combined with the regular sampling of environmental surfaces, that is designed to detect bacteria and food residues, and which is already in use to ensure food hygiene is maintained in many countries (201), including Finland (based on the General food hygiene regulation 852/2004/EU)(202). Similarly, regular sampling of surfaces in hospitals, in the military and other instances prone to HuNoV outbreaks would help the staff to prepare to the HuNoV outbreaks by, for instance, requesting diligent hand washing and by increasing the cleaning effords on surfaces.

6.3 Inactivation of noroviruses on surfaces by UV irradiation (IV)

The European Commission regulation on microbiological criteria for foodstuffs (EC No 2073/2005 2005) declares that food should not contain micro-organisms, including HuNoV, in quantities that present an unacceptable risk for human health (203). The very low infective dose of HuNoVs, and their easy transfer from environmental surfaces to foodstuffs, raises the urgent need for effective inactivation methods for reducing the levels of these viruses upon food contact- and upon contact with other environmental surfaces. UV is considered to be an important alternative to chemical disinfection of micro-organisms and it has been used to inactivate bacteria in a hospital environment (204-206), including air, surfaces, and instruments (140). Although UV has been reported to inactivate HuNoV surrogates and probably HuNoV itself, efficiently both in suspension and on environmental surfaces (chapter 2.10.4), existing HuNoV outbreak management guidelines still require more research to be able to recommend their effective use for the disinfection of HuNoV on environmental surfaces (21, 207). Targeted studies on the effectiveness of UV towards HuNoVs would help in determining practical applications for the use of UV on surfaces and would propably lead to recommendations of its use by the authorities preparing the outbreak management quidelines.

The efficacy of UV in reducing the levels of HuNoV on surfaces has been shown to be dependent on many different factors such as irradiation intensity, exposure time, lamp placement, and air

movement patterns (167). Since short-wavelength UV is easily blocked by solid objects, viruses may not be inactivated if sufficient faecal matter and other impurities are present on the surface at the time of disinfection, as also happened in our study. UV inactivation has been shown to follow so called 'one-hit kinetics', as reviewed by Cutler and Zimmerman. (167). This occurs when if a photon is absorbed during UV, one photoproduct is formed in the virus structure, which inactives the virus. Thus, raising the UV dose to viruses would result in a proportional increase in virus inactivation. Nuanualsuwan and co-workers observed this for FCV in 2002 when there was a straight correlation between the UV dose and FCV inactivation (171). Similar straight line curve of MuNoV inactivation was recorded in study IV, whereas the PCR reduction of the virus levels for both MuNoV and HuNoV occurred as a tailing reduction curve. A similar tailing off has also been observed for other viruses, such as polioviruses and rotaviruses (208). Experimental bias, two different subpopulations of viruses and an aggregation of these micro-organisms have been suggested as being a cause for the tailing, but no conclusive evidence has been presented (167). In our study, HuNoVs may have been protected from the UV on the surfaces by the blocking due to organic matter that originated in the diluted faecal sample. Therefore, initial cleaning of those surfaces has to be done before UV disinfection.

Unlike many chemical agents, UV does not leave harmful residues on the disinfected sites (208), such as food contact surfaces. Instead it affects to air oxygen forming ozone, a gas with high oxidizing potential. Although the levels of ozone in rooms disinfected by UV are usually low, they may rise to concentrations that harm mucus and respiratory tissues in humans. The destructive nature of UV and it's by product ozone not only towards microbes but also towards human cells severely limits their use in such a way that humans must never be exposed to them (140). Therefore disinfection of food contact surfaces in food industry, restaurants, hospitals and other facilities could be executed after a working shift has ended.

6.4 The efforts to distinguish infective and inactivated noroviruses from each other by pre-PCR treatments (IV)

RT-PCR and QRT-PCR methods only detect a specific sequence of the genomic material of HuNoVs, thus the infectivity of HuNoVs in samples found positive by these assays will remain unknown and the correlation between viral particles and genomic copies will not be clear. Several approaches have been developed to overcome the interpretation problems of these PCR assays, including the characterization of capsid changes that cause or accompany a loss of viral infectivity (209) and the detection determination of genome integrity (176, 210).

Since the HuNoV virion is constructed simply of a shell formed by the capsid protein shielding the interior viral genome, a damaged capsid would be, in theory, more susceptible to protease degradation than capsids of undamaged viruses. Protease treatment would then result in an exposure of viral RNA, thus enabling the degradation of RNA by RNase and subsequently a negative RT-PCR result. In 2002, Nuanualsuwan and Cliver managed to reduce the PCR signal of inactivated FCV samples compared to the inactivated, non-treated virus samples by treatment with proteinase K and RNAse (211). Similar to that found by Nuanualsuwan and Cliver, it was also observed in study IV that enzymatic pre-PCR treatment distinctly accelerated the reduction of both MuNoV and HuNoV levels after UV treatment, although the reduction of pre-PCR treated MuNoV and HuNoV particles was not as great as the inactivation of MuNoV. Lamhoujeb and co-workers observed in 2008 that concentrations of proteinase and RNAse enzymes present in the FCV sample affected the capacity of

the enzymes to digest the sample (123). The enzyme concentrations and the balance between proteinase and RNAse enzymes also seemed to affect the digestion capacity of the enzymes in study IV.

In 2010, Parshionikar and co-workers used propidium monoazide to penetrate the damaged or compromised virus capsids and bind covalently to viral RNA upon exposure to visible light (181). Those authors also observed that this bound RNA was unavailable for amplification, although the determination of the actual number of infectious HuNoVs was not successful in all cases. Sano and co-workers (2010) introduced a method in which the viruses that were under oxidative stress could be separated from intact virions by avidin-immobilized affinity chromatography (183). The method only measured the oxidative products on the viral capsid protein, consequently it may have overestimated the infectivity of HuNoV after treatments that mainly target the viral RNA (211). The enzymatic treatment that was used in study IV may have similarly failed to digest the viral particles which only had damaged RNA, but not the protein capsid, which would have given an overestimation of the persistence of MuNoV and HuNoV towards UV.

Another approach to discriminating between infectious and non-infectious HuNoVs is based on the binding properties of infective viruses to porcine gastric mucins, which results in the unbound virus particles being discarded and thus not copied in the PCR assays (172). In these experiments, a small amount of HuNoVs were still bound to gastric mucins after the sample had been treated by a 500 mJ/cm^2 dose of UV. After the same UV dose, enzymatically treated MuNoV and HuNoV samples still produced positive signals after RT-QPCR runs also in our study. Li and co-workers (2011) used Caco-2 cells as also used by Dancho and co-workers to bind to infective HuNoVs (180). As a result, those authors observed that the method could decrease the lower limit of thr detection levels in a 10-fold dilution series of HuNoVs by 1–3-log_{10}, but the detection of non-infectious viral particles could not be eliminated totally, as was also found in our study.

Food and environmental samples are usually tested for HuNoVs by using RT-PCR or RT-QPCR (102). Samples that give a positive RT–PCR test result, although the viruses in the samples are actually inactivated, are likely to be wrongly perceived as threats to public health. Furthermore, detected HuNoV genome in food products, such as in frozen berries, can result wide withdrawal of the products and thus large costs to the society. Therefore, pre-PCR treatments that could help in distinguishing between infective and inactivated HuNoVs would theoretically facilitate the public health authorities' decision making when HuNoVs have been detected in food or environmental samples. In practice, HuNoV contamination from food is difficult to detect with the present methods and the need for treatments distinguishing infective and inactivated HuNoVs is under debate. The small infective dose of HuNoV leads to the requirement that even a very small infectious proportion of these viruses must be identified in order to be able to declare the sample free on these pathogens. At present, pre-PCR treatments may still be not sensitive enough to be routinely used in the field.

7 CONCLUSIONS

1 The swabbing method used in this study is suitable for the detection of HuNoV on environmental surfaces and hard food surfaces. It is easy to perform, is time-saving and the RNA of the virus remains detectable in the swabs for days after the sampling. Currently available methods for identifying HuNoV in food products are often laborious and time-consuming, and food as well as faecal samples may not be available for analysing. Thus, sampling of food contact surfaces and environmental surfaces offers a rapid alternative for detecting the presence of HuNoV during outbreak investigations, surveillance and risk assessment.

2 HuNoV is easily transferred from gloves of a food handler to RTE foods during manual preparation of food items, such as cucumber sandwiches. The transfer rates of the virus and the number of food servings onto which the virus is likely to transfer to are dependent on the initial HuNoV load on the hands of food handlers. The wearing of protective gloves while preparing RTE foods did not prevent HuNoV contamination of the foods, when the food handler had virus-contaminated hands before gloving. HuNoV-contaminated gloves were found to transfer the virus to the food servings more efficiently than a single contaminated food ingredient during the preparation of a deli sandwich.

3 When HuNoVs are circulating in the population, usually during early spring, their genome can be detected on environmental surfaces in places where humans have been in close proximity to one another. HuNoV is mostly detected on surfaces most often touched by bare hands, such as door handles. During an outbreak, the HuNoV load on environmental surfaces can be high enough to enable sequencing of the outbreak virus genome from environmental swabs taken from the outbreak site.

4 Results for the HuNoV surrogate MuNoV imply that HuNoV is inactivated quite effectively on environmental surfaces, by UV. The results of UV inactivation as determined by a viability assay are not comparable to the results of viral genome persistence after UV treatment. Viral RNA can even be detected on environmental surfaces treated with very high UV doses, while infectivity is lost with much lower doses. Faecal matter or other impurities facilitate the persistence of HuNoV.

5 Pre-PCR treatments such as enzymatic digestion by pronase and RNAse enzymes can reduce positive signals due to damaged virus particles upon RT-QPCR. However, attaining a level at which all inactivated virus particles would be enzymatically digested is not feasible using the present methods.

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Swabs as a Tool for Monitoring the Presence of Norovirus on Environmental Surfaces in the Food Industry

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ABSTRACT

Human norovirus (HuNoV), which causes gastroenteritis, can be transmitted to food and food contact surfaces via virus-contaminated hands. To investigate this transmission in food processing environments, we developed a swabbing protocol for environmental samples, evaluated the stability of HuNoV in the swabs, and applied the method in the food industry. Swabs made of polyester, flocked nylon, cotton wool, and microfiber were moistened in either phosphate-buffered saline (PBS) or glycine buffer (pH 9.5) and used to swab four surfaces (latex, plastic, stainless steel, and cucumber) inoculated with HuNoV. HuNoV was eluted with either PBS or glycine buffer and detected with quantitative reverse transcription PCR. HuNoV recoveries were generally higher with an inoculation dose of 100 PCR units than 1,000 PCR units. The highest recoveries were obtained when surfaces were swabbed with microfiber cloth moistened in and eluted with glycine buffer after a HuNoV inoculation dose of 100 PCR units: 66% ± 18% on latex, 89% ± 2% on plastic, and 79% ± 10% on stainless steel. The highest recovery for cucumber, 45% ± 5%, was obtained when swabbing the surface with microfiber cloth and PBS. The stability of HuNoV was tested in microfiber cloths moistened in PBS or glycine buffer. HuNoV RNA was detected from swabs after 3 days at 4 and 22°C, although the RNA levels decreased more rapidly in swabs moistened with glycine buffer than in those moistened with PBS at 22°C. In the field study, 172 microfiber and 45 cotton wool swab samples were taken from environmental surfaces at three food processing companies. Five (5.6%) of 90 swabs collected in 2010 and 7 (8.5%) of 82 swabs collected in 2012 were positive for HuNoV genogroup II; all positive samples were collected with microfiber swabs. Three positive results were obtained from the production line and nine were obtained from the food workers' break room and restroom areas. Swabbing is a powerful tool for HuNoV RNA detection from environmental surfaces and enables investigation of virus transmission during food processing.

Human noroviruses (HuNoVs) are the most common cause of nonbacterial gastroenteritis worldwide *(21, 26, 28)*. In 2002, over 1,000 HuNoV infection outbreaks associated with settings such as hospitals and schools were reported in Europe *(26)* and 199 foodborne HuNoV infection outbreaks appeared in the United States *(28)*. Although HuNoV infection is usually mild and self-limiting, prolonged symptoms requiring hospitalization can occur in children and elderly or immunocompromised persons.

HuNoVs are transmitted from person to person mainly through the fecal-oral route, either directly or indirectly via water, food, or contaminated surfaces *(22)*. Shedding can also occur during vomiting and lead to transmission of the virus through air droplets *(29)*. HuNoVs are shed in large numbers, up to 10^9 genomic copies per g, in feces of infected individuals *(2)*, whereas the infective dose is believed to be as low as 10 to 100 virus particles *(42)*. The virus can be shed in feces for more than 1 month in a normal infected person and for several months in a person whose immune system has been compromised *(2, 27)*.

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In addition to being highly contagious, HuNoV is very resistant to environmental stresses, as shown by both experimental and epidemiological studies *(9, 36)*. Because HuNoV is extremely hard to culture in cell lines, most of the persistence studies have been performed with surrogate viruses *(8, 12, 33)* or virus reduction in experiments has been measured by changes in RNA concentrations *(7, 23)*. Results obtained in these studies indicate that HuNoV tolerates well relatively high or low temperatures, high or low pH, UV light, and several disinfectants. HuNoV can persist on various surfaces for days at room temperature *(11)* and for hours on human hands *(24)*.

The role of food workers in the transmission of HuNoV is prominent *(10, 37, 43)*. Greig et al. *(19)* reviewed more than 300 foodborne HuNoV infection epidemics connected to food workers around the world from 1960 through 2006. In 28% of these outbreaks, food workers had contaminated the food by touching it with their hands *(43)*. HuNoV contamination of food represents an important threat to consumers. Because of mild symptoms and a short illness period, many foodborne outbreaks are not reported, leading to underestimation of the number of outbreaks. The most common food items causing foodborne outbreaks are those

eaten without further cooking, such as sandwiches and salads *(19)*. In many countries, food is not routinely tested for viruses in epidemiological investigations; therefore, proof of viruses as the causative agents of the outbreak often cannot be obtained. Other reasons for the failure to confirm HuNoV involvement in foodborne outbreaks are low virus levels, heterogeneous distribution of viruses in samples, and a lack of effective methods for the detection of viruses in food *(21, 22, 35)*.

Environmental swabs have been successfully applied to detection of HuNoV in settings associated with foodborne outbreaks, such as restaurants and cruise ships *(5, 6, 45, 46)*. The virus has also been detected in swab samples from various catering companies that have not been associated with recent outbreaks of gastroenteritis *(6)*. In this article, we present a swab sampling method with quantitative reverse transcription PCR (qRT-PCR) for the detection of HuNoV on contaminated environmental surfaces. We investigated the effect of glycine buffer compared with commonly used phosphate-buffered saline (PBS) to elute viruses when swabs made of various materials were used for sampling. Persistence of HuNoV in the swabs after sampling was also studied because several days may elapse before environmental virological samples reach the testing laboratory. The swabbing method was then applied in a field study for detection of HuNoV on surfaces in food processing areas and break rooms in three food industry companies manufacturing ready-to-eat food products.

MATERIALS AND METHODS

Viruses. For artificial contamination of test surfaces and as a positive control in the PCR assay, we used a human stool preparation containing HuNoV genogroup II (GII.4). A 10% fecal suspension was prepared in PBS (pH 7.2), cooled at 5°C for 2 h, and frozen at −70°C in aliquots. A stool preparation containing HuNoV genogroup I (GI.6) was used as a PCR-positive control and prepared similarly. The endpoint dilution method with viral RNA revealed a virus levels of 10^{10} and 10^{8} PCR units per ml for HuNoV GII and HuNoV GI stocks, respectively. A standard curve was plotted for sequential 10-fold dilutions of RNA, and one qRT-PCR detectable virus unit (pcr-u) was defined as the highest 10-fold dilution of the sample that produced a positive result with a cycle threshold (C_T) of <40.

Murine norovirus (MuNoV; Herbert [Skip] Virgin, Washington University, St. Louis, MO) was used as a process control. The virus was cultured in RAW 264.7 cells (ATCC CRL-2278) in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 10 mM HEPES, and 1% glutamine-penicillin-streptomycin. After viruses had been cultivated on confluent RAW 264.7 cell monolayers for 2 to 3 days, the infected cells were subjected to freezing and thawing three times to release the viruses. Virus stock was prepared according to Park et al. *(33)*. To concentrate the MuNoV, the supernatant was subjected to ultrafiltration (Amicon Ultra-15, Millipore, Billerica, MA) at 4,500 × *g* for 10 min at 4°C. The supernatant was recovered, diluted with PBS to 2 ml, pressed through a 0.2-μm-pore-size syringe filter prehandled with Polysorbate 20 solution, and stored in aliquots at −70°C. The titer of MuNoV released from cells was determined to be approximately 10^{7} PFU/ml by viability assay *(44)*. The PCR titer of the stock was 10^{10} pcr-u/ml.

Swabs. The following swabs were used in the experiments: polyester swabs (175KS01, Mekalasi Oy, Helsinki, Finland), flocked nylon swabs (500C50, Mekalasi Oy), cotton wool swabs (Selefa Trade, Spånga, Sweden), and microfiber cloths (Taski Microcare, Novakari Oy, Helsinki, Finland). Two lots of microfiber cloths were used. The first (microfiber 1) was manufactured before 2010, and the second (microfiber 2) was manufactured in 2011. Both microfiber cloths were made of 85% polyester and 15% polyamide. These cloths were cut into pieces (1.5 by 5 cm) and riveted to a link to allow better handling with tweezers.

Optimization of swabbing method. Three environmental surfaces and one food surface were used for testing the swabbing protocol for virus detection. A low-density polyethylene (PELD plastic) tray and a stainless steel tray were bought from the local general store. Latex surfaces were prepared by cutting powder-free latex gloves *(1)* with sterilized scissors to the proper size (see below) and by taping the edges to fix them onto the surface of the fume hood. The cucumbers were washed with tap water, dried with a paper towel, cut in the longitudinal direction, and then cut into the 5-cm-long pieces used for artificial contamination with HuNoV.

HuNoV GII stock solution, which was used for contamination of test surfaces, was diluted to 10^{-4} and 10^{-6} in sterilized water and divided into 100-μl portions. The 25 cm^2 of the test surface was inoculated evenly with 100 μl of either virus dilution, except for those on cucumber, which were inoculated with the 10^{-4} virus dilution only. For each swabbing tool, two identical squares were made, and for each experiment a square inoculated with 100 μl of sterile water, which served as a negative control, was prepared. Samples were allowed to dry overnight at room temperature in a fume hood before sampling, except for the cucumber samples, which were allowed to dry for 1 h.

A semidirect lysis method was used for virus elution in the swabbing protocol. Each swab was moistened with 2 ml of either PBS or 50 mM glycine buffer (pH 9.5) in a 50-ml flask and used for swabbing one area (5 by 5 cm) of the test surface. Swabbing was done by wiping the surface rapidly in an up-and-down motion while steadily moving across the surface. The entire area was first swabbed with one side of the swab, the swab was turned over, and the same contaminated area was swabbed again. After careful wiping for 1 min, swabs were placed back in their flasks, which were shaken in an orbital shaker (IKAKS 2060 basic, Patterson Scientific, Cambridge, UK) at 250 rpm for 10 min at room temperature. A 4-ml volume of NucliSENS miniMAG lysis buffer (bioMérieux, Boxtel, The Netherlands) was added to each 50-ml flask with shaking to ensure elution of viruses from the swabs. RNA extraction was continued using the fluid content of the flasks. Each experiment was performed twice on different days for the two virus dilutions. A negative control surface was swabbed with a cotton wool swab.

Persistence study setting. On two consecutive weeks and four consecutive days per each week, PELD plastic surfaces were inoculated with 100 μl of 10^{-6} dilution of HuNoV GII as described, and the samples were dried in a fume hood for 1 h. Swabbing was performed with microfiber 2 cloths moistened with PBS or glycine buffer on three parallel surfaces on each day of the experiment. After swabbing, the tubes containing swabs were placed on a horizontal plane at either 4 or 22°C. The 4 ml of miniMAG lysis buffer was added on the fourth day, and the experiment was continued as described.

Application of the swabbing method in field experiments. A total of 152 swab samples, 90 in May 2010 and 62 in April

2012, were collected from three Finnish food manufacturing companies that had not had HuNoV outbreaks. In 2010, we focused the swabbing on production lines, where the temperature is kept at 7 to 8°C or at 20°C, depending on the production phase. We took a total of 60 samples from the production line surfaces such as control panels and door handles. During the same visit, we took 12 samples from microwave ovens, refrigerators, and coffee machines from break rooms and 18 samples from door handles, taps, and toilet seats from restroom areas. In 2012, 7 swab samples were taken from the production lines, 27 from break rooms, and 28 from restroom areas. After 2 weeks, swabbing was repeated in one of the break room areas from the same places as sampled in the previous round to monitor the effects of cleaning measures between sampling rounds.

Field samples in 2010 were collected by swabbing parallel surfaces with either microfiber 2 cloth (45 samples) or cotton wool (45 samples) swabs moisturized in glycine buffer. In 2012, only microfiber 2 swabs were used. Sampling was done according to same protocol used for the optimization study with the same surface area (25 cm^2) and the same sampling protocol. Target surfaces, such as door handles, were swabbed so that half of the surface was swabbed with a microfiber swab and half was swabbed with a cotton wool swab. All swabs were placed in 50-ml tubes containing 2 ml of the same buffer and kept in a Styrofoam box with freezer blocks until the samples reached the laboratory on the same day. One process control with a standardized amount of MuNoV (10^4 pcr-u) was included with each lot of samples. Upon arrival at the laboratory, 4 ml of miniMag lysis buffer was added, and samples were shaken for 10 min and then frozen at −70°C. The next day, samples were thawed and processed for RNA extraction.

RNA extraction and qRT-PCR. RNA extraction was performed according to NucliSENS miniMAG kit instructions with the following modifications: 60 μl instead of 50 μl of magnetic beads was added to the sample tube, and the samples were shaken in an orbital shaker at room temperature at 150 rpm for 10 min instead of being incubated without shaking. Amplification of GII HuNoV was performed using primers QNIF2d (+) and COG2R (−) and probe QNIFS (+) *(25)* following a protocol described by Summa et al. *(39)*. The same protocol was used for GI HuNoV, using primers QNIF4 (+) and QNIF3 (−) and probe JJV1P as described previously *(40)*. For MuNoV, the same protocol used for HuNoV GII detection was used with primers MNVfor and MNVrev and probe MNV *(20)*. Amplification was performed with the Rotorgene 3000 detection system (Corbett Life Science, Sydney, Australia) under the following conditions: initial activation for 25 min at 50°C, second activation for 15 min at 95°C, and then 45 cycles of 95°C for 15 s and 60°C for 60 s. Fluorescence was measured after the annealing step. In the data analysis, the threshold of the PCR was set to 0.02 with a cutoff of 40.

A standard curve was prepared from 10-fold dilutions of HuNoV GII and used to calculate the pcr-u counts of the samples. Every PCR run included duplicates of RNA samples, a positive PCR control containing HuNoV (GI or GII), a positive PCR control containing the same MuNoV as used as a process control, a negative control for RNA extraction, a PCR control containing distilled water, and a nontemplate control.

Sequencing. The swab samples taken in the field study that were positive for HuNoV GII in the qRT-PCR assay were screened for genotype GII.4 using a specific set of primers recently published by Maunula et al. *(30)*. From the same positive samples, a capsid portion of 517 nucleotides was amplified using conventional RT-PCR with primers SR2-2F and JT2R *(5)* and the One Step RT-PCR kit (Qiagen, Valencia, CA). The nucleic acid sequences of the amplicons were determined by the DNA Sequencing Service (Institute of Biotechnology, University of Helsinki, Helsinki, Finland).

Statistical analysis. Statistical analysis was performed using a one-way analysis of variance and a Bonferroni posttest with a cutoff of $P < 0.05$. The analysis was performed using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA; www.graphpad.com).

RESULTS

Optimization of swabbing. The impact of four swab materials and two elution buffers on the sensitivity of the swab sampling method was evaluated using a semidirect lysis technique, in which both elution buffer and lysis buffer are used to detach viruses from swabs. The swabs were moistened and eluted with PBS or glycine buffer before proceeding with RNA extraction and HuNoV qRT-PCR. Swabbing was performed on three environmental surfaces and one food surface.

The mean virus recovery rates of four independent tests of swabbing from latex, PELD plastic, and stainless steel surfaces contaminated with 100 pcr-u of HuNoV GII are shown in Figure 1. Only slight variation in performance between swabs was observed in this trial, with the exception of microfiber 1 swabs with glycine buffer elution, which yielded the highest recoveries ($P < 0.05$) on all surfaces. Particularly high recoveries, 88.7% ± 2.7% (glycine buffer) and 79.6% ± 3.7% (PBS), were obtained when swabbing a plastic surface with microfiber 1 swabs. Microfiber 2 swabs (manufactured in 2011) did not yield comparable results. Characteristics of elution buffers did not seem to play a major role in virus recovery, except when swabbing with microfiber 1 swabs; glycine buffer eluted viruses more efficiently than did PBS ($P < 0.05$). On the latex surface, a slight positive effect (4.8 to 28.9%) was also seen when eluting viruses from any swab with glycine buffer, although the difference was not significant. The recoveries in the study were in general, highest from stainless steel (26.1% ± 12.6% to 79.0% ± 10.2%; mean = 37.7%) and lowest from the latex surface (11.1% ± 5.2% to 66.2% ± 18.0%; mean = 24.4%). All swab materials performed better ($P < 0.05$) for stainless steel than for latex surfaces when PBS was used for elution, but no significant difference was observed with glycine buffer.

When the trial was repeated with an inoculation dose of 1,000 pcr-u of HuNoV GII, virus recovery rates were generally lower (Fig. 2). The highest mean recovery was 34.3% for swabbing stainless steel and the lowest was 12.2% for swabbing latex. Thus, no significant difference in swab performance was observed, except on the latex surface ($P < 0.05$), where polyester swabs produced a recovery of 33.3% ± 10.5% and other swabs produced recoveries of less than 17%. Only microfiber 2 swabs were available for this trial (microfiber 1 material was no longer available). With these swabs, eluting viruses with glycine buffer instead of PBS did not produce higher recoveries from any

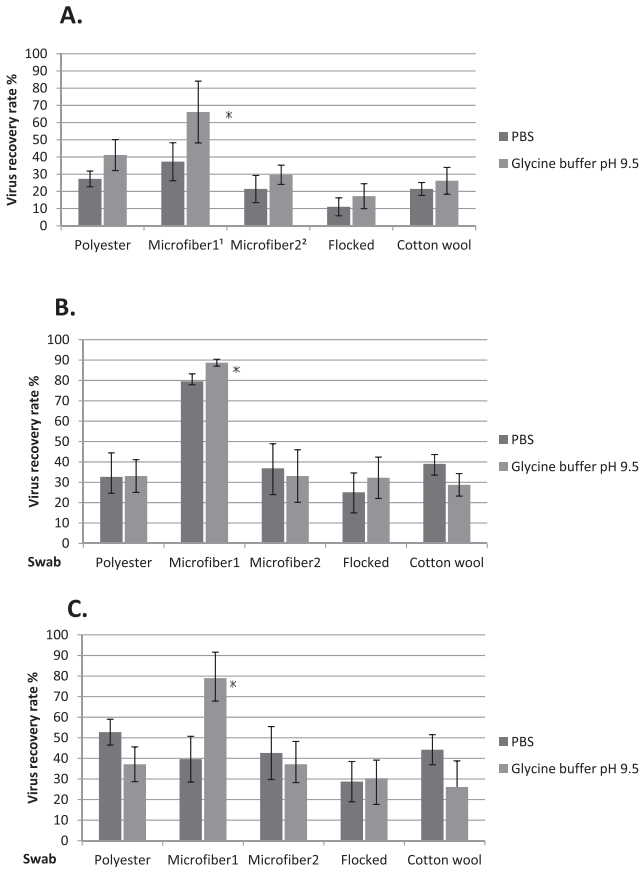

FIGURE 1. *Virus recoveries (%; mean ± SD) of inoculated HuNoV GII.4 (10^2 pcr-u) using the surface swab sampling technique. Inoculated surfaces were latex (A), PELD plastic (B), and stainless steel (C).* [1] *Microfiber 1, first lot of microfiber cloth, manufactured before 2010;* [2] *microfiber 2, second lot of microfiber cloth, manufactured in 2011; * swab implicated with statistical significance (*$P < 0.05$*).*

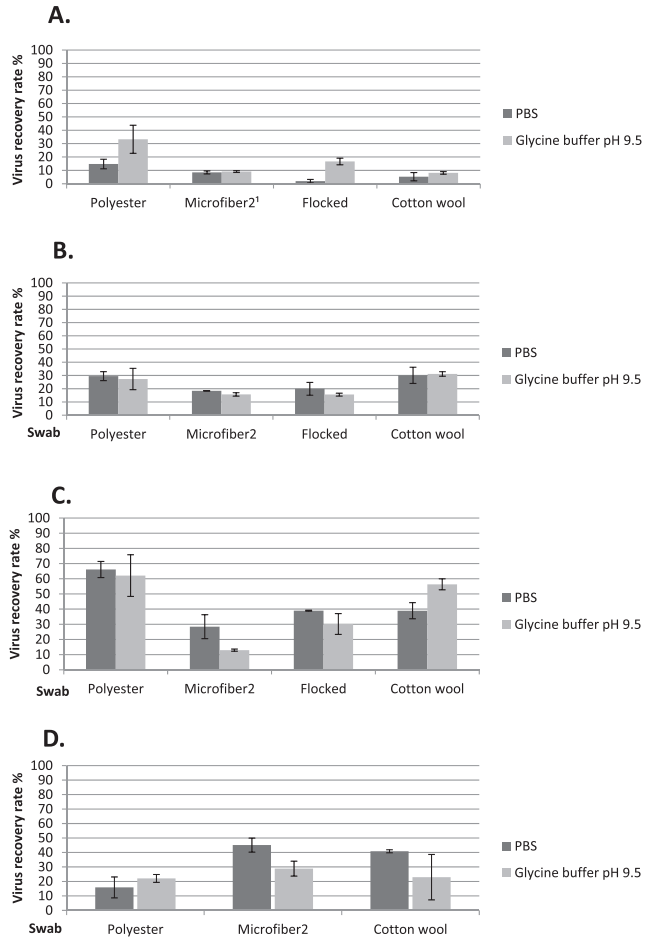

FIGURE 2. *Virus recoveries (%; mean ± SD) of inoculated HuNoV GII.4 (10^4 pcr-u) using the surface swab sampling technique. Inoculated surfaces were latex (A), PELD plastic (B), stainless steel (C), and cucumber (D).* [1] *Microfiber 2, second lot of microfiber cloth, manufactured in 2011.*

surface. When swabbing the outer surface of the cucumber, the highest virus recoveries (45.2% ± 5.2%) were achieved with microfiber 2 swabs using PBS buffer for elution.

Persistence of HuNoV in swabs. Plastic surfaces contaminated with 100 pcr-u of HuNoV GII were swabbed with microfiber 2 swabs moistened with PBS or glycine buffer. The stability of HuNoV in the swabs was evaluated as changes in C_T values at 4 and 22°C on days 0, 1, 2, and 3 (Fig. 3). All samples remained positive for HuNoV during the 3 days of incubation regardless of the temperature or buffer solution used, and the reduction in viral RNA was less than 1.4 log units (less than 4.5 PCR cycles) during this time. The most consistent results were obtained with PBS buffer at 4°C; glycine buffer samples had more variation in virus levels. At 22°C in glycine buffer, virus decreased rapidly to close to the detection level. Between days 0 and 1, the reduction curves at 22°C were steeper than those at 4°C with both buffers used, although the data from swabs moistened in PBS produced a less steep curve than did the data from swabs moistened in glycine buffer.

On day 2, the difference of RNA reduction between PBS- and glycine buffer–moistened swabs was almost 0.5 log units (1.5 PCR cycles) at 22°C, with a higher RNA recovery in PBS-moistened swabs.

HuNoV on surfaces in the food industry. Environmental swabs collected in May 2010 and April 2012 from three food industry companies during an HuNoV epidemic season in the community were analyzed for the presence of HuNoV GI and GII RNA. Twelve (9.4%) of 127 microfiber swab samples were positive for HuNoV (GII) by qRT-PCR. No positives results (0 of 45) were detected in 2010 from samples taken with cotton wool swabs. In May 2010, swabbing with microfiber 2 revealed HuNoV on three surfaces of a food production line: a handle of a knife (C_T = 36.54), a cover of a box containing raw produce (C_T = 38.02), and gloves of an employee who directly handled raw produce (C_T = 36.64). HuNoV was also found on the handles of a coffee machine (C_T = 36.56) and a microwave oven (C_T = 34.95), both used in the break room. HuNoV findings in April 2012 were from coffee machines (C_T = 38.64 and 39.15) in a break room and from door knobs (C_T = 36.56, 36.76, and 35.5) and toilet knobs (C_T = 35.09) in the restroom next to the break room. When the same sites were swabbed 2 weeks later, HuNoV was still found on a

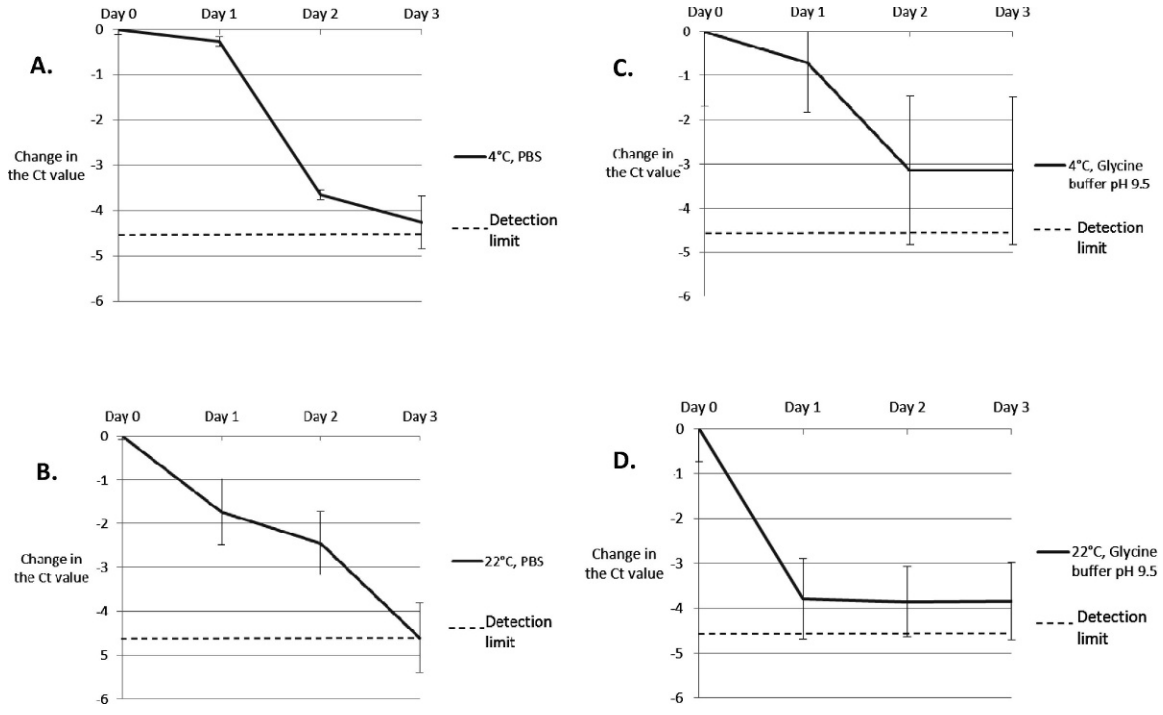

FIGURE 3. *Reduction of HuNoV GII.4 RNA levels in microfiber swabs that were moistened with PBS and stored at 4°C (A) or 22°C (B) or that were moistened with glycine buffer (pH 9.5) and stored at 4°C (C) or 22°C (D).*

toilet knob (C_T = 36.98). Ten of the 12 positive samples were HuNoV GII.4 positive with screening primers. However, none of the products could be sequenced because of the low amount of viral RNA in the samples. No PCR inhibitors were detected in sample eluates at any point during the study, as indicated by the C_T values of an internal MuNoV control from undiluted RNA and a 1:10 dilution (data not shown).

DISCUSSION

Outbreaks of gastroenteritis caused by HuNoV have become a major public health concern in recent years. Effective control of outbreaks depends in part on the identification of the source of contamination. In this article, we describe an efficient and sensitive swabbing protocol that can be used to detect HuNoV on environmental surfaces and demonstrate that HuNoV is readily detected in swabs stored for up to 4 days at room temperature. The presence of HuNoV RNA was also revealed on surfaces of break rooms and on equipment and the gloves of a food worker working in a food processing company that had not been associated with an HuNoV infection outbreak.

Independent of the route of contamination, environmental and food surfaces usually contain very low levels of HuNoV. Therefore, methods used for detection must be both sensitive and reliable. Detection of virus from foods using such methods as polyethylene glycol precipitation and/or ultrafiltration involves many steps during which virus particles can be lost *(34),* whereas with simpler swabbing protocols virus loss seems less likely. High levels of virus recovery have been achieved with two approaches to surface sampling: swabbing *(5, 15, 35)* and aspiration *(11, 41).* The swabbing technique appears to be more appropriate for use with small and vertical surfaces such as door knobs and handles. Because this method is fast and easy to follow, staff at sampling sites can perform the swabbing themselves, widening the applicability of the protocol to larger follow-up studies and enabling routine monitoring of environmental surfaces in food processing and catering companies, hospitals, and elder care facilities *(6, 15, 16).*

Factors such as swab material, features of elution buffers, and surface type have an effect on the recovery of HuNoV from surfaces *(35, 41).* Swab material plays an important role in both the removal of viruses from surfaces and the successful elution of viruses from the swab. Gibson et al. *(18)* tested removal of norovirus surrogates MuNoV and feline calicivirus from solid surfaces by wiping the surfaces with microfiber cloths, obtaining removal of 3.5 log PFU of both viruses. These results suggest that microfiber cloth is a reliable swabbing material for virus sampling, a finding confirmed by our results. Several types of microfiber exist, some of which are more suitable for virus sampling than others *(31).* In our study, microfiber 1 cloth was clearly more efficient than microfiber 2 cloth for collecting viruses and releasing them in the elution step. The reason for this difference is unknown because the ingredients and the weaving of both microfiber cloths were reported to be identical, and the fibers of both cloths looked the same under a light microscope. One possible explanation is that the production process, including the dyeing phase, of microfiber cloths designed for cleaning rather than surface sampling may have changed, perhaps altering the net surface charge and thus influencing virus attachment or release.

Viruses are usually eluted from swab material by using a separate elution step *(46)* or by using lysis buffer directly *(5).* When a separate elution step is used, a considerable amount of virus particles may be lost if the elution is inefficient. In contrast, with direct lysis the transportation of swabs from the sampling site to the laboratory is challenging. With a semidirect lysis method, swabs can be immediately stored under humid conditions, and virus elution and detection can be continued in the laboratory. A neutral solution such as PBS *(15, 35)* and alkaline or slightly acidic buffers such as glycine *(41)* have been used for elution of viruses from swabs. Taku et al. *(41)* obtained higher virus recovery (42%) when using a slightly acidic buffer than when using an alkaline glycine buffer (28%) or a pH neutral solution (10%). In our study, slightly higher recoveries were obtained when eluting viruses with an alkaline glycine buffer than when eluting them with PBS, although the reduction of HuNoV levels was smaller when swabs were stored in PBS than when they were stored in glycine buffer. The elution buffer should therefore be selected case by case, taking into consideration the time between swabbing and virus analysis.

The virus recoveries from latex surfaces were low, especially when a higher inoculation dose of HuNoV was used. According to the manufacturer, powder-free latex gloves are treated with chlorine gas, which may have an impact on virus recovery from this material. The uneven surface of the latex gloves also may have had an impact. On uneven or porous surfaces, the viruses seemed to be trapped in the material and thus were out of reach of the sampling tool, whereas virus removal by swabs seemed to be easier on nonporous surfaces. Our higher virus recovery on nonporous stainless steel surfaces agree with the results of Taku et al. *(41)* and Scherer et al. *(35).* Most norovirus findings in epidemiological studies also come from nonporous surfaces such as toilet seats and toilet faucets *(5, 15).* Scherer et al. *(35)* reported virus recovery of more than 30% when swabbing the outer surface of a cucumber. We also recovered high levels of virus from cucumber surfaces. The swabbing method seems to be more suitable for virus detection from fruits and vegetables with a nonporous outer surface structure than are other more laborious protocols *(38),* as also suggested in the European Committee for Standardization draft *(14).*

Despite our efforts to achieve a controlled test environment, relatively high standard deviations (SDs) were obtained in every test. High SDs may be due to the heterogeneous structure of test surfaces, variable attachment of the virus to these surfaces, and the small number of repeated experiments. As suggested by Scherer et al. *(35),* who also obtained high SDs in their experiments, these values may indicate errors in the sampling mechanism, including collecting and processing errors in the protocol, making it difficult to draw conclusions about the superiority of a particular swab material or elution buffer.

Several factors can influence the stability of enteric viruses on food contact surfaces, including exposure to sunlight *(33),* surface material *(11),* and temperature *(8, 13).* Escudero et al. *(13)* observed that HuNoV persisted in a dried state on solid surfaces for more than 40 days at ambient temperature, with a 1.5- to 2.3-log reduction in virus copies, as determined by qRT-PCR. Compared with these results, we observed more rapid reduction of HuNoV on swabs, although the samples remained PCR positive during the entire trial. The reason for this difference may be that Escudero et al. used undiluted fecal specimens, whereas our virus inoculum was diluted with sterile water. Also, during the mechanical action of swabbing, some of the virus capsids may have been broken down, exposing the viral RNA. Consequently, our results resemble previous findings that RNA on surfaces is degraded rapidly (i.e., in 3 to 7 days) without the protective protein capsid of the virus *(11, 13).*

In this study, HuNoV-positive samples were collected from staff break rooms and from production lines in food processing companies manufacturing ready-to-eat food products. To our knowledge, this is one of the first studies reporting norovirus genome findings from food processing premises. Nevertheless, the relevance of these findings remains unclear because no HuNoV outbreaks relating to these food processing companies were reported before or after this study. PCR-based methods detect only the genome of pathogens, not the infectivity. Some decontamination protocols, such as heat treatment, may leave the capsid of the norovirus intact, and thus the viral genome remains detectable by RT-PCR *(17, 23, 32),* which can lead to false interpretations of risks. The low temperature of the production lines prolongs the survival of viral RNA, making the time of surface contamination and possible efficiency of decontamination hard to define.

Because symptoms of HuNoV gastroenteritis are short term but virus shedding continues for 1 to 2 weeks, an infected food worker is likely to come back to work long before virus shedding has ended *(2).* As reviewed by Todd et al. *(43),* inadequate hand hygiene practices of these food workers can lead to contamination of food ingredients, even when the food worker wears gloves. In our study, HuNoV was found in both restroom areas and break rooms, suggesting that the virus has spread to surfaces via employees' hands. Contamination via hands can be transferred to up to seven clean surfaces, as shown by Barker et al. *(3),* with an estimated viral load of at least 50 to 80 infectious viral units *(4).* Thus, even one HuNoV-shedding food worker can significantly contribute to virus contamination at food processing companies.

In conclusion, this study revealed that swabbing is a powerful tool for detection of norovirus on environmental surfaces. The method is fast and easy to perform, and the RNA of the virus remains detectable in the swabs for days after the sampling. Although the presence of HuNoV RNA on the production lines suggests a possible risk of food contamination, several factors should be considered before making conclusions about the real risk of foodborne outbreaks in these situations. However, education of food workers on the contamination routes of enteric viruses and strict hand hygiene procedures combined with efficient cleaning and possible disinfection of break rooms and production lines are important for reducing the risk of norovirus contamination.

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Norovirus Transmission between Hands, Gloves, Utensils, and Fresh Produce during Simulated Food Handling

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Human noroviruses (HuNoVs), a leading cause of food-borne gastroenteritis worldwide, are easily transferred via ready-to-eat (RTE) foods, often prepared by infected food handlers. In this study, the transmission of HuNoV and murine norovirus (MuNoV) from virus-contaminated hands to latex gloves during gloving, as well as from virus-contaminated donor surfaces to recipient surfaces after simulated preparation of cucumber sandwiches, was inspected. Virus transfer was investigated by swabbing with polyester swabs, followed by nucleic acid extraction from the swabs with a commercial kit and quantitative reverse transcription-PCR. During gloving, transfer of MuNoV dried on the hand was observed 10/12 times. HuNoV, dried on latex gloves, was disseminated to clean pairs of gloves 10/12 times, whereas HuNoV without drying was disseminated 11/12 times. In the sandwich-preparing simulation, both viruses were transferred repeatedly to the first recipient surface (left hand, cucumber, and knife) during the preparation. Both MuNoV and HuNoV were transferred more efficiently from latex gloves to cucumbers (1.2% ± 0.6% and 1.5% ± 1.9%) than vice versa (0.7% ± 0.5% and 0.5% ± 0.4%). We estimated that transfer of at least one infective HuNoV from contaminated hands to the sandwich prepared was likely to occur if the hands of the food handler contained 3 $\log_{10}$ or more HuNoVs before gloving. Virus-contaminated gloves were estimated to transfer HuNoV to the food servings more efficiently than a single contaminated cucumber during handling. Our results indicate that virus-free food ingredients and good hand hygiene are needed to prevent HuNoV contamination of RTE foods.

The effective transmission routes of human noroviruses (HuNoVs) are one of the major reasons why these viruses are recognized as the most common nonbacterial cause of gastroenteritis worldwide (1). HuNoVs spread via the fecal-oral route among humans but can also easily be transmitted to food via inanimate and animate surfaces, such as food preparation equipment and human hands (2, 3). In addition, food such as vegetables and soft fruit can be contaminated earlier in the food chain, e.g., via virus-contaminated irrigation water (2, 4). Once in food ingredients, HuNoV can probably persist on food for extended periods under frozen and cooled conditions, as well as at room temperature, as was shown in HuNoV surrogate studies (5, 6). Several attributes of HuNoV, such as a high virus load in the vomit and feces of infected individuals, a prolonged virus-shedding time, a small infective dose of the virus, and high environmental stability, all facilitate virus transmission from the environment and foods to humans (7).

Virus contamination during preparation of ready-to-eat (RTE) foods that are not heated before consumption, such as delicatessen sandwiches, result in risk to consumers. For instance, the data reported during 2001 to 2008 to the CDC Food-Borne Disease Outbreak Surveillance System showed that 40% (328/813) of the HuNoV outbreaks investigated implicated sandwiches, salads, or other foods eaten raw or lightly cooked (8). A review by Todd and coworkers (9) revealed that HuNoV-associated food-borne gastroenteritis outbreaks are frequently linked to food handlers. In over 60% of the 376 reviewed outbreaks, direct food handling by an infected person or carrier of HuNoV was associated with the spreading of these outbreaks. Furthermore, in almost 30% of the HuNoV outbreaks analyzed in the study, food handlers did not wear gloves while preparing the foods, contrary to the recommendations by the Codex Alimentarius (10). Inadequate hand hygiene and gloving seem, therefore, to play major roles in HuNoV transmission linked to food handlers.

Recent studies have indicated that HuNoV is transmitted efficiently between hands, food items, and environmental surfaces during donor surface-recipient surface interaction (11). Models for simulating the transmission of HuNoV during food preparation have been developed (12, 13), but information on the transmission routes and quantities of HuNoV transferred during the actual food preparation events, such as manual preparation of RTE foods, is still limited. With more accurate knowledge of the transmission routes of HuNoV, intervention measures, such as changing gloves, can be targeted more efficiently.

In the present study, our objective was to determine whether and to what extent HuNoVs or their surrogate, murine noroviruses (MuNoVs), are transferred from virus-contaminated hands (or underneath gloves in the case of HuNoV) to clean latex gloves during gloving. The second objective was to determine the transmission of HuNoV or MuNoV, either from the food ingredient (cucumber; *Cucumis sativus* L.) or the food handlers' hands, by simulating manual preparation of a cucumber sandwich. Virus transfer was investigated by swabbing donor and recipient surfaces with polyester swabs. Environmental and food surfaces were monitored for viruses with reverse transcription-quantitative PCR (RT-QPCR). Using a predictive transfer model, the lowest

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level of HuNoV contamination on hands that would lead to transfer of at least one infective virus particle from hands to gloves and to the final product was estimated. The quantity of the sandwich servings onto which HuNoV could be transferred from either virus-contaminated hands or food ingredients via the gloves of the food handler was also calculated.

MATERIALS AND METHODS

Viruses. For artificial contamination of powder-free latex gloves [manufactured according to standard D3578-05(2010), Standard Specification for Rubber Examination Gloves] or cucumber surfaces, we used murine norovirus 1 (MNV-1), which was obtained from Herbert W. Virgin at the Washington University School of Medicine (St. Louis, MO, USA), or a human stool preparation containing HuNoV genogroup II cluster 4 (GII.4).

HuNoV. A 10% fecal suspension was prepared from the stool containing the HuNoV GII.4 in phosphate-buffered saline (PBS) (pH 7.2), cooled to 5°C for 2 h, and frozen at −70°C in aliquots. A standard curve was plotted for serial 10-fold dilutions of RNA, and one RT-QPCR-detectable virus unit (pcr-u) was defined as the highest 10-fold dilution of the sample showing a positive result with a cycle threshold (C_T) of <40 (14). The endpoint dilution from the lowest dilution of the sample to the first dilution of the sample giving a negative result in RT-PCR revealed a virus concentration of 10 $\log_{10}$ pcr-u/ml in RT-QPCR, equal to 9.7 $\log_{10}$ genome copies/ml.

MuNoV. MuNoV was cultured in RAW 264.7 cells (American Type Culture Collection [ATCC] CRL-2278) in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 10 mM HEPES (Sigma-Aldrich, St. Louis, MO, USA), and 1% glutamine-penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA). After the viruses had been cultivated on confluent RAW 264.7 cell monolayers for 2 to 3 days, the infected cells were subjected to freezing and thawing three times to release the viruses. The titer of MuNoV released from the cells was determined to be approximately 7 $\log_{10}$ PFU/ml by viability assay (15). The PCR titer of the MuNoV stock was defined as 10 $\log_{10}$ pcr-u/ml. A standard curve was plotted for serial 10-fold dilutions of RNA, and one RT-QPCR-detectable pcr-u was defined as the highest 10-fold dilution of the sample showing a positive result with a C_T of <40.

Preparations for trials. All trials were performed in a class I biosafety cabinet on a disposable cover (Nalgene Versidry; VWR International, Radnor, PA, USA). Disposable latex gloves were used in the trials (SemperGuard latex IC; Sempermed, Clearwater, FL, USA), straight from a package. Knives, made entirely of stainless steel, were washed and then sterilized in an autoclave before use and between the trials. Plastic pipette tip box (ART; Thermo Fisher Scientific, San Diego, CA, USA) covers (7.5 by 12.5 by 3 cm), made of polypropylene, were used as surrogates for slices of bread. They were used to enable detection of viruses by a swabbing method. These plastic box covers, referred to below as "bread," were washed with soap and water before use and were discarded after every trial. The cucumbers were washed with tap water, dried on a paper towel, and slit vertically before beginning the experiments. One volunteer, referred to as the test person, who was part of the research group, was used in the transfer studies. The hands of the test person were washed with soap and water and allowed to dry before inoculating MuNoV on them or donning latex gloves for inoculation of HuNoV. All items needed in the trials were placed in a biosafety cabinet before beginning the experiments.

Transfer of MuNoV and HuNoV while donning latex gloves. The transfer of MuNoV from artificially contaminated hands to clean latex gloves was tested as follows. MuNoV (6 $\log_{10}$ pcr-u) was inoculated on the right or left clean bare hand of a single test person from the research group. The 100-μl dose of virus was spread evenly on every fingertip and on the palm of the left or right hand. The virus was allowed to dry on the hand at room temperature for 60 min, during which time the test person was not allowed to use the inoculated hand. After the incubation period, the test person performed the gloving. Gloving was performed the same way in every trial: the test person took the gloves from a container with the right hand and then donned the gloves, first on the left and then on the right hand. After gloving, swab sampling was immediately performed from the outside of the gloved left and right hands separately, using a polyester swab (175KS01; Mekalasi Oy, Nurmijärvi, Finland) moistened in glycine buffer, pH 9.5, according to the protocol described by Rönnqvist and coworkers (16).

The transfer of HuNoV (6 $\log_{10}$ pcr-u) from hands to gloves was performed in a similar manner, with two differences. First, for safety, HuNoV was inoculated on a latex glove, not a bare hand, after which the test person donned a clean pair of gloves. Second, the virus transfer during gloving was not only tested after a drying period of 60 min postinoculation, but also as wet without drying.

MuNoV and HuNoV transfer during manual preparation of the delicatessen sandwich. To test virus transfer between surfaces in the process of manually preparing a cucumber sandwich, the test person performed the preparation, after which the food and environmental surfaces were swabbed. An inoculation dose of 3.5 $\log_{10}$ pcr-u (100 μl) of MuNoV or HuNoV was seeded on the test person's latex-gloved right or left hand evenly as droplets across the entire surfaces of the hands (palms and fingertips) or on half of a cucumber (the top half of the outer surface of the cucumber lying horizontally). After an incubation period of 60 min at room temperature (21 ± 1°C), the preparation was performed as follows: (i) the right-handed test person grasped the cucumber with the left hand; (ii) took the knife into the dominant right hand; (iii) cut six slices of the cucumber, each 5 mm thick and 40 mm in diameter; and (iv) placed the slices on top of the bread with the right hand.

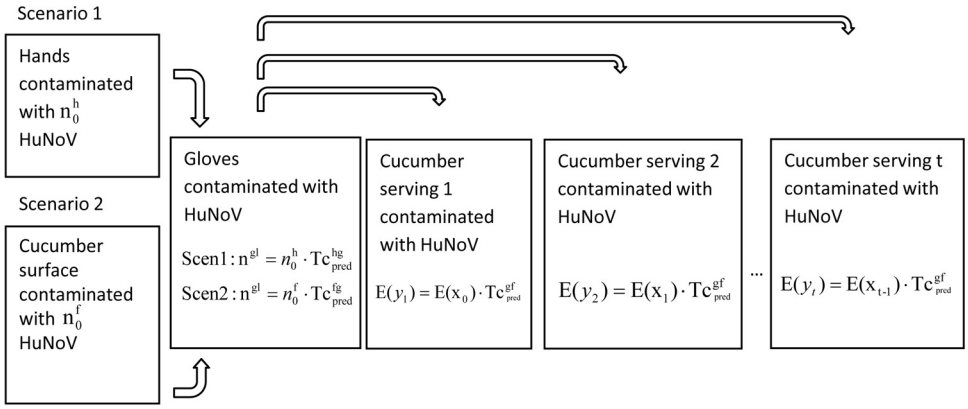
Swab samples were taken from the following surfaces: (i) palm and fingers of the glove of the right hand, (ii) palm and fingers of the glove of the left hand, (iii) the entire knife for cutting the cucumber, (iv) the outer surface of the cucumber, (v) the inner and outer surfaces of cucumber slices placed on the bread, and (vi) the top and sides of the bread. Surface sampling was performed with polyester swabs moistened in glycine buffer (pH 9.5) by carefully swabbing the entire area of the target surfaces. All the trials were performed three times. After sampling, the swabs were processed directly.

Virus elution, RNA extraction, and RT-QPCR. A semidirect lysis method was used to elute the viruses and to prepare the sample for RNA extraction, according to the method of Rönnqvist and coworkers (16). Briefly, the viruses were eluted from the swabs, first with 2 ml of glycine buffer, pH 9.5, and after an incubation of 10 min in an orbital shaker (IKAKS 2060 basic; Patterson Scientific Camlab Ltd., Cambridge, United Kingdom) at 250 rpm, 4 ml NucliSens miniMag (bioMérieux, Boxtel, The Netherlands) lysis buffer was added. After the second 10-min incubation, RNA extraction was performed.

RNA extraction was performed according to the instructions for the NucliSens miniMag kit (input volume, 6 ml). Amplification of MuNoV and HuNoV were performed, using a TaqMan RT-QPCR for the polymerase-gene-capsid-gene junction, according to the protocols recently described by Rönnqvist and coworkers (16). In brief, the detection was performed using a QuantiTect Probe RT-PCR kit (Qiagen, Venlo, The Netherlands) with the Rotorgene 3000 detection system (Corbett Life Science, Sydney, Australia), using the primers MNVfor and MNVrev and the probe MNV for MuNoV (17) and COG2R (−) and QNIF2d (+) and the probe QNIFS (+) for HuNoV (18).

Standard curves were prepared from 10-fold serial dilutions of MuNoV and HuNoV RNAs in water (starting concentration, 10 $\log_{10}$ pcr-u/ml), which were analyzed simultaneously with the samples and used to calculate the pcr-u counts of the samples. Duplicates of RNA samples, a negative control for RNA extraction, a negative PCR control containing distilled water, and a nontemplate control were included in every PCR run.

Virus recovery rate and transfer coefficient calculations. The remnant recovery rates were calculated as the observed pcr-u counts of the



n_0^h =the initial amount of HuNoV on hands
n_0^f =the initial amount of HuNoV on food
n^{gl} =the initial expected amount of HuNoV on the gloves
Tc_{pred}^{hg} =the predicted transfer coefficients observed from hand to glove
Tc_{pred}^{fg} = the predicted transfer coefficients observed from food to glove
Tc_{pred}^{gf} = the predicted transfer coefficients observed from glove to food

FIG 1 Model for evaluating the extent of HuNoV contamination by a food handler's gloved hand or by a food ingredient during manual preparation of a cucumber sandwich.

donor surfaces (left hand, right hand, and outer surface of the cucumber) divided by the observed original pcr-u count of the inoculation dose multiplied by 100%. The cucumber slices were regarded as donor surfaces when the cucumber was inoculated with the viruses, because the virus was inoculated onto the same area where the slices were cut. The transfer coefficients were calculated similarly by comparing the pcr-u count of the acceptor surface with the observed original pcr-u count of the inoculation dose, which was incubated in the test tube for the same amount of time as the samples on the donor surfaces. If the pcr-u count of the acceptor surface was not positive in all repetitions, the pcr-u counts of the negative samples from acceptor surfaces were included in the calculations as 0 pcr-u. The pcr-u counts in this study were normalized over the initial pcr-u count of the virus inoculation dose (3.5 $\log_{10}$ pcr-u per 100 μl for MuNoV and HuNoV). Since one 100-μl inoculation dose was included as a sample in every test series and the virus recovery rates were always calculated in relation to that sample, normalization did not impact the virus recovery rates. The estimations for true transfer coefficients were calculated from the observed transfer coefficients by the following formula: transfer × (100/observed virus recovery rate of the acceptor surface). The observed virus recovery rates of the acceptor surfaces (latex, 33% ± 10%; plastic, 27% ± 8%; stainless steel, 62% ± 13%; and cucumber, 22% ± 7%), established under the same experimental setup/conditions published previously (16), were used in these calculations.

The HuNoV and MuNoV transfer coefficients were analyzed statistically with Student's *t* test in SPSS software (SPSS Statistics; IBM). The significance was determined at a *P* value of <0.05.

Data for the statistical model. The data for the statistical model consisted of the estimates for true MuNoV and HuNoV transfer efficiencies calculated from the raw pcr-u transfer data. The trial was repeated in three categories according to the direction of virus transfer: from hands to gloves, from gloves to food ingredients, and from food ingredients to gloves. Eleven trials were performed for the hands to gloves, five trials for the food ingredients to gloves, and six for the gloves to food categories. The transfer results obtained with dried HuNoV from hands to gloves were used only to describe more accurately the conditions during the preparation of RTE food. The following assumptions were made prior to modeling: HuNoV transferred to the glove during gloving came in contact with the cucumber when the sandwich was prepared, one contaminated food ingredient (half of a cucumber) was designated one contaminated RTE food serving, the food serving was considered contaminated if at least one infective virus particle was transmitted to the food, and there was no direct contact or transfer of HuNoV between cucumbers. MuNoV and HuNoV transfer data were combined in the model based on the finding that no statistical difference was found for the transfer coefficients and recoveries of the two viruses.

Statistical model. The following Bayesian statistical model is available by request. The computations of the model were performed using OpenBUGS software (http://www.openbugs.net/w/FrontPage).

The aim of the modeling was to evaluate the extent of HuNoV contamination in the prepared cucumber sandwiches and their contact surfaces when the virus contamination originated either from the hands of the food handler or from a single food ingredient. We assumed that the transfer coefficients (Tcs) observed from hand to glove during the glove changing (Tc^{hg}), from glove to food ingredient during contact (Tc^{gf}), and from food ingredient to glove during contact (Tc^{fg}) followed a beta distribution (data model):

$$\begin{aligned} Tc_i^{hg} &\sim \text{beta}(\alpha_1, \beta_1),\ i = 1, \ldots, 11 \\ Tc_j^{gf} &\sim \text{beta}(\alpha_2, \beta_2),\ j = 1, \ldots, 6 \\ Tc_k^{fg} &\sim \text{beta}(\alpha_3, \beta_3),\ k = 1, \ldots, 5 \end{aligned} \quad (1)$$

where i, *j*, and *k* denote the number of trials and the Tcs are observed proportions in the trials. A conventional uninformative exponential (0.01) distribution was used as a prior for both parameters of the beta distributions.

The predicted transfer coefficient from a food handler to food was $Tc_{pred}^{hg} \times Tc_{pred}^{gf}$ (the probability that a single virus moves from a food handler to food), where the values of Tc_{pred}^{hg} and Tc_{pred}^{gf} were simulated from their posterior predictive distributions based on the observed Tcs. The predicted transfer coefficient from food ingredient to food was $Tc_{pred}^{fg} \times Tc_{pred}^{gf}$, in which the values of Tc_{pred}^{fg} and Tc_{pred}^{gf} were similarly simulated from their posterior predictive distributions.

Next, we modeled the predicted number of HuNoV-contaminated food servings after repeatedly preparing cucumber sandwich servings in two scenarios (Fig. 1). In the first scenario, the hands of the food handler were as-

TABLE 1 Virus transfer coefficients from MuNoV-contaminated hands or gloved hands inoculated with HuNoV to a clean pair of latex gloves when donning the gloves[a]

Virus	Inoculation site	Drying time (min)	Hand	Virus concn ($\log_{10}$ pcr-u/ml)	Transfer coefficient (%) (no. positive/total)	Calculated transfer coefficient (%)[b]
MuNoV	Left hand	60	Left	5.6 ± 5.2	1.5 ± 0.5 (3/3)	4.4 ± 1.5
		60	Right	6.0 ± 5.2	2.6 ± 3.2 (3/3)	7.8 ± 9.6
	Right hand	60	Left	0.0 ± 3.0	0.0 ± 0.0 (1/3)	0.0 ± 0.0
		60	Right	4.5 ± 4.4	0.1 ± 0.1 (3/3)	0.3 ± 0.2
HuNoV	Left gloved hand	0	Left	6.0 ± 5.9	11.4 ± 8.5 (3/3)	34.6 ± 25.7
		0	Right	5.3 ± 5.3	2.1 ± 2.4 (2/3)	6.5 ± 7.2
		60	Left	4.0 ± 2.9	0.1 ± 0.0 (3/3)	0.3 ± 0.0
		60	Right	4.2 ± 4.0	0.1 ± 0.1 (2/3)	0.4 ± 0.3
	Right gloved hand	0	Left	6.5 ± 5.9	32.4 ± 8.6 (3/3)	98.0 ± 26.0
		0	Right	6.0 ± 5.5	8.7 ± 5.6 (3/3)	26.2 ± 17.0
		60	Left	5.7 ± 5.5	3.6 ± 3.6 (3/3)	11.0 ± 10.9
		60	Right	4.4 ± 4.5	0.2 ± 0.3 (2/3)	0.7 ± 1.0

[a] The inoculation dose of MuNoV and HuNoV was 6 $\log_{10}$ pcr-u.
[b] Estimate of the true transfer coefficient. In estimation calculations, a following recovery rate of 33% from the surface of latex gloves was used.

sumed to initially contain 1 to 4 $\log_{10}$ virus particles before gloving and preparing a series of sandwich servings. In the second, the first single food ingredient (cucumber) that the food handler touched before preparing a series of sandwiches was assumed to contain, likewise, from 1 to 4 $\log_{10}$ virus particles. The amount of HuNoV on the gloves was assumed to decrease during every contact, so that the expected number of virus particles remaining on the gloves after the preparations with the same gloves, $E(x_t)$, was

$$E(x_t|n^{gl}, Tc^{gf}_{pred}) = n^{gl} \times e^{\left[t \cdot \log\left(1 - Tc^{gf}_{pred}\right)\right]} \quad (2)$$

where n^{gl} is the initial expected amount of HuNoV on the gloves, transferred either from the hands or from an initial single food ingredient, $n^{h}_{0} \times Tc^{hg}_{pred}$ or $n^{f}_{0} \times Tc^{fg}_{pred}$, depending on the scenario chosen (1 or 2, respectively). The expected number of viruses in the next food serving, y_{t+1}, is then

$$E(y_{t+1}) = E(x_t) \times Tc^{gf}_{pred} \quad (3)$$

RESULTS

Transfer of MuNoV and HuNoV during gloving. The transfer of MuNoV from either the left or right hand to latex gloves during gloving by a right-handed person was investigated by testing swabs taken from gloves for the presence of MuNoV RNA by RT-QPCR. These swabs repeatedly tested positive, overall, 10/12 times (Table 1), indicating the transfer of MuNoV. When the nondominant left hand was contaminated by the virus, MuNoV RNA was detected on the glove swabs in 6/6 experiments, whereas when the dominant right hand was artificially contaminated, MuNoV RNA was detected on the gloves in 4/6 experiments. The transfer coefficients of MuNoV RNA to gloves varied from 0.1% to 7.0% when the left hand was contaminated with virus and from 0.0% to 0.2% when the right hand was contaminated. In calculating the estimates of true transfer coefficients of MuNoV, we considered the individual recovery rates from latex, plastic, stainless steel, and cucumber surfaces, obtained in a previous study with the same sampling protocol (16). Calculations of the estimations for true transfer coefficients of viruses revealed the difference in transfer coefficients between the contaminated hands, although it could not be verified statistically. In the case of the contaminated left hand, the average true transfer coefficient, calculated from a virus recovery rate of 33% obtained in the previous study (16) for both recipient gloved hands, was 6.1% ± 5.6%, whereas when the right hand was contaminated, the average coefficient was only 0.2% ± 0.1%.

When HuNoV was used instead of MuNoV in the experiments, the virus was inoculated onto the gloved hands before donning a clean pair of latex gloves (Table 1). This time, the viruses were either dried on the gloves for 60 min before gloving or the right-handed person donned clean gloves immediately after the inoculation. HuNoV RNA was transferred from the gloved hands to clean gloves as effectively as MuNoV was transferred from hands to gloves: 10/12 times when dried for 60 min and 11/12 times when wet. The virus was transferred to the gloves 10/12 times when the left gloved hand had been artificially contaminated and 11/12 times when the right gloved hand was contaminated. The transfer coefficients of HuNoV varied from 0.0% to 44.4% regardless of the drying time or the inoculation site (left or right hand), but the average concentration of viruses on the swabbed gloves (6.1 $\log_{10}$ pcr-u per hand) was higher ($P < 0.05$) when the virus inoculation remained wet than when it was left to dry for 60 min (5.0 $\log_{10}$ pcr-u per hand). No statistical difference in the transfer coefficients was observed between the HuNoV and MuNoV results in these trials.

Transfer of MuNoV and HuNoV between contact surfaces in manual preparation of a delicatessen sandwich. MuNoV transfer between a donor surface (left-hand glove, right-hand glove, or outer surface of a cucumber) and acceptor surfaces was investigated in the process of simulating manual preparation of a cucumber sandwich (Fig. 2). In calculating the remnant recovery rates of raw data from donor surfaces after the simulation experiment, we observed that the average remnant recovery rate from artificially virus-contaminated cucumber surfaces was higher (18.4%) than that from contaminated glove surfaces (5.8% and 6.6% for the left and right hand, respectively). The remnant MuNoV recovery rates from cucumber surfaces (3.0% to 48.9%) varied more than those from left- and right-hand gloves (0.4% to 13.7%) (Table 2). During the simulation study, we observed that MuNoV was transferred to more acceptor surfaces if the virus contamination had occurred on the surface of the cucumber than if it had occurred on either of the hands of the food worker (Table 2). However, we observed MuNoV transfer from the donor surface only to the first

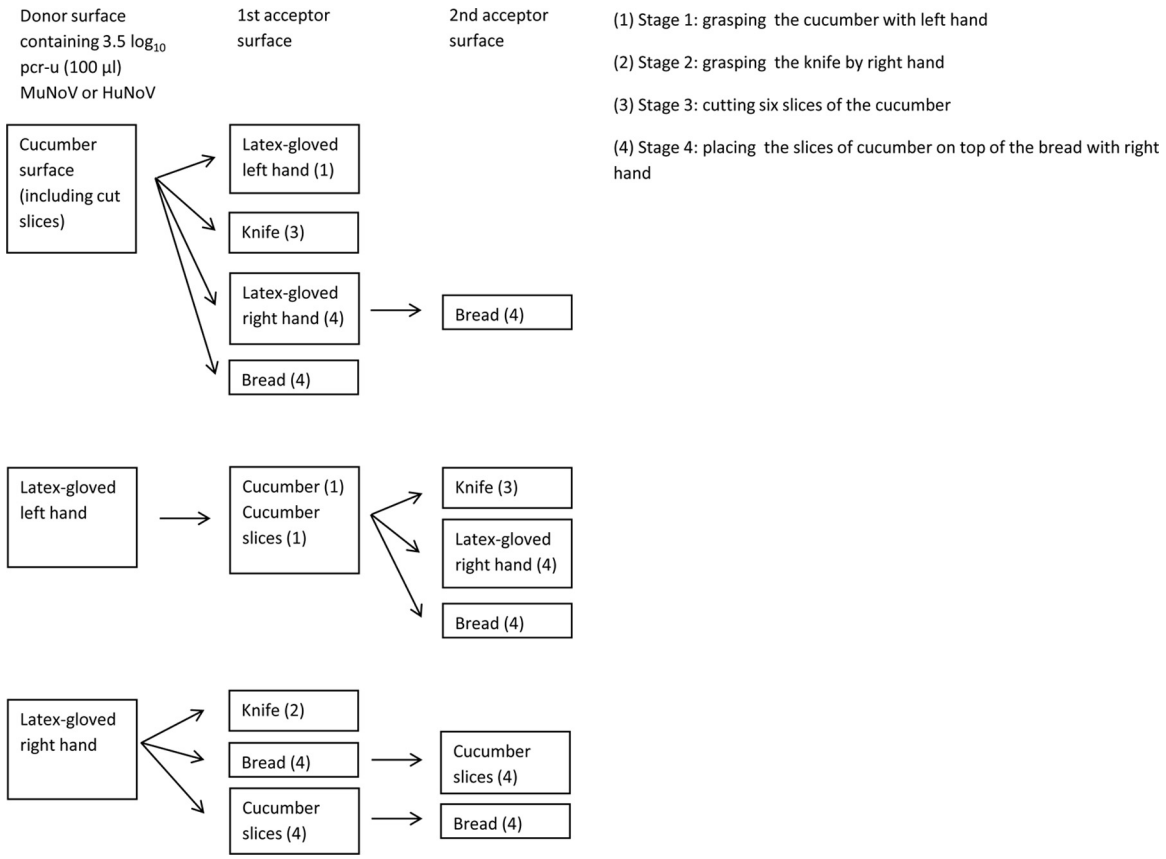


FIG 2 Possible contamination routes of MuNoV and HuNoV and stages 1 to 4, during which the contamination may occur when preparing a delicatessen sandwich.

contact surface, but not to the second, on each repetition of the simulation. In independent experiments, we observed MuNoV transfer from the artificially contaminated cucumber surface to the gloved hand holding the cucumber in 3 out of 3 experiments and to the knife blade used for cutting the cucumber in only 1 out of 3 experiments. From the contaminated left hand, we observed virus transfer to the cucumber in all three experiments. MuNoV transfer from the right gloved hand to the knife handle was observed in all three experiments, while transfer from hand to bread was observed in 2 out of 3 experiments. MuNoV was transferred more efficiently from the glove to the surface of the cucumber than from the glove to the knife handle ($P < 0.05$). Virus transfer was also more efficient from the glove to the cucumber than vice versa, although this was not supported statistically. Indeed, the highest transfer coefficient, 1.2% ± 0.6%, was observed when the virus was transferred from the glove to the cucumber surface. When estimates of the true transfer coefficients from the glove to the cucumber surface were calculated, the coefficient was even more pronounced (5.4% ± 3.1%), although its difference from other transfer events was not statistically significant. The virus transfer coefficients from the glove to the knife were low, even when the surface-specific recovery rate of the knife was taken into account in the calculations: less than 1% in all the repetitions.

The average remnant recovery rates of HuNoV from the cucumber surface (6.6%) and gloves (10.6% and 8.5% for the left and right hand, respectively) were more alike than the corresponding rates for MuNoV (Table 2). There was also less variation in the recovery rates between the trials—2.9% to 13.0% for the cucumber surface and 6.4% to 15.6% for the gloves—than in the MuNoV trials. In the transfer experiments, HuNoV transferred from the donor surfaces to the acceptor surfaces in quantities similar to those of MuNoV. We observed that HuNoV was transferred to more acceptor surfaces from the cucumber surface than MuNoV. In addition to the left gloved hand (3 out of 3 experiments) and knife blade (1 out of 3 experiments), we observed transfer to the right gloved hand in 1 out of 3 experiments and to the bread in 1 out of 3 experiments. From the left gloved hand, the virus was transferred to the cucumber in 3 out of 3 experiments and from the right-gloved hand to the knife handle in 2 out of 3 experiments. As in the MuNoV tests, more viruses were transferred from glove to cucumber than vice versa, although this could not be confirmed statistically.

Statistical model. In the Bayesian analysis, data from both the gloving experiment and the food-handling study were used in modeling. As a result of this analysis, we estimated that HuNoV contamination on the hands should be more than 3.4 $\log_{10}$ infective virus particles to result in contamination of a single prepared cucumber sandwich serving (probability, 50.0%). With 4.2 $\log_{10}$ virus particles on the hands, the probability of the sandwich becoming contaminated would already have risen to 70.0%.

In this analysis, we also calculated that HuNoV on food handlers' contaminated hands/gloves would be transferred to far more cucumber sandwich servings than by sporadic HuNoV contamination of a single food ingredient. We calculated that if 3 $\log_{10}$

TABLE 2 Virus remnant recovery rates, transfer coefficients, and estimated true transfer coefficients of MuNoV and HuNoV between surfaces in manual preparation of a cucumber sandwich after inoculation of 3.5 $\log_{10}$ pcr-u (5.5 $\log_{10}$ pcr-u/ml) of MuNoV or HuNoV on cucumber, right hand, or left hand

Virus	Inoculation site	Surface	Virus concn ($\log_{10}$ pcr-u/ml)	Remnant recovery rate (%) (no. positive/total)	Transfer coefficient (%) (no. positive/total)	Calculated transfer coefficient (%)[a]
MuNoV	Cucumber	Cucumber	4.7 ± 4.8	18.4 ± 26.4 (3/3)		
		Right hand	<1[b]			
		Left hand	3.3 ± 3.1		0.7 ± 0.5 (3/3)	2.1 ± 1.6
		Knife	1.7 ± 1.9		0.0 ± 0.0 (1/3)	0.3 ± 0.5
		Cucumber slices	2.9 ± 2.4	0.3 ± 0.1 (3/3)		
		Bread	<1			
	Left hand	Cucumber	3.6 ± 3.1		1.2 ± 0.6 (3/3)[d]	5.4 ± 3.1
		Right hand	NC[c]			
		Left hand	4.2 ± 4.1	5.8 ± 5.7 (3/3)		
		Knife	<1			
		Cucumber slices	<1			
		Bread	<1			
	Right hand	Cucumber	NC			
		Right hand	4.3 ± 4.2	6.6 ± 6.1 (3/3)		
		Left hand	NC			
		Knife	2.9 ± 2.7		0.2 ± 0.2 (3/3)[d]	0.4 ± 0.3
		Cucumber slices	<1			
		Bread	0.0 ± 1.6		0.0 ± 0.0 (2/3)	0.1 ± 0.1
HuNoV	Cucumber	Cucumber	4.2 ± 4.2	6.6 ± 4.7 (3/3)		
		Right hand	1.9 ± 2.1		0.0 ± 0.1 (1/3)	0.1 ± 0.2
		Left hand	2.9 ± 2.7		0.5 ± 0.4 (3/3)	1.4 ± 0.7
		Knife	3.3 ± 0.3		0.4 ± 0.1 (1/3)	0.1 ± 0.1
		Cucumber slices	2.3 ± 2.5	0.6 ± 0.3 (3/3)		
		Bread	1.9 ± 2.1		0.0 ± 0.1 (1/3)	0.2 ± 0.2
	Left hand	Cucumber	3.7 ± 3.8		1.5 ± 1.9 (3/3)	6.9 ± 8.8
		Right hand	NC			
		Left hand	4.3 ± 3.7	10.6 ± 3.6 (3/3)		
		Knife	<1			
		Cucumber slices	<1			
		Bread	<1			
	Right hand	Cucumber	NC			
		Right hand	4.3 ± 3.7	8.5 ± 2.3 (3/3)		
		Left hand	NC			
		Knife	2.7 ± 2.8		0.3 ± 0.2 (2/3)	0.4 ± 0.5
		Cucumber slices	<1			
		Bread	<1			

[a] Estimate of the true transfer coefficient. In estimation calculations, the following recovery rates were used: outer surface of cucumber, 22%; surface of plastic, 27%; surface of stainless steel, 62%; and surface of latex gloves, 33%.
[b] Under the detection limit of 0.1 $\log_{10}$ pcr-u.
[c] NC, no contact with virus.
[d] More efficient transfer from the glove to the surface of the cucumber than from the glove to the knife handle ($P < 0.05$).

HuNoV particles were present on the hands of the food handler before gloving and food preparation, the probability of the expected value for HuNoVs being 1 or more in the 8th cucumber sandwich serving would still be over 50% (Fig. 3). If, however, the same number of particles were present on the surface of a cucumber, the probability of transfer to even the first serving would be less than 5%. According to the calculations, the HuNoV level on a single contaminated cucumber would have to be 3.7 $\log_{10}$ virus particles to have a probability of virus transfer similar to that for hands containing 3 $\log_{10}$ HuNoV particles.

DISCUSSION

The importance of HuNoVs as the causative agents of food-borne gastroenteritis outbreaks has been understood for several decades (1). The contamination routes of these viruses, however, are still being investigated with the aid of novel techniques. In the present study, the transfer of HuNoVs and their surrogate MuNoVs was measured for the first time during gloving and simulated preparation of a sandwich. Subsequently, the data were used in a model to calculate the number of servings contaminated as a result of transfer between hands and surroundings, demonstrating that contaminated gloves transferred HuNoV to the food servings more efficiently than sporadic food ingredient contamination during handling.

In the present study, the transfer coefficients of both MuNoV and HuNoV RNA or virus from glove to cucumber were suggested to be higher than the transfer coefficients from cucumber to glove, although the difference could not be verified statistically. Previously, a similar finding was observed for feline calicivirus (FCV), which was transferred more efficiently (46%) from fingertips to ham than vice versa (6%) (19). More recently, HuNoVs were

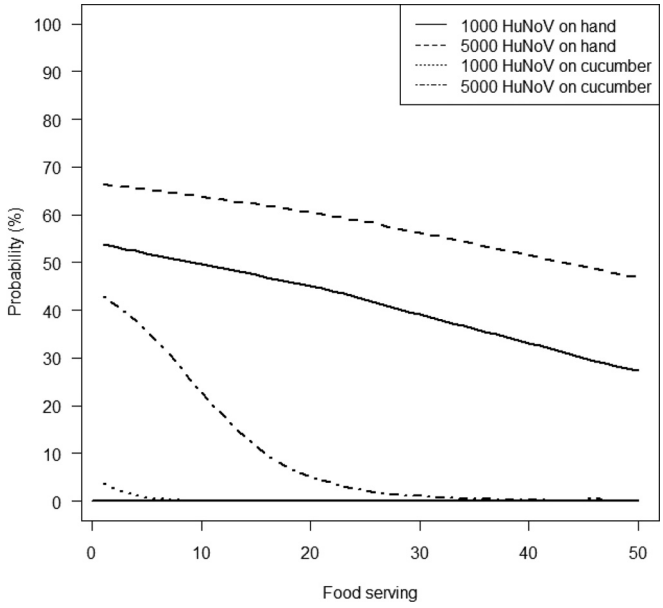


FIG 3 Probabilities of the expected value of HuNoV particle transfer from contaminated hands of a food handler or from a contaminated single cucumber food ingredient to food servings being ≥1 in a food serving when the initial numbers of HuNoVs on the hands and on the cucumber are 3 $\log_{10}$ and 3.7 $\log_{10}$ virus particles.

transferred more efficiently from gloves to food ingredients (lettuce [*Lactuca* L.], 2.7%; ham, 16.2%) than from gloves to stainless steel (0.1%) (20). This is in agreement with the present study, in which HuNoV and MuNoV were transferred more efficiently from gloves to cucumber than from gloves to a stainless steel knife. The transfer coefficients were similar, despite differences in the virus concentrations used: 5.5 $\log_{10}$ PFU per surface for an inoculation dose of FCV (19), 6.8 $\log_{10}$ genomic copies of HuNoV or 4.3 $\log_{10}$ PFU of MuNoV per surface (20), or 6 $\log_{10}$ pcr-u of HuNoV and MuNoV in the gloving experiment and 3.5 $\log_{10}$ pcr-u of HuNoV and MuNoV in the present food-handling study. Stals and coworkers (20) showed that HuNoV and MuNoV were transferred from gloves to sandwich bun. Similarly, we observed in our study that MuNoVs were sometimes transferred from gloves to bread, even though the transfer coefficient obtained cannot be considered accurate because of the structural differences between actual bread and the plastic surface we used as a model. Tuladhar and coworkers (21) observed that the transfer coefficients of MuNoVs from bare fingertips to cucumbers were approximately 50%, much higher than our estimate of the true transfer of MuNoV from gloves to cucumber, 5.4%. Although we had different settings, since in our study the virus was distributed to the entire palm of the hand instead of a fingertip, it may be that bare fingers transfer noroviruses more efficiently than latex gloves. No statistical difference was found for the transfer coefficients and remnant recovery rates of MuNoV and HuNoV in the present study or in the study by Sharps and coworkers (22), suggesting that MuNoV serves as a suitable surrogate for HuNoV in virus transfer studies.

Surface materials affect the recovery rates of HuNoV obtained by swabbing (20, 23). Therefore, in the present study, estimates of the true transfer coefficients of MuNoV and HuNoV were calculated. The highest single remnant recovery rate from a cucumber surface for MuNoV, 49%, was similar to the HuNoV recovery rate of 32% (inoculation dose, 4.3 $\log_{10}$ pcr-u) from cucumber reported by Scherer and coworkers (23) and the 50% MuNoV recovery rate from the lettuce surface obtained by Stals and coworkers (20). This indicates that only a small portion of MuNoVs were transferred onward from the cucumber in that single trial. The recovery rates Stals and coworkers (20) obtained from a nitrile glove surface (38%) and the recovery rate we obtained from latex gloves without any preparation in a previous study (33%) (16) were much higher than the remnant recovery rates we obtained in this study from latex gloves for HuNoV after preparing the cucumber sandwich: 6.5% to 15.6%. This suggests that a high proportion of HuNoVs were transferred from the latex glove surfaces to cucumbers and other contact surfaces. Wang and coworkers (24) reported much lower MuNoV recovery rates, averaging 11.4%, from knives than the 62% we used in the true transfer coefficient calculations. These unequal recovery rates may have resulted from the difference in virus recovery methods: Wang and coworkers (24) reported that the viruses were eluted from the knives by stomaching. The recovery rates for viruses published in other studies seem to be comparable only when identical recovery methods are used.

Barker and coworkers (25) showed that when the initial dose of the virus on the fingertips was approximately 4.3 $\log_{10}$ pcr-u, HuNoV was transferred from contaminated fingertips sequentially to as many as seven clean melamine surfaces. This contamination level was actually obtained in a volunteer study by Liu and coworkers (26): the HuNoV levels on the rinse samples of the hands of six HuNoV-infected volunteers ranged from 2.81 to 4.45 $\log_{10}$ genomic equivalent copies. In the present study, 4 $\log_{10}$ pcr-u or larger amounts of HuNoV on contaminated hands were estimated to lead, despite covering of the hands with gloves, to contamination of essentially all the sandwich servings prepared after gloving on the same working shift. In the transfer model used in the present study, a sandwich serving was defined as contaminated when at least one HuNoV genome was transferred to the sandwich. The definition is based on the calculations of Teunis and coworkers (27), who estimated the probability of even one HuNoV infecting a human as being 50%. Although RT-QPCR cannot discriminate between infectious and noninfectious particles transferred between hands, gloves, and food products during sandwich preparation, the estimate gives direct information on the risk of sandwich contamination with infective viruses during food preparation. The quantitative exposure model of Mokhtari and Jaykus (13) and the recent HuNoV transfer model of Verhaelen and coworkers (28) lend support to the concept that hands are a significant vehicle in HuNoV transmission during the processing of RTE foods, in line with the present study.

Protective gloves are considered to aid in preventing the transfer of food-borne viruses during food preparation (10). However, in the present study, contamination of hands with MuNoV and HuNoV prior to gloving led to virus contamination of the protective gloves in the majority of experiments. If infective, enough HuNoVs could transfer to the cucumber sandwiches prepared and cause infection when consumed. The present study supports the findings by Mokhtari and coworkers (13) that proper hand washing prior to gloving would result a significant drop in virus levels on the hands, thus preventing transfer of HuNoV from hands to RTE foods more efficiently than use of only one of these prevention measures. Recently, the Codex Alimentarius guidelines on the application of general principles of food hygiene to the control of viruses in food was adopted by the Codex Alimentarius

Commission (29). The document clearly states that wearing gloves or the use of hand sanitizers does not exempt food handlers from having thoroughly washed their hands before donning gloves. Such practices require good compliance and are dependent on, among other factors, education and facilities.

The present study has demonstrated that HuNoV is transferred broadly both from food ingredients to the environment and from food handlers' hands to food ingredients and to prepared RTE foods. Contamination of fresh food ingredients by HuNoV during crop production can be reduced by using clean water for irrigation of the crops and washing. Our study showed that wearing gloves reduces the risk of virus transfer from contaminated hands and can partially, but not completely, protect foods from contamination by the food handler. Therefore, effective hand hygiene, including hand washing with soap and water, is crucial in preventing contamination of otherwise HuNoV-free food ingredients by these viruses.

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Outbreak of gastroenteritis caused by norovirus GII.4 Sydney variant after a wedding reception at a resort/activity centre, Finland, August 2012

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SUMMARY

In August 2012, an outbreak of gastroenteritis occurred among 88 persons attending a wedding reception at a resort/activity centre in Ylöjärvi, Finland. Of 39 interviewed guests, 23 met the case definition. Two persons were hospitalized. Epidemiological, laboratory and environmental investigations were conducted to characterize the outbreak and to recommend control measures. Investigation confirmed the presence of a new strain of norovirus GII.4 Sydney variant in stool specimens obtained from two wedding guests and on several environmental surfaces in the centre. In the questionnaire study, none of the foods or beverages served during the reception were significantly associated with the illness. Additional cases of gastroenteritis that occurred at the centre before and after the wedding reception supported the hypothesis of environmental transmission of norovirus. After thorough cleansing and disinfection and 1 week's quarantine, no new cases with symptoms typical for norovirus infection were identified at the centre.

Key words: Epidemics, gastroenteritis, Norwalk agent and related viruses.

INTRODUCTION

Infections with norovirus (NoV) are one of the leading cause of viral gastroenteritis worldwide [1–4]. Typically, infection with NoV is self-limiting and is characterized by nausea, vomiting, abdominal pain and diarrhoea. The incubation period ranges from 24 to 48 h [5]. The most important routes of transmission are faecal–oral, vomit–oral and from person to person. The main vehicle of infection is contaminated food or water. The virus is highly contagious, an estimated dose $\geqslant$18 viral particles is sufficient to cause infection [6]. Noroviruses are environmentally stable. They can survive freezing, heating (30 min at 60 °C) and are resistant to relatively high concentrations of chlorine [1, 7]. Several outbreaks with widespread contamination of environments have been reported, particularly in closed settings [8–10].

In Finland, the municipal authorities report suspected foodborne and waterborne outbreaks to the national online registry (FWD registry) developed and maintained by the National Institute for Health and Welfare (THL) and the Finnish Food Safety Authority Evira. In 1995, Finland initiated

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nationwide laboratory-based surveillance for NoV infections. Between 1998 and 2011, the annual number of cases of NoV infection reported to the National Infectious Diseases Register ranged from 125 to 2807. Since 1997, NoV has been the most common cause for foodborne and waterborne outbreaks [11]. During 1998–2002, the most common NoV genogroup causing gastroenteritis outbreaks was genogroup II accounting for 219 (87%) of 252 outbreaks, genogroup I caused 33 (13%) outbreaks [12].

On 20 August 2012, an infectious disease nurse from the Epidemiology Unit at the Helsinki City Health Department informed Pirkkala Environmental Health about gastroenteritis in two guests at a wedding buffet that was held on 18 August 2012. The wedding reception took place at a resort/activity centre (hereafter referred to as the centre) in Ylöjärvi, Finland. On 20 August, the municipal authorities were notified of the outbreak through the FWD registry. We investigated the outbreak in order to identify the source and aetiology of the infections, undertake control measures and prevent similar outbreaks in the future.

METHODS

Description of the location

The centre is located on Lake Näsijärvi, in the municipality of Ylöjärvi, in western Finland. The centre consists of a main building with an event hall, kitchen, sauna, 10 guest rooms with toilets, and three cottages.

Epidemiological investigation

NoV was suspected as the cause of the outbreak since the incubation period, and the description and duration of symptoms of the three guests with gastroenteritis were consistent with NoV infection. We defined a case as a person who attended the wedding buffet on 18 August 2012 at the centre and developed at least one of the following symptoms between 18 and 21 August 2012: diarrhoea ($\geqslant$3 loose stools a day), vomiting, nausea or abdominal pain.

Health inspectors obtained email addresses of 54 wedding guests and on 30 August, we sent them a web link to a standardized online questionnaire. The self-administered questionnaire gathered information on demographic details, food and beverages consumed during the reception, date and time of onset, duration and characteristic of clinical symptoms, collection of stool specimens and hospitalization.

We performed a descriptive analysis of cases. We compared the exposed with the unexposed through the calculation of attack rates, with 95% confidence intervals also calculated. All statistical analyses were conducted using Stata v. 12 (StataCorp., USA).

Environmental investigation

On 21 August, the municipal health inspectors contacted the wedding organizers, visited the centre, and investigated the general hygiene of the kitchen. The employees of the centre were asked to provide faecal specimens for bacterial and viral analysis and they were supplied with sampling containers. Food samples were not collected since they were no longer available.

Laboratory investigation

Stool specimens were provided by three guests of the wedding buffet with gastrointestinal symptoms. The specimens were tested for *Salmonella, Shigella, Campylobacter, Yersinia* spp., *S. aureus, B. cereus, C. perfringens* by routine methods [13] and for NoV using real-time reverse transcription (RT)–PCR assay in a local clinical microbiology laboratory and a virology laboratory, respectively. The specimens of the staff were not tested since the laboratory did not receive a referral.

Water specimens were obtained from the tap in the kitchen, from the lake (on 22 August), and from the ice cube machine (on 4 September). Specimens were tested for gut-derived enterococci, *E. coli* and coliform bacteria.

On 21 August, the municipal health inspectors collected environmental specimens from the baking board, cutting board and cold pantry handle, to test for aerobic bacteria and *Salmonella*.

In total, 36 swabs from surfaces at the premises in the centre were taken for NoV analysis. On 22 August, the municipal health inspectors obtained 27 surface specimens for NoV analysis from the main building in the centre. Surfaces were brushed with swabs, which were inserted into a tube containing 5 ml phosphate buffered saline (PBS). The swabs were subjected to nucleic acid extraction using NucliSENS® miniMAG® kit (bioMérieux, The Netherlands) while viral RNA from polymerase-capsid gene junction was amplified using primers and probes specific for NoV genogroups I and II, and QuantiTect probe RT–PCR kit (Qiagen, Germany) in real-time

RT–PCR according to the methods previously described [14, 15]. On 28 August, after the first cleansing, five specimens were taken from places where noroviruses were detected previously. The final collection of four environmental specimens was performed after the second cleansing, on 4 September.

Genotyping analysis was done for three NoV isolates from swabs and from two NoV isolates from patients' stools. Viral RNA was amplified in polymerase region A using a one-step RT–PCR kit (Qiagen) according to Vinjé *et al.* [16]. Nucleic acid sequences of the PCR products were determined. A genotyping tool (www.rivm.nl) and BLAST search in Genbank were used for genotype determination. In addition, one NoV isolate from a swab specimen was amplified in region D and used for genotype determination [16].

RESULTS

Epidemiological investigation

Eighty-eight guests from various countries attended the wedding buffet, of which 54 (61%) had an email address. Thirty-nine (72%) responded to the survey (59% female). The median age of respondents was 37 years (range 27–68 years). Twelve respondents were from abroad (France, $n=6$; Italy, $n=4$; Switzerland, $n=2$). Seven guests had travelled abroad (to France, Italy, Germany, Denmark, Sweden) in the 2 weeks before the wedding reception. Twenty-three (59%) of respondents met the case definition. The highest attack rate (71%) was in the 20–30 years age group. The attack rate was 65% in females and 50% in males (Table 1).

All 23 cases became ill within a 3-day period, 19–21 August 2012 (Fig. 1). The symptoms of the first case started 12 h after the reception ended. The peak of the outbreak was on 20 August, when 15 (65%) cases fell ill, and the outbreak ended on 21 August.

The most commonly reported symptoms were nausea (91%), abdominal pain (74%), diarrhoea (70%), vomiting (48%), headache (39%), and fever (13%). Two cases (9%) were hospitalized.

None of the foods or beverages served at the wedding reception was significantly associated with the illness (Table 2).

Environmental investigation

In the kitchen, the general hygiene was according to requirements. During the visit to the centre on 21 August and during the phone interview with the manager of the centre on 22 August, the health inspector identified three staff members (two customer service staff and a cleaner) who were ill with symptoms consistent with NoV infection. All had become ill on 21 August, following the wedding, and had not been symptomatic at work. The investigation also indicated that the day before the wedding buffet (17 August 2012), nine people who were staying at the centre, and had received food service, had subsequently developed gastrointestinal illness with symptoms typical for NoV infection after leaving the centre. None of these were accommodated in the same rooms as the wedding guests. Three days after the wedding, the next group of 20 people attended and ate at centre.

Table 1. *Attack rate (AR) of gastrointestinal symptoms by age group and gender, wedding reception, Ylöjärvi, Finland, August 2012*

Demographic characteristics	No. of persons	No. ill	AR (%)
Age group (yr)*			
20–30	7	5	71
31–40	19	11	58
41–50	0	–	–
51–60	4	2	50
>60	8	5	62
Gender			
Male	16	8	50
Female	23	15	65
Total	39	23	60

* One person did not report their age.

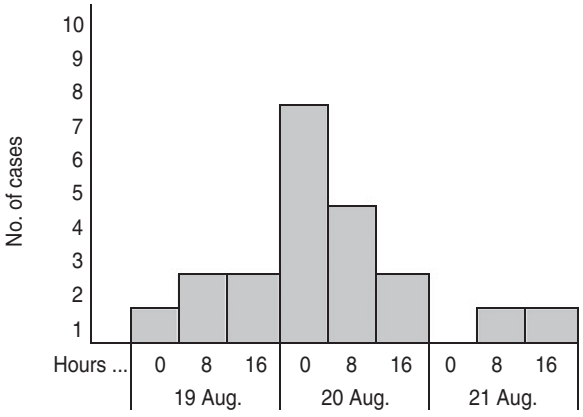

Fig. 1. Distribution of cases with acute gastroenteritis ($n=20$) in guests at the wedding reception according to date and time of symptoms onset (three persons did not indicate the time of symptoms onset), Ylöjärvi, Finland, August 2012.

Table 2. *Attack rates and relative risk of acute gastroenteritis associated with specific food items and beverages consumed during a wedding reception, Ylöjärvi, Finland, August 2012*

	Attack rate in exposed	Attack rate in non-exposed	RR	95% CI	Cases exposed, %
Food items					
Salmon with basil	59 (22/37)	0 (0/0)	–	–	96
Shrimp salad	63 (17/27)	33 (2/6)	1·9	0·59–6·07	74
Baltic herring rolls	70 (14/20)	42 (5/12)	1·7	0·81–3·48	61
Olive rosemary focaccia	75 (12/16)	55 (6/11)	1·4	0·75–2·53	52
Country salad with chicken meat	65 (15/23)	50 (4/8)	1·3	0·61–2·77	65
Castle roast beef	62 (15/24)	50 (6/12)	1·2	0·66–2·38	65
Garlic potatoes	61 (19/31)	50 (2/4)	1·2	0·44–3·40	83
Watermelon feta salad	63 (17/27)	57 (4/7)	1·1	0·55–2·23	74
Rye buttons	67 (6/9)	62 (10/16)	1·1	0·59–1·94	26
Olive oil and white balsamic vinegar	64 (7/11)	61 (8/13)	1·0	0·56–1·92	30
Sugar frosting	61 (20/33)	60 (3/5)	1·0	0·47–2·17	87
Vanilla cream puffs	67 (14/21)	67 (8/12)	1·0	0·61–1·65	61
Sour cream herb sauce	60 (9/15)	70 (7/10)	0·9	0·48–1·53	39
Baked root vegetables	68 (19/28)	100 (1/1)	0·7	0·53–0·88	83
Beverages					
Water	63 (22/35)	0 (0/1)	–	–	96
Tea	100 (5/5)	57 (16/28)	1·7	1·27–2·41	22
Juice	62 (10/16)	59 (13/22)	1·1	0·63–1·77	43
Mineral water	60 (12/20)	61 (8/13)	1·0	0·56–1·71	52
Coffee	58 (18/31)	71 (5/7)	0·8	0·47–1·42	78
Homemade rye beer	33 (2/6)	64 (16/25)	0·5	0·16–1·68	9
Milk	0 (0/1)	64 (21/33)	0·0	–	0

RR, Risk ratio; CI, Confidence interval.

Within 36 h of their arrival, five persons had fallen ill with gastrointestinal symptoms.

Laboratory investigation

Two stool specimens from the wedding guests were positive for NoV. *Salmonella, Shigella, Campylobacter, Yersinia* spp., *S. aureus, B. cereus, C. perfringens* were not found in any of the specimens tested.

Water specimens from the lake and the tap in the kitchen were negative for gut-derived enterococci, *E. coli* and coliform bacteria (0 MPN/100 ml). The level of heterotrophic spore-forming bacteria [240 colony-forming units (c.f.u.)/ml] in the ice-cube specimen was over the recommended limit (100 c.f.u./ml) of the Finnish Food Safety Authority Evira.

The microbiological quality of the surface hygiene and water specimens collected on 21 and 22 August was satisfactory. The level of aerobic microorganisms was: 76 c.f.u./cm^2 for the baking board, 3 c.f.u./cm^2 for the cutting board, and 80 c.f.u./cm^2 for the cold pantry door handle. All specimens were negative for *Salmonella*.

Out of 27 environmental specimens collected on 22 August, nine were positive for NoV. NoV was detected in the kitchen, two hotel rooms and in the main building of the centre (Table 3). The results of five specimens obtained on 28 August taken after cleansing of previously contaminated surfaces, confirmed the presence of NoV in one room. On 4 September, after further cleansing, NoV was no longer detected.

NoV isolates from two patients and from three swab specimens taken from the surfaces of one guest room were characterized further by sequence analysis. All sequences were identical and were characterized as NoV genotype GII.4. The virus strain was 98·9% and 98·5% identical with GII.4 Sydney variant (accession no. JX459908) in polymerase region A and capsid region D, respectively.

DISCUSSION

The results of this investigation indicated that the outbreak of NoV gastroenteritis occurred in persons who attended a wedding reception at the centre. NoV was

Table 3. *Environmental specimens tested for norovirus at the centre, Ylöjärvi, Finland, 2012*

Sampling date	Sampling site	No. of specimens positive for norovirus/no. of specimens taken	Surfaces with positive results
22 Aug. 2012	Kitchen	2/5	Freezer door handle Tap handle
	Room A	4/4	Surface with vomit Mattress Tap handle Toilet seat
	Room B	2/3	Soap devices Pooled specimen
	Storehouse	0/2	–
	Meeting hall	0/7	–
	Sauna	0/3	–
	Main building	1/3	Door handles
28 Aug. 2012 (after first cleaning)	Kitchen	0/2	–
	Room A	1/1	Pooled specimen
	Room B	0/1	–
	Main building	0/1	–
4 Sept. 2012 (after second cleaning)	Room A	0/4	–

detected in two stool specimens obtained from wedding guests and in 10/36 environmental specimens collected from the centre. The high attack rate and clinical picture reported by the cases are typical for NoV infection. The distribution of cases with a rapid increase and decline and a single peak suggest a point-source outbreak. The statistical analysis of data on food and beverage consumption by the guests did not indicate any specific source of infection. The high attack rate (100%) and risk ratio (1·75) for drinking tea was not considered relevant, since only five cases had been drinking tea at the buffet.

The general hygiene of the centre's kitchen was visually good and none of the kitchen staff had been symptomatic at work. However, NoV was detected on the freezer handle and the kitchen tap handle, indicating that contaminated hands had been touching them. No stool samples from the staff were tested for NoV and none of the food that had been served was available for analysis. In order to assess the microbiological causality, samples from staff members and food served should be available for testing, and the laboratories should be informed about receiving outbreak samples.

Although the microbiological quality of water specimens was consistent with the norm, the level of heterotrophic spore-forming bacteria in the ice-cube machine was over the recommended limit. The investigation indicated that the machine had been out of order for a long time before the wedding buffet and had not been used. It is probable that the number of heterotrophic bacteria in the ice cube machine had increased after the water flow in the machine ceased.

During the control visits the health inspectors identified that several staff members at the centre were ill with gastrointestinal symptoms, although they had not been symptomatic while at work. NoV shedding can continue for several weeks in symptomatic and asymptomatic cases [17, 18]. In hotels, where new cohorts of susceptible guests often change, the staff members that excrete the virus, as well as contaminated surfaces, may prolong the outbreak for several weeks [19].

The extensive environmental investigation indicated the presence of NoV on several surfaces at the centre. The contaminated surfaces in the main building were easily accessible and commonly used. Since NoV infection has a very low infectious dose and noroviruses were detected on door handles and tap handles, contaminated hands could have played a key role in the environmental transmission cycle. Additional cases of gastroenteritis that occurred before and after the wedding reception supported the hypothesis of an environmental transmission of NoV.

In order to prevent NoV transmission at the centre, we recommended ill staff members stay at home for 2 days after the symptoms had ceased. Careful hand hygiene, always washing with soap and water after toilet visits, and before preparing, serving or eating food decreases the transmission risk [20]. In the kitchen, we drew attention to the recommendation of the Finnish Food Safety Authority that 200 g frozen samples of all served foods should be stored for 2 weeks at institutional kitchens to enable microbiological investigations after possible outbreaks.

Since several cases of gastrointestinal illness were reported in three guest groups, the accommodation was left unused for a period of 1 week to cut the environmental transmission cycle. During this period, the accommodation was thoroughly cleansed and disinfected. Control specimens for NoV analyses were taken before the accommodation was returned to use. However, NoV was still isolated in several sites in a hotel room that had been severely contaminated with vomit. The premises were cleansed with detergents and the surfaces disinfected with hypochlorite solution according to THL guidelines [20]. We recommend that: contaminated materials should be treated with water and ordinary detergents and disinfected with 1000 ppm hypochlorite or by steam cleaning; materials with vomit or faecal stains can be disinfected with 5000 ppm hypochlorite, with disposable cleaning cloths used. To avoid droplet infection, the use of disposable gloves, eye-nose mask and apron are necessary during cleaning. Closing the centre for 1 week was recommended. After thorough cleansing and disinfection and 1 week's quarantine, no new cases with symptoms typical for NoV infection were identified at the centre.

The sequence analysis of isolates from two patients and three environmental specimens indicated the same genotype in all specimens. The NoV GII.4 Sydney variant was identified. In March 2012, a new variant of GII.4, designated Sydney, was reported in Australia. Since then, increased activity of this variant has been observed worldwide [21–23]. The NoV GII.4 Sydney variant was identified for the first time in Finland during this outbreak. It is possible that one of the wedding guests from abroad had imported the new strain to the centre. However, since symptoms typical for NoV infection were also reported in other guests prior to the wedding reception, it is possible that the centre had been contaminated prior to the wedding event. NoV may remain infectious for over 2 weeks on environmental surfaces and in water for over 2 months [24–27].

Noroviruses belonging to GII.4 have been the predominant strain worldwide for over a decade [28]. In 2002, 2004, 2006 and 2010 the number of outbreaks caused by NoV increased markedly [29]. Increased NoV activity was associated with the emergence of new variants of GII.4 associated with the multiple outbreaks in the USA and Europe: the Farmington Hills variant in 2001–2002, the Hunter virus at the end of 2004, the GII.4-2006a/2006b viruses in 2006, and New Orleans virus in 2010 [30–33]. In 2012, an increased activity of NoV was observed in Australia, New Zealand, France, and Scotland, which may indicate a new epidemic wave caused by the new variant [21].

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DECLARATION OF INTEREST

None.

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ORIGINAL PAPER

Ultraviolet Light Inactivation of Murine Norovirus and Human Norovirus GII: PCR May Overestimate the Persistence of Noroviruses Even When Combined with Pre-PCR Treatments

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Abstract Transmission of gastroenteritis-causing noroviruses may be significant via contaminated surfaces. Measures for control, e.g. disinfection with ultraviolet irradiation (UV), are therefore necessary for interrupting this transmission. Human norovirus (HuNoV) *GII.4* and Murine norovirus (MuNoV) were used to study the efficacy of UV for virus inactivation on dry glass surfaces. MuNoV inactivation was measured using viability assay and the reduction in viral RNA levels for both viruses using reverse transcription quantitative PCR (RT-QPCR). For each UV dose, two parallel sample groups were detected using RT-QPCR: one group was enzymatically pre-PCR treated with Pronase and RNAse enzymes, while the other was not treated enzymatically. In the viability assay, loss of infectivity and a 4-log reduction of MuNoV were observed when the viruses on glass slides were treated with a UV dose of 60 mJ/cm^2 or higher. In the RT-QPCR assay, a steady 2-log decline of MuNoV and HuNoV RNA levels was observed when UV doses were raised from 0 to 150 mJ/cm^2. A distinct difference in RNA levels of pre-treated and non-pretreated samples was observed with UV doses of 450–1.8 $\times$ 10^3 mJ/cm^2: the RNA levels of untreated samples remained over 1.0 $\times$ 10^3 PCR units (pcr-u), while the RNA levels of enzyme-treated samples declined below 100 pcr-u. However, the data show a prominent difference between the persistence of MuNoV observed with the infectivity assay and that of viral RNA detected using RT-QPCR. Methods based on genome detection may overestimate norovirus persistence even when samples are pretreated before genome detection.

Keywords Norovirus · Murine norovirus · UV irradiation · Environmental surfaces

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Introduction

Human noroviruses (HuNoV) are the most common viral agents associated with outbreaks of gastroenteritis (Lopman et al. 2003; Lynch et al. 2006; Koopmans 2008), which spread rapidly in semi-closed facilities, such as hospitals (Gallimore et al. 2006). A faecal–oral transmission route, a high viral load in the vomit and faeces of infected persons (up to 10^9 genomic copies/g, Atmar et al. 2008), lack of long-term immunity following previous infection and a low infective dose (10–100 virus particles, Teunis et al. 2008) all enhance the spreading of HuNoV in the population. In addition to being transmitted directly from person to person, the viruses spread easily via contaminated surfaces (Koopmans and Duizer 2004).

HuNoV outbreaks typically begin with a sudden illness peak with high secondary attack rates (Cheesbrough et al. 1997). According to epidemiological studies, secondary infections have occurred not only in those in close contact with infected persons but also in those having only touched surfaces contaminated by infected persons (Cheesbrough et al. 1997; Thornley et al. 2011). Environmental fomites thus seem to play a major role in sustaining a succession of

outbreaks, and therefore control measures, e.g. cleaning and disinfection, are important for interrupting transmission chains. Thorough disinfection of surfaces is especially important in hospitals, geriatric care facilities and daycare centres, where residents are in close contact with one another and the infection pressure is high (Ludwig et al. 2008).

Ultraviolet light (UV) irradiation is widely used for the disinfection of drinking water in water treatment plants and the disinfection of surfaces and air in research facilities and hospitals. Promising results in the effectiveness of UV irradiation towards bacteria (Martin et al. 2011) and cultivable Human viruses (Meng and Gerba 1996; Nuanualsuwan et al. 2002; de Roda Husman et al. 2004) have also increased the interest to investigate the efficacy of UV towards HuNoV.

Despite various efforts to cultivate HuNoV in cell lines (Duizer et al. 2004a, b), no functional protocol exists for the purpose. RT-QPCR techniques, which are commonly used to investigate HuNoV inactivation, are unable to discriminate between infectious and non-infectious viruses (Richards 2012). Inactivation studies on HuNoV are, therefore, usually carried out using surrogate viruses, e.g. Feline and Canine caliciviruses (Duizer et al. 2004a, b) and more recently Murine norovirus (MuNoV) (Lee et al. 2008; D'Souza and Su 2010; Park et al. 2010), which resemble HuNoV in morphology (Wobus et al. 2004). Although a lot of important information is gained with the use of surrogates, there is concern that they are less resistant towards environmental stresses compared to the viruses of interest (Richards 2012).

Several attempts have been made to develop pre-PCR treatments which would ensure that the PCR is only copying genomes of infectious viruses in the sample. A widely used approach is enzymatic treatment, where proteinase breaks up the coat protein of damaged viruses and RNAase digests the viral RNA (Nuanualsuwan et al. 2002; Lamhoujeb et al. 2008; Diez-Valcarce et al. 2011). Protocols also exist were RNAase is used alone (Topping et al. 2009; Nowak et al. 2011). Although the results of these applications have been somewhat successful, they still require confirmation before enzymatic treatments can be used reliably in predicting the infectivity of non-cultivable viruses.

The objective of our study was to evaluate the inactivation of MuNoV and the virus stability of MuNoV and HuNoV on dry surfaces when exposed to UV irradiation. We also investigated whether enzymatic pre-PCR treatment with Pronase enzyme mix and RNAse enzyme can affect virus particles so that the damaged virus particles do not show a positive signal in RT-QPCR, while the infectious viruses show equal positive signal with and without pre-enzymatic treatment.

Materials and Methods

Viruses

For the artificial contamination of glass surfaces, we used MuNoV 1, which was obtained from Dr. Herbert W. Virgin at the Washington University School of Medicine (St. Louis, MO, USA) or a human stool preparation containing HuNoV genogroup II (GII.4). A 10 % faecal suspension was prepared from the stool containing HuNoV in balanced salt solution (PBS, pH 7.2), cooled at 5 °C for 2 h and frozen at −70 °C in aliquots. RNA extraction was performed according to instructions given in the NucliSENS® miniMAG® (Biomerieux, Boxtel, The Netherlands) kit with a sample size of 100 µl. The end point dilution of the extracted virus sample revealed a concentration of 10^{10} PCR units (pcr-u)/ml in RT-QPCR. A standard curve was plotted for sequential 10-fold dilutions of RNA, and one RT-QPCR-detectable virus unit (pcr-u) was defined as the highest 10-fold dilution of the sample, showing a positive result with Ct < 40. The PCR titre of MuNoV was determined as 10^{10} pcr-u/ml.

MuNoV Preparation and Viability Assay

MuNoV was cultured in RAW 264.7 cells (ATCC-CRL-2278) in high-glucose Dulbecco's Modified Eagle's Medium (DMEM), containing 10 % heat-inactivated foetal bovine serum (FBS, Gibco), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 1 % glutamine–penicillin–streptomycin. RAW 264.7 cells were grown and maintained according to standard animal cell culture protocols and kept at 37 °C with 5 % carbon dioxide (CO_2). Cells from passage 2 to passage 20 were used for the experiments.

To produce virus stock, MuNoV was cultivated on confluent cell monolayers for 2–3 days, and after appearance of the cytopathic effect (CPE), the infected cells were thrice subjected to freezing and thawing to release the viruses. The virus stock was prepared according to Park et al. (2011). Briefly, to concentrate the MuNoV, the supernatant was subjected to ultrafiltration (Amicon Ultra-15; Millipore, Billerica, MA, USA) at 4.5×10^3 g for 10 min at 4 °C, to remove the cell debris. The supernatant from the ultrafiltration unit was recovered, adjusted to 2 ml with PBS, pressed through a 0.2-µm syringe filter prehandled with Polysorbate 20 solution (Sigma, Saint Louis, MO, USA) and stored in aliquots at −70 °C.

The titre of MuNoV released from the cells was determined in a viability assay to be approximately 10^6 plaque-forming units (PFU)/ml (Verhaelen et al. 2012). In short, RAW 264.7 cells were diluted in DMEM to a concentration of 2×10^5 cells/ml and 100 µl of cell suspension was

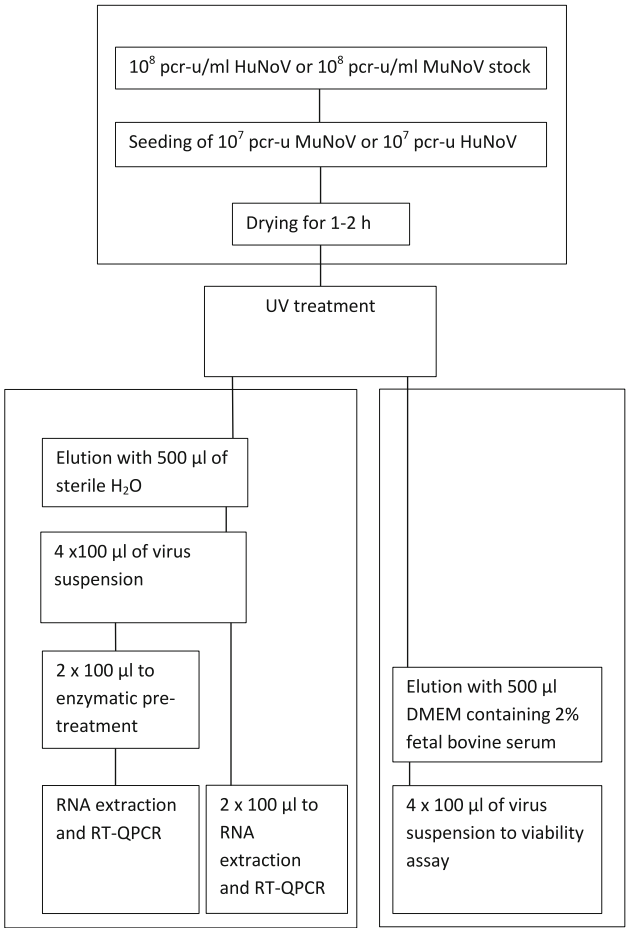

Fig. 1 Flow chart of the protocol for the UV irradiation treatment of HuNoV and MuNoV

seeded into each well in a 96-well plate (Nunc A/S, Roskilde, Denmark). After 4 h of incubation at 37 °C under 5 % CO_2, 100 µl aliquots of each 10-fold serial dilution of concentrated MuNoV, prepared in DMEM with 2 % FBS, 10 mM HEPES and 1 % glutamine–penicillin–streptomycin suspension were added to six parallel wells on the plate per dilution. The plates were incubated at 37 °C under 5 % CO_2 and checked daily for the presence of CPE using a light microscope. The wells with CPE were recorded as positive. The final reading was performed after 5 days and the tissue culture infectious dose ($TCID_{50}$/ml) and PFU/ml were calculated using the protocol described by Morales (2006 http://www.urmc.rochester.edu/mbi/resources/Xenopus/protocols/TCID50-protocol.pdf. Access date 30.8.2012).

UV Inactivation

UV irradiation was generated by an ozone-free low-pressure mercury-vapour discharge lamp (Sylvania G15T8, London, UK). The lamp emitted short-wavelength UV irradiation with a peak wavelength at 253.7 nm and an output of 0.48 ± 0.02 mW/cm^2. The lamp was switched on for 10 min at room temperature (RT) to reach its maximum output before starting the experiments. UV irradiation intensity was measured by a digital UVX radiometer (IL Metronic Sensortechnik GmbH, Germany).

The virus stock suspensions were diluted 10-fold with sterile H_2O to prepare the working virus suspension. 100 µl of virus suspension was dispersed to form a circular thin layer of ϕ 100 mm on a glass slide. The suspension was left to dry in a flow hood for 1–2 h at RT. The distance between the slides and the UV lamp was 11 cm and the lamp was positioned directly above the slides to ensure uniform radiation. The UV irradiation doses, which increased with time, used in the viability assays were 0, 7.5, 15, 30, 60, 90, 120 and 150 mJ/cm^2, whereas doses in the PCR assay were 0, 30, 60, 90, 120, 150, 300, 450, 600, 750, 900 and 1.8×10^3 mJ/cm^2. The experiments were repeated thrice. For each UV dose, four parallel samples were inoculated: half of the samples were to be analysed using RT-QPCR detection and the other half using the viability assay (Fig. 1). For HuNoV, only RT-QPCR detection was used.

Virus Elution and Viability Assay

MuNoVs that were used for the viability assay were eluted from slides with 500 µl of DMEM containing 2 % foetal bovine serum 100 µl at a time with back and forth pipetting. The elute was divided into four 100-µl portions, one of which was used to prepare a dilution series of 1:1, 1:2.5, 1:5, 1:7.5, 1:10, 1:50, 1:100 and thereafter 10-fold dilutions to reach $1:1.0 \times 10^6$ dilution. Three of the 100-µl portions were inoculated to the assay wells as such to represent the parallel samples of the 1:1 dilution. The viability assay was performed according to the viability assay protocol explained in the chapter 'MuNoV preparation and viability assay'.

Virus Elution and Enzymatic Pretreatment

HuNoV and MuNoV used for RT-QPCR detection were eluted from the slides with 500 µl of sterile H_2O. Four 100-µl quantities were separated from the elute, two of them for RNA extraction and RT-QPCR and two for enzymatic pretreatment preceding RNA extraction and PCR steps.

Enzymatic digestion was carried out as described by Nuanualsuwan and Cliver (2002) and Lamhoujeb et al. (2008), with major modifications. Briefly, 6 mg of Pronase enzyme (Roche Diagnostics, Indianapolis, IN, USA, Cat. No. 10 165 921 001), mixture of proteinases isolated from the extracellular fluid of *Streptomyces griseus*, dissolved in sterile H_2O (200 mg/ml, Roche) was mixed with 100 µl of the virus sample and the samples were shaken at 37 °C for 10 min. The reaction was stopped by adding 2 µl of

200 μM phenylmethane sulfonyl fluoride (Aldrich Sigma Canada Ltd., Oakville, Ontario, Canada) and the suspensions were left at RT for 15 min. RNAse (0.04 mg, Roche Diagnostics, Indianapolis, IN, USA) was added and the mixtures were incubated at 37 °C for 40 min, after which 80 U of RNAse inhibitor solution (Promega US, Madison, WI, USA) was added.

The effects of Pronase and RNAse were tested before the UV inactivation study to verify that enzymatic pre-PCR treatment would show negative results when the dried surface MuNoV sample was treated with the maximal, 1.8×10^3 mJ/cm^2, UV dose. Three doses of Pronase, 3, 6 and 9 mg, and two doses of RNAse, 0.02 and 0.04 mg, were tested prior to the suitable doses of 6 mg of Pronase and 0.04 mg of RNAse being chosen for the UV irradiation study. Enzymatic effects were tested with two MuNoV concentrations: 10^7 and 10^8 pcr-u. Identical MuNoV samples were treated with enzymes without UV irradiation to confirm that the loss of virus tested by RT-QPCR was not prominent.

RNA Extraction and RT-QPCR

RNA extraction was performed according to instructions given in the NucliSENS® miniMAG® kit. Amplification of HuNoV and MuNoV was performed using a TaqMan RT-QPCR for polymerase-gene-capsid-gene junction according to protocols recently described by Rönnqvist et al. (2013). In brief, the detection was performed using the QuantiTect Probe RT-QPCR kit (Qiagen, Hilden, Germany), primers and a probe QNIF2d (+) and COG2R (−) and QNIFS (+) for HuNoV (Loisy et al. 2005), and MNVfor and MNVrev and a probe MNV for MuNoV (Hewitt et al. 2009). Amplification was performed with the Rotorgene 3000 detection system (Corbett Life Science, Sydney, Australia).

Standard curves were prepared from 10-fold dilutions of HuNoV and MuNoV and used to calculate the pcr-u counts of the samples. RNA sample duplicates, a negative control for RNA extraction, a negative PCR control containing distilled water and a non-template control were included in every PCR run.

Data for the Statistical Model

The data consisted of MuNoV and HuNoV concentration measurements at the zero point and after different UV irradiation doses that were measured using RT-QPCR as described above. The maximum UV dose used was 1.8×10^3 mJ/cm^2. The trial was repeated in four categories according to virus type (HuNoV or MuNoV) and enzyme treatment (yes or no). Four to seven trials for each category were performed. All the data used in our study were based on these experiments.

Statistical Model

The aim of the modelling was to represent the relationship between UV irradiation doses and the reduction of MuNoV and HuNoV particles on dry surfaces. Based on the data and the information from the literature (Song et al. 2004) (Eq. 1), we used a Bayesian linear regression model to determine this relationship. Deviance Information Criterion (DIC) (Spiegelhalter et al. 2002, 2003) was used to compare different regression models. After testing several competing regression models, the following model fitted best to the data:

$$\begin{aligned} Y_{i,j} &\sim \mathrm{N}(\mu_{i,j}, \tau) \\ \mu_{i,j} &= \alpha + \beta_{\mathrm{uv}} \cdot \log(X_{i,j} + 1) \end{aligned} \quad (1)$$

The response variable ($Y_{i,j}$) used is a $\log_{10}$ inactivation of the MuNoV or HuNoV. A $\log_{10}$ inactivation equals a negative logarithm of the remaining fraction. For example, a $1 - \log_{10}$ reduction means that 90 % of the viruses are removed, 2 $\log_{10}$ corresponds to 99 % and so on. The values of the response variable (inactivation in the i^{th} UV dose level in the j^{th} trial within category) were analytically calculated from the data. These values are presented in Fig. 2. The direct MuNoV or HuNoV concentration was not used as the response variable, because the concentration at the zero point varies significantly between different trials. A log-transformed UV dose ($\log(X_{i,j})$) was used as an explanatory variable instead of a direct UV dose. A log transformation was performed because of the much better model fit. The intercept parameter (α) of the model was fixed at zero, which means that no inactivation should take place when the UV dose is zero.

The slope parameter (β_{uv}) and the precision parameter (τ) were estimated from the data. Both parameters were given practically uninformative prior distributions:

$$\begin{aligned} \beta_{\mathrm{uv}} &\sim \mathrm{Norm}(0, 100^2) \\ \tau &\sim \mathrm{Gamma}(0.01, 0.01) \end{aligned} \quad (2)$$

The model was separately applied to the category datasets because the shapes of the data distributions were dissimilar in different categories. Computations of the model were performed with WinBUGS 1.4.3 software (Spiegelhalter et al. 2003). The number of Markov Chain Monte Carlo (MCMC) iterations was 10,000.

Results

Inactivation of MuNoV by UV Irradiation Measured by Viability Assay

The inactivation levels of MuNoV dried on glass surfaces by UV irradiation were investigated in RAW 264.7 cells.

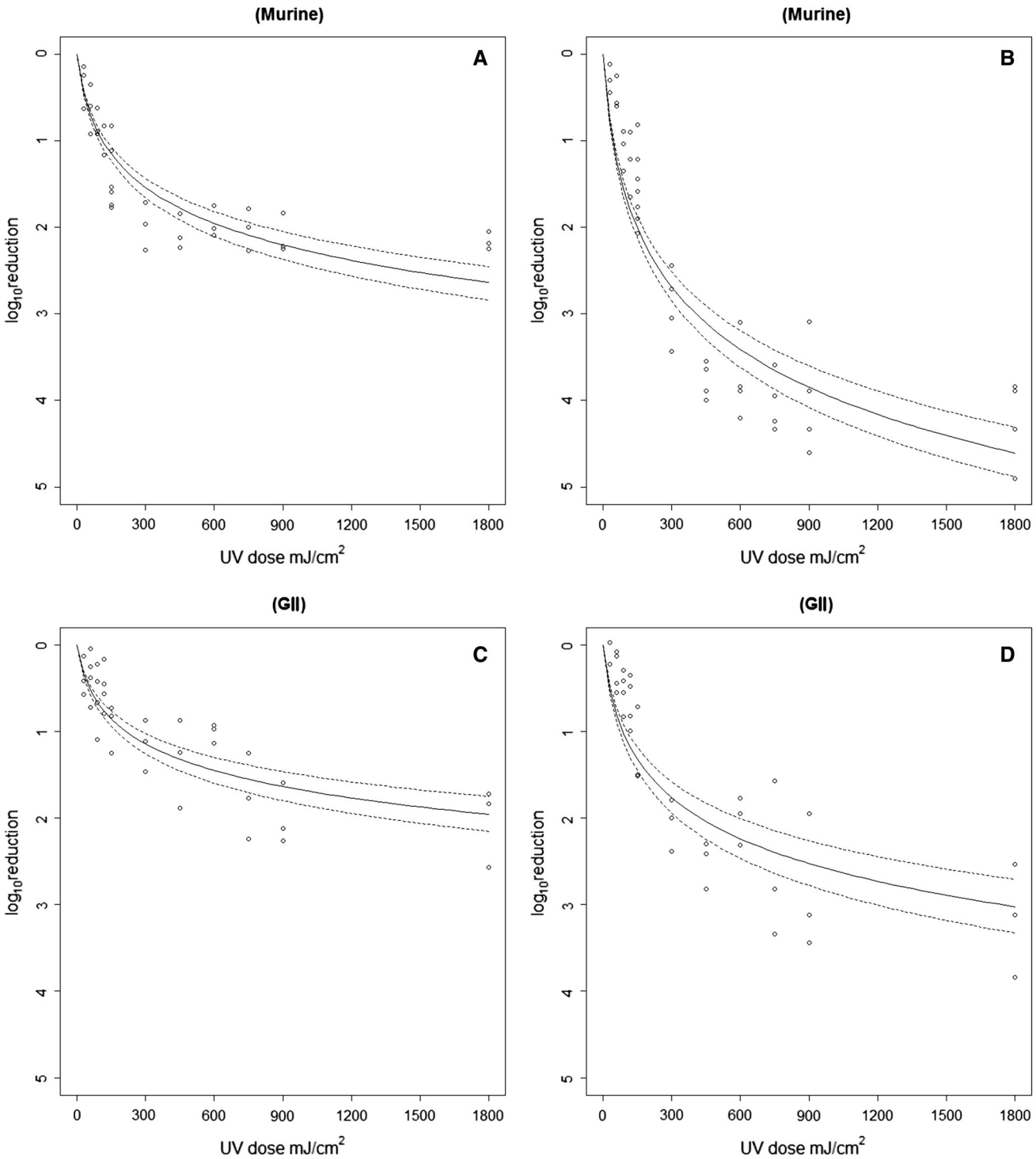

Fig. 2 Observed reduction rates (*circles*) and prediction distributions (*dashed lines*) for the expected reduction rates of MuNoV and HuNoV GII, either with no enzymatic pre-PCR treatment (**a**, **c**) or with enzymatic pre-PCR treatment (**b**, **d**)

MuNoV was found to be sensitive to UV irradiation in the experiments. The inactivation curve (Fig. 3) was observed to be two phased: during the first phase, the virus titre decreased rapidly from 1×10^5 to 2.7×10^2 PFU when the UV fluence was increased from 0 to 7.5 mJ/cm^2, after which the decrease was more moderate. Total loss of infectivity was achieved at a 3-min time point at a fluence of 90 mJ/cm^2.

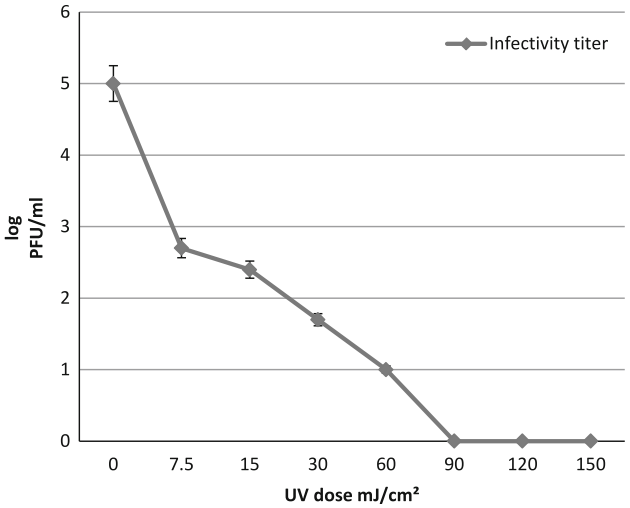

Fig. 3 UV inactivation of MuNoV dried on a glass slide measured by viability assay

Stability of MuNoV and HuNoV RNA After UV Treatment Measured Using RT-QPCR

The decrease in virus levels detected using RT-QPCR, when the virus-contaminated surfaces were treated with UV irradiation, was found to be UV dose dependent and comparable for both viruses, MuNoV (Fig. 2a) and HuNoV (Fig. 2c). During the first 10 min when UV fluence was increased gradually from 0 to 300 mJ/cm^2, MuNoV levels decreased rapidly, from approximately 3.0×10^4 to $<3.0 \times 10^3$ pcr-u (Fig. 2a). A tailing of the virus level curve was observed with UV fluences higher than 300 mJ/cm^2, resulting in positive PCR signals even with a UV contact time of 60 min and fluence of 1.8×10^3 mJ/cm^2. HuNoV showed more resistance towards UV irradiation than MuNoV: the levels of HuNoV decreased from 2.5×10^4 to 6.0×10^3 pcr-u when UV fluence was increased from 0 to 300 mJ/cm^2 (Fig. 2c).

Validation of Enzymatic Pre-RT-QPCR Treatment

Enzymatic pre-PCR treatment using Pronase, RNAse or both, and two concentrations, 10^8 and 10^9 pcr-u/ml (10^7 and 10^8 pcr-u/individual 100-µl sample), of MuNoV was validated for our study by testing the protocol using either MuNoV samples that did not receive UV treatment or samples that received maximum UV irradiation, 1.8×10^3 mJ/cm^2 (Table 1). First, we observed that the loss of RNA when non-irradiated virus samples were treated according to enzymatic pre-PCR protocol was less than one log unit, which was regarded as tolerable and thus the protocol was deemed applicable to use. We next observed that the combination of Pronase and RNAse enzymes to UV-treated virus samples was the only pretreatment option not showing a positive signal in RT-QPCR after treatment when tested with 10^7 pcr-u of MuNoV, although sample treatment with RNAse alone also resulted in a negative signal in one out of three times. None of the enzymatic pre-PCR treatments resulted in negative signals when tested with 10^8 pcr-u of MuNoV (Data not shown).

The Effect of Enzymatic Pre-PCR Treatment on the Stability of MuNoV and HuNoV RNA After UV Treatment

The addition of enzymatic pre-PCR treatment to the UV protocol produced a total decrease of 4–5 $\log_{10}$ in MuNoV levels when UV fluence was 1.8×10^3 mJ/cm^2 (Fig. 2b). The difference between enzymatically pre-PCR-treated and untreated samples was seen with UV doses higher than 450 mJ/cm^2: the concentration of MuNoV samples untreated with enzymes remained at over 1.0×10^3 pcr-u regardless of the rise in UV dose, whereas the virus concentration of samples treated with enzymes fell under 100 pcr-u. The effect of enzymatic pre-PCR treatment on HuNoV samples treated with UV irradiation was less prominent compared to the effect on MuNoV samples: the total decrease in HuNoV levels was 2.5–3 $\log_{10}$ with UV fluence of 1.8×10^3 mJ/cm^2 (Fig. 2d). Nevertheless, according to the results, the enzyme treatment significantly accelerated the reduction of both MuNoV and HuNoV levels.

Statistical Analysis

As a result of the Bayesian analysis, we obtained posterior distributions for the regression model parameters (β_{uv} and τ) and prediction distributions for the mean reduction rates (μ) as well as for the observable reduction rates of viruses. Prediction distributions for the mean virus reduction rates are shown in Fig. 2 (dashed lines). The times needed to achieve specified levels of MuNoV and HuNoV reduction rates are shown in Table 2. The highest reduction rate was estimated to occur among the enzyme-treated MuNoV viruses, where 4-$\log_{10}$ inactivation was achieved with 95 % probability (Table 3).

Discussion

Because of the importance of HuNoVs as pathogens spreading via the environment, much effort has been put in investigating their persistence against various sanitizing methods (reviewed by Koopmans and Duizer 2004). While most studies have been performed as infective assays with surrogate viruses, we investigated the effects of UV irradiation on the viruses with a method based on PCR

Table 1 Validation of the effect of enzymatic pre-PCR treatment on 10^7 pcr-u of MuNoV dried on glass slides

UV treatment 1,800 mJ/cm^2	Enzymatic pre-PCR treatment		MuNoV Ct	ΔCt < 3[a] with or without enzyme treatment	Number of negative results after UV treatment[b]
	Pronase 6 mg	RNAse 0.04 mg			
−	−	−	25.0 ± 0.8	nc	nc
−	+	−	27.0 ± 1.2	3/3	nc
−	−	+	28.2 ± 0.9	2/3	nc
−	+	+	28.0 ± 0.6	3/3	nc
+	−	−	30.2 ± 0.6	nc	0/3
+	+	−	34.1 ± 4.9	nc	0/3
+	−	+	38.2 ± 2.2	nc	1/3
+	+	+	−(no Ct)	nc	3/3

nc Not calculated

[a] Ct-values have been compared with the Ct-value of the sample that has not been treated (-UV, -enzymes)

[b] Ct 40 was the cutoff limit in this study and samples with Ct > 40 were interpreted as negative

Table 2 Estimated time (in minutes) needed to achieve specified levels of MuNoV and HuNoV pcr-u reduction rates with 95 % probability when treating viruses on dry surfaces with UV irradiation of 0.5 mW/cm^2

Virus	Pre-PCR enzyme treatment	Time for $\log_{10}$-inactivation (min)				
		1 $\log_{10}$	2 $\log_{10}$	3 $\log_{10}$	4 $\log_{10}$	5 $\log_{10}$
MuNoV	−	5	27	–	–	–
MuNoV	+	2	6	16	43	–
HuNoV	−	10	–	–	–	–
HuNoV	+	4	19	–	–	–

detection of the HuNoV genome. In the absence of a practical method for cultivating HuNoV, we used various pretreatments that have been introduced to distinguish damaged HuNoV particles from those that may still be infective after inactivation treatments (Nuanualsuwan et al. 2002; Topping et al. 2009; Mormann et al. 2010; Nowak et al. 2011, Diez-Valcarce et al. 2011). Our study showed that although UV irradiation is effective towards MuNoV present on surfaces, the RT-QPCR method overestimated the infectivity of treated MuNoVs, even when combined with enzymatic pre-PCR treatment. HuNoV behaved similarly as MuNoV in our tests, which implies that the method also overestimates the infectivity of HuNoV after UV irradiation.

Previous studies have shown that MuNoV is inactivated effectively when treated with perpendicular UV irradiation, both in suspension and when dried on a surface (Lee et al. 2008; Jean et al. 2011). Lee et al. (2008) observed that MuNoV was inactivated in suspension after it had been treated with 30 mJ/cm^2 of UV irradiation. We found that a similar input is enough to inactivate MuNoV on surfaces. This UV fluency is easily reached in surface-sanitizing devises, e.g. self-sanitizing keyboards, which deliver 120 mJ/cm^2 doses of UV, thus making such devises useful not only against bacteria as reported by Martin et al. (2011) but also against enteric viruses. Although effective against microorganisms, UV rays are easily blocked by solid objects. UV irradiation is therefore most effective when used so that UV is directly targeted at close proximity towards the surface that is to be disinfected.

The results obtained when MuNoV stability was investigated with RT-QPCR without pretreatment differed considerably from those obtained using the viability assay. MuNoV RNA was detectable even at the last time point of 60 min (1.8×10^3 mJ/cm^2) of the experiment, although according to the viability assay, no viable MuNoVs were present after 3 min of UV irradiation. Lee et al. (2008) also

Table 3 Probability (%) to achieve 3 $\log_{10}$-virus pcr-u reduction rate with specified doses of UV light

Virus	Pre-PCR enzyme treatment	Probability (%)				
		150 mJ/cm^2	300 mJ/cm^2	900 mJ/cm^2	1,350 mJ/cm^2	1,800 mJ/cm^2
MuNoV	−	0.00	0.00	0.01	0.01	0.02
MuNoV	+	0.00	0.02	87.31	99.99	100.00
HuNoV	−	0.00	0.00	0.01	0.01	0.01
HuNoV	+	0.00	0.01	0.04	10.86	56.62

reported that while 232 s (approximately 25 mJ/cm^2) of UV irradiation inactivated MuNoV by more than 3 $\log_{10}$, the reduction in virus particles observed with RT-QPCR was less than 0.5 $\log_{10}$. Differences between the results obtained by the viability assay and RT-QPCR can at least partially be explained by the UV inactivation mechanism (Nuanualsuwan and Cliver 2002): small UV doses may induce the chemical dimerization and cross-linking of viral RNA, which do not prevent gene amplification leading to positive signalling. While high UV doses were used in our experiments, which should attack both the RNA and capsid of viruses reducing positive signals in RT-QPCR amplification, we still observed a tailing on both MuNoV and HuNoV reduction curves. Some virus particles in our samples may in this case have been protected by proteins and other physical elements, such as cell debris from bacteria (HuNoV) or RAW 264.7 cells (MuNoV), from UV irradiation so effectively that only minor damage to the virus could have occurred.

Nuanualsuwan and Cliver (2002) used proteinase K enzyme to break down the damaged virus particles, resulting in negative signals in traditional RT-PCR from inactivated Feline calicivirus (FCV) samples. Diez-Valcarce et al. (2011) used similar pre-enzymatic treatment to quantify the infectivity of MuNoV after different treatments, including UV irradiation. We, instead, used a Pronase enzyme mix to achieve more effective protein digestion though a wider range of cleavage sites compared to the proteinase K enzyme (Roche Diagnostics). We observed that enzymatic pre-PCR treatment distinctly accelerated the reduction of both MuNoV and HuNoV levels after UV treatment. The reduction of MuNoV particles, however, was not at the same level as the decline in the infectivity, more similar than reported by Diez-Valcarce et al. (2011). Lamhoujeb et al. (2008) observed that concentrations of proteinase and RNAse enzymes present in the FCV sample affected the effectiveness of enzymatic digestion. The enzyme concentrations and the balance between proteinase and RNAse enzymes also seemed to be important in our study, as too high concentrations of proteinase resulted in the loss of presumably viable viruses, whereas too low concentrations of either enzyme were not enough to digest the damaged virus particles (data not shown).

One approach to discriminating between infectious and non-infectious HuNoVs is based on the binding properties of infective viruses to porcine gastric mucins, resulting in the unbound virus particles being discarded and thus not copied in PCR assays (Dancho et al. 2012). In Dancho's experiments, a small amount of HuNoVs still bound to gastric mucins after the sample had been treated with 500 mJ/cm^2. We, very similarly, saw a positive RT-QPCR signal of MuNoV and HuNoV samples at the same UV irradiation level, although virus levels were much lower in the enzymatic pre-PCR-treated samples than in untreated samples. These results indicate that these pretreatment methods do not necessarily exclude viruses that have been inactivated due to minor changes in RNA while the capsid has been left intact. The same problem was observed by Parshionikar et al. (2010) with a pre-PCR method, where propidium monoazide is allowed to penetrate through damaged or compromised virus capsids and bind covalently to viral RNA upon exposure to visible light, making this RNA unavailable for amplification, although the use of this method displayed negative RT-QPCR results with heat-treated HuNoV samples.

FCV and MuNoV are currently commonly used as HuNoV surrogates in inactivation studies (reviewed by Richards 2012). The recently described Tulane virus (Tian et al. 2013) has been suggested to be more closely related to HuNoV than MuNoV, but the benefits of using it as a surrogate for HuNoV instead of MuNoV remain unclear (Hirneisen and Kniel 2013). MuNoV has, however, been shown to be more resistant towards UV irradiation than FCV (Park et al. 2011), which was the main reason for using it in our study. Despite of its rather close relativity to HuNoV, MuNoV reacted somewhat differently to UV irradiation and to enzymatic pre-PCR treatment. This may be partly due to the starting material: MuNoV samples contained cell debris from RAW 264.7 cells, whereas HuNoV samples were prepared from stools which usually contain high concentrations of other viruses and bacteria. This could make the samples more resistant towards both UV irradiation and enzymatic pre-PCR treatment.

The regression model performed in our study is commonly used to describe the relationship between UV dose and the reduction rate of microbes (Song et al. 2004, Nuanualsuwan et al. 2008). However, the corresponding research frame has not been carried out before according to our knowledge. Our results show a significant difference in reduction rates between enzyme-treated and non-enzyme-treated situations in both MuNoV and HuNoV. This is an obvious conclusion despite the results being based on a rather small number of trials.

According to our findings and previous research, UV irradiation can be a powerful tool for the disinfection of MuNoV and probably also of HuNoV on inanimate surfaces. Although the virus stability curves of HuNoV and MuNoV were non-identical, both viruses showed equivalent kinetics patterns of virus particle reduction when treated with UV irradiation. This suggests that MuNoV is a suitable surrogate for testing the general effects of UV irradiation on HuNoV. Enzymatic pre-PCR treatment reduced positive signals due to damaged virus particles in RT-QPCR, but it did not reach a level at which all inactivated virus particles would have been enzymatically

digested. It seems that finding a practical method for cultivating HuNoV or in other ways determining its infectivity is needed to reliably determine the reduction in HuNoV infectivity when exposed to disinfecting treatments. Until such an assay is available, directional information on the matter can be achieved by using surrogate viruses. The most important inactivation studies on HuNoV may, however, have to be carried out as clinical trials.

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