Sources and persistence of human noroviruses in fresh produce chains and associated public health risks

Katharina Verhaelen

Sources and persistence of human noroviruses in fresh produce chains and associated public health risks

Thesis Utrecht University, Institute of Risk Assessment Sciences (IRAS)/National Institute for Public Health and the Environment (RIVM)

Author:	Katharina Verhaelen, 2014
ISBN:	978-94-6259-230-8
Lay-out:	Eelco Roos
Cover:	Katharina Verhaelen, Isi Kunath, Ankie Sterk and Eelco Roos
Printed by:	Ipskamp

Sources and persistence of human noroviruses in fresh produce chains and associated public health risks

Besmettingsbronnen en persistentie van humane norovirussen in de productieketen van verse groenten en fruit en het geassocieerde volksgezondheidsrisico door consumptie

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen
op maandag 7 juli 2014 des avonds te 6.00 uur

door

Katharina Verhaelen

geboren op 25 februari 1983 te Kleve, Duitsland

Promotoren:	Prof.dr. A.M. de Roda Husman Prof.dr. ir. A. H. Havelaar Prof. dr. F. van Knapen
Copromotor:	Dr. M. Bouwknegt

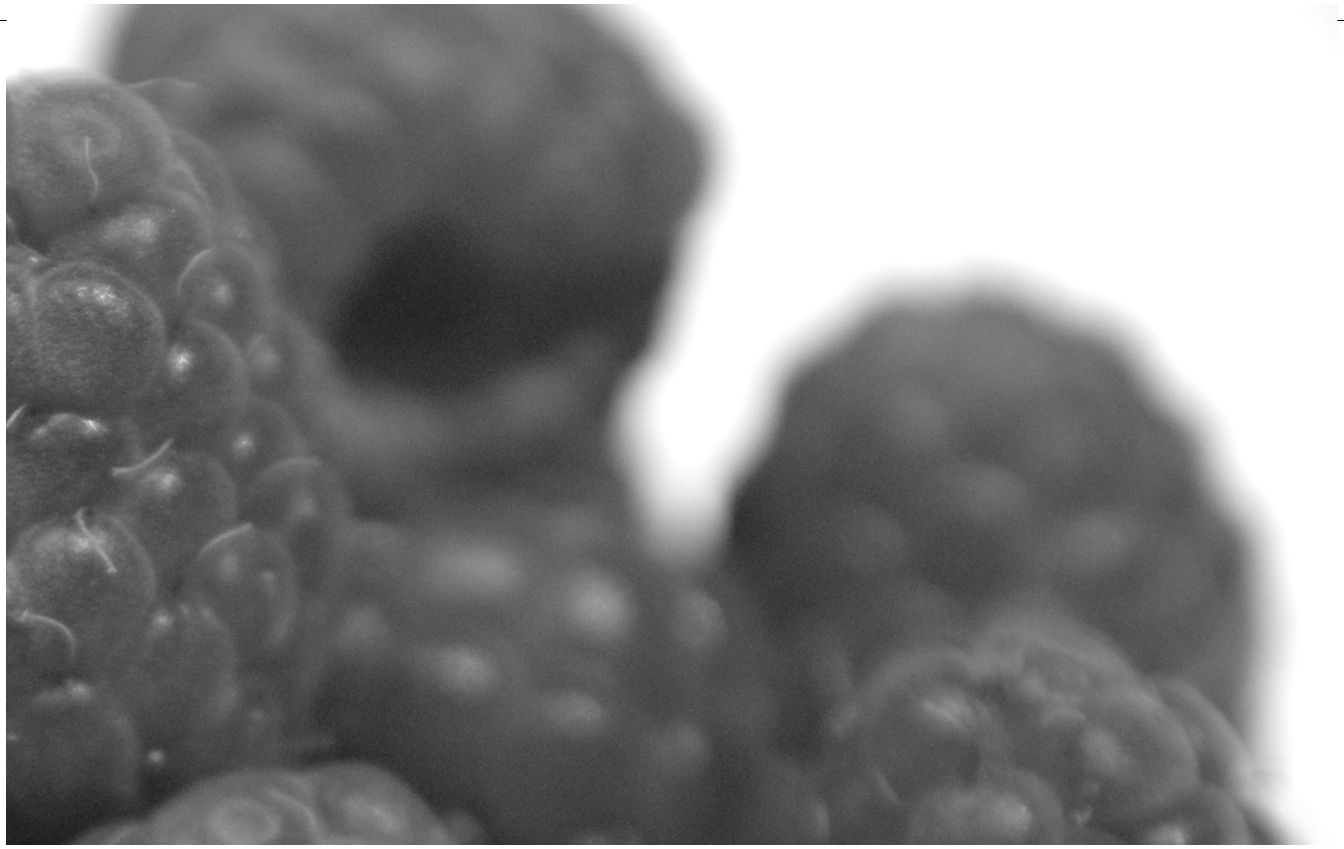
"What seems to us as bitter trials are often blessings in disguise"

Oscar Wilde

Contents

Chapter 1	General introduction	9
Chapter 2	Persistence of human norovirus GII.4 and GI.4, murine norovirus, and human adenovirus on soft berries as compared with PBS at commonly applied storage conditions	33
Chapter 3	Virus transfer proportions between gloved fingertips, soft berries, and lettuce, and associated health risks	55
Chapter 4	Persistence of human norovirus in reconstituted pesticides – Pesticide application as a possible source of viruses in fresh produce chains	75
Chapter 5	Quantitative farm-to-fork risk assessment model for norovirus and hepatitis A virus in European leafy green vegetable and berry fruit supply chains	91
Chapter 6	Wipes coated with a singlet oxygen producing photosensitizer are effective against human influenza virus but not against norovirus	115
Chapter 7	General discussion	133
Chapter 8	Summary	151
	Nederlandse samenvatting	157
	Acknowledgements	161
	Curriculum vitae	165
	List of Publications	167

1



General introduction

Foodborne viruses

Microbiological food safety has traditionally concerned non-viral pathogens and detection systems for viruses on foods were long lacking. Viruses are now recognized as the main agents causing foodborne disease [1-3]. Nevertheless, current food safety concepts such as Hazard Analysis Critical Control Point (HACCP) systems rarely include viral pathogens [4] and the great numbers of viral foodborne outbreaks show that current food safety protocols may not suffice to guarantee viral food safety [5]. Unlike bacteria and fungi, viruses cannot cause food spoilage or produce toxins, as they cannot replicate outside a living host cell. However, they are generally highly persistent in the environment and are commonly infectious to humans even in low numbers. Most foodborne viruses are transmitted by the fecal-oral route and fall into one of two categories: viruses that cause hepatitis and viruses that cause gastrointestinal illness. Hepatitis A and E viruses belong to the first group, whereas several viruses cause foodborne gastroenteritis such as noroviruses, astroviruses, rotaviruses, sapoviruses, adenoviruses, and coronaviruses [6]. Of all these viruses, noroviruses are associated with foodborne viral disease most frequently.

Foodborne norovirus epidemiology

Human norovirus is a leading cause of gastroenteritis worldwide and is associated with sporadic and outbreak cases of gastroenteritis in all age groups [7-9]. Formerly called Norwalk virus, it is infamous as a cause of gastroenteritis outbreaks, especially in settings with a vulnerable population, such as hospitals and nursing homes [10-12]. Outbreaks are also commonly reported in settings with shared common facilities and food sources, such as cruise ships [13-15] and canteens [16-18]. The burden of institutional norovirus outbreaks is nevertheless suggested to be relatively small compared to the burden of community-acquired norovirus infections [19, 20]. About 17 to 26% of norovirus disease is estimated to be foodborne [3, 20, 21] and the virus has been identified as the most common cause of foodborne disease. In the US, for example, more than 50% of foodborne disease is attributed to norovirus, which translates to about 5 million cases per year [2, 3]. It is followed by *Salmonella* with 11% of foodborne disease [3]. Of foodborne pathogens, norovirus is the second most common cause of hospitalization and the fourth most common cause of death in the US [3]. In The Netherlands, noroviruses were estimated to be the fourth most important food related pathogen based on the incidence and the severity of illness following infection [22]. Foodborne norovirus outbreaks have the potential to result in large outbreaks associated with food items not heated before consumption, such as fresh or frozen produce [5, 23-27]. This was demonstrated in Denmark and Finland, where contaminated frozen raspberries and fresh lettuce led to a series of outbreaks affecting up to a thousand individuals [28-30]. Another striking example is an outbreak in Germany associated with imported frozen

strawberries, which included more than 11,000 diseased people [31]. Compared to fresh produce, frozen produce represents an additional public health risk, because its shelf-life of two years allows a prolonged and spatially extended virus spread that complicates linkage of resulting outbreaks to a common food source. Globalization and the resulting complexity of international food trade networks likewise may lead to large and diffuse outbreaks that are difficult to trace [30, 32, 33]. Despite the great genetic diversity of norovirus only a few strains, primarily genogroup II genotype 4 (GII.4), are responsible for most cases of human gastroenteritis [34, 35]. Foodborne outbreaks, however, are relatively more often associated with non-GII.4 strains as compared to outbreaks in e.g., health care settings [36-38]. Moreover, foodborne outbreaks can be associated with mixtures of norovirus strains on fresh produce [31, 39] posing the additional risk of recombination and development of novel strains after ingestion [40].

Norovirus disease

Norovirus disease is mostly described as self-limiting, with an incubation time of 24 to 72 hours after ingestion of the virus. Common clinical manifestations comprise diarrhea and acute onset of vomiting. The viruses are non-invasive pathogens, and bloody diarrhea or high fever are uncommon [41]. Symptoms may last 1 to 3 days, with longer recovery times for young, old, and immunocompromised patients [19, 41, 42]. The disease is generally not life-threatening, but severe outcomes, including hospitalization and death, may occur in vulnerable populations [43, 44]. For The Netherlands it is estimated that norovirus causes annually several hundreds of hospitalizations of children and elderly and approximately 57 deaths [22, 45, 46]. These numbers are higher in developing countries, and an estimated 200,000 children under 5 years of age die from norovirus infections each year [44]. The virus replicates in the upper intestinal tract, but its preferred host cell remains unknown. Noroviruses bind to ABO, Lewis and Secretor histo-blood group antigens (HBGAs), exhibiting strain-dependent patterns [47-51]. Based on genetic predisposition, individuals can thus be resistant to infection by certain norovirus strains, and nonsecretors have been shown to be highly protected from infections with several norovirus genotypes, including the common GII.4 and the Norwalk virus prototype strain (GI.1) [49, 52-55]. Norovirus immunity is most likely of short duration [56-58], and antigenic variation allows the virus to re-infect individuals [50].

Relevant foodborne norovirus characteristics

Noroviruses are a group of morphological similar but genetically and antigenically diverse viruses. They belong to the family *Caliciviridae* and possess a single-stranded, positive-sense, RNA genome of about 7.7 kb [59]. The genome contains three open reading frames (ORFs). The first ORF encodes a polyprotein precursor that is further processed into non-structural proteins. ORF2 encodes the capsid protein VP1, and ORF3 encodes

the minor structural protein, VP2 [60]. Based on the amino acid sequence of the capsid protein, norovirus genotypes can be classified into five genogroups (GI–GV) and are further subdivided into genotypes. Genogroups GI, GII, and GIV are known to infect humans. The great success of the virus as a foodborne pathogen is explained by its viral properties such as persistence, the great number of excreted viruses, and the high probability of infection per ingested virus.

Persistence

The longer a virus stays infectious in its environment, the higher the probability of its transmission to a human host. Noroviruses are small, round viruses of approximately 38 nm in diameter; they are not enveloped, i.e., they lack an outer membrane consisting of a lipid structure around their capsid [59]. Both characteristics, the small size and the absence of an envelope, render the virus resistant to environmental conditions; it is therefore persistent on e.g. surfaces [61] and relatively impervious to intervention measures such as disinfectants [62, 63]. In addition, noroviruses are persistent in a wide range of pH, as it must withstand the low pH of the stomach and the high pH of intestine. The norovirus GI strains are reportedly more persistent than GII strains against disinfection treatments and environmental conditions [64, 65].

Shedding and Infectivity

The spread of norovirus particles is facilitated by the vast numbers in which noroviruses are shed, ranging from 10^8 to 10^{10} genomic copies per gram of feces [66-68], and the high probability of infection by a single particle [69]. On the basis of a dose response model for NoV GI.I, a probability of infection of 0.5 per ingested virus particle and an ID_{50} (50% infectious dose) at 18 virus particles was estimated [69]. A recent study confirmed the high infectivity of norovirus particles based on data from oyster related outbreaks and showed that GI and GII norovirus strains present similar probabilities for infection and illness [70]. Assuming a load of 10^8 infectious particles per gram of stool and an ID_{50} of 18, one gram of stool would theoretically suffice to infect 2.7 million individuals. It should be noted that up to 1.8 kg of watery diarrhea can be excreted during the first 24 hours of disease [71]. Prolonged shedding and asymptomatic infectious also contribute to foodborne norovirus disease [72-74]. Viral shedding continues after symptom resolution and might last for weeks up to months [66, 75]. A recent study defined regular shedding and prolong shedding periods with a mean of 14–16 days and 105–136 days, respectively [76]. Projectile vomiting in the acute disease phase adds as well to the spread of the virus [77],with a release of 10^4 genomic norovirus copies/mL in a total excreted volume of up to 1.7 L [71].

Norovirus transmission

Knowledge on the various types of transmission routes and their contribution to the spread of noroviruses is crucial to prioritize appropriate control measures to reduce virus transmission and disease. Noroviruses are transmitted via the fecal-oral route and by ingestion of vomit or fecal aerosols. As proof is lacking for zoonotic transmission of human pathogenic noroviruses, humans are believed to be the only reservoir [8]. Person-to-person transmission is consequently the only transmission route, but it may be direct or indirect (e.g. by surfaces and foods). The relative contribution to the public health risk of direct and indirect transmission remains unknown [5].

Direct transmission

Direct person-to-person transmission is considered the main transmission route of norovirus spread in outbreaks [5, 36]. It is defined as the transmission of noroviruses from one person to another, for example, by shaking hands with a person who has noroviruses on his hand, and subsequently ingesting those viruses. Direct transmission is of particular concern in healthcare settings and others in which humans are routinely in close physical contact [78]. Indirect transmission from surfaces may be underestimated, however, because whether viruses were transferred directly from a person's hand or from a doorknob previously touched by that person is often unclear [79]. Moreover, in an outbreak situation, especially in closed settings, person-to-person transmission may quickly take over initial foodborne introduction of viruses [38]. Thus, even though direct person-to-person may be the dominant transmission route, the primary transmission route of norovirus outbreaks may be foodborne.

Indirect transmission

Surfaces and foods, including water, can transmit noroviruses, if contaminated with the feces or vomit of infected individuals. Surface transmission is also referred to as environmental transmission. Norovirus contamination of hard surfaces (steel, glass, or plastic) such as doorknobs, kitchen knives, plastic bags, and work surfaces has been described in various facilities [80-83]. In norovirus transmission via surface contamination, vomiting is often the initial contamination event [84-87]. Surface contamination may extend the period of a norovirus outbreak and sustain a succession of outbreaks in closed settings like airplanes, cruise ships, and concert halls [81, 87-90]. Transmission by food is particularly attributed to products that are not heated before consumption, such as manually prepared ready-to-eat foods like sandwiches and cakes, shellfish, and fresh produce [5, 23-27]. The focus of this thesis is on fresh produce. Sales of minimally processed ready-to-eat fruits and vegetables have grown rapidly in the last decade [91] and globalized markets make a wide variety of fresh produce available throughout the year. In norovirus outbreaks related to fresh produce, mainly soft berries (especially raspberries) and lettuce

were involved [5, 24, 26, 27, 30, 92]. In the US, leafy vegetables (33%), fruits and nuts (16%), and molluscs (13%) were implicated most commonly in outbreaks that could be attributed to a single commodity [26]. In monitoring studies not associated with outbreaks, the prevalence of norovirus contamination on fresh produce varied widely between 1 to 50% for lettuce [93-95], and 0 to 30% for soft berries [93, 96], with norovirus GI being more prevalent than GII [93, 94, 97]. The high prevalence of norovirus genomes on fresh produce indicated a frequent exposure of produce to norovirus sources.

Norovirus sources in fresh produce chains

Fresh produce can become contaminated at any stage in the farm-to-fork continuum: primary production, processing, retail, and lastly in food service operations or private households. Contamination at stages before retail are the most likely to result in diffuse and international outbreaks, especially if produce is distributed widely [28-30, 98]. Contamination is always a result of human fecal or vomit pollution, either by contact of foods with contaminated water, manure or soil, or by contact with infected food handlers or contaminated equipment. Cross-contamination may add to the spread of viruses on foods. Knowledge on the kind of norovirus sources and the relative importance of sources on consumer health risks is crucial to the implementation of appropriate measures to reduce contamination on fresh produce.

Water

In the primary production of fresh produce, water is used for several applications: for irrigation, for reconstitution of pesticides and water-soluble fertilizers, and for hydroponic cultures. To this end, farmers use a great diversity of water sources, including well water and different types of surface water, such as river water or lake water [97, 99-102], all of which can harbor noroviruses [94, 103-110]. Water may become contaminated with the virus by, for example, discharges of partially treated wastewater and sewage or by combined sewer overflows [111, 112]. Additionally, the increasing scarcity of potable water in several regions in the world results in increased use of reclaimed wastewater in agriculture [113, 114]. Norovirus contaminated water may cause external contamination of fresh produce or internal contamination by uptake of noroviruses via roots or leaves through stomata and cut edges [115-119]. Internal contamination of viruses is poorly studied, and findings are not yet conclusive; for example, norovirus RNA was detected within lettuce leaves after challenge with very high norovirus titers in the water of hydroponic cultures, but not if low norovirus levels were added to water used to irrigate soil in which lettuce is conventionally grown [117, 118, 120, 121]. In terms of production procedures relevant to the introduction of noroviruses in fresh produce chains, the focus in the literature is mostly on irrigation water [5, 94, 97, 101, 111, 122-125].

Nevertheless, studies that actually tested the presence of noroviruses in irrigation water are scarce. Surface water used for irrigation was found positive for noroviruses GI and GII in 4% to 30% of the tested samples [94, 96, 125] but in one study no virus was detected [97]. Nor was norovirus detected in a study of groundwater used for irrigation [124]. Other production processes, such as the application of pesticides, are less often considered, and no studies are available on the potential presence of norovirus in water used to reconstitute pesticides. However, water sources used for pesticide dilutions may be the same as used for irrigation. Once past primary production, water is used to rinse fresh produce at various points in processing, but rinsing involves potable water [95], which is less likely to contain noroviruses than surface water. However, rinsing water may become contaminated with noroviruses released from contaminated produce and may consequently contribute to the spread of viruses by cross-contamination.

Surfaces

Surfaces in food settings can become contaminated with noroviruses by vomit or by feces of infected food handlers or by cross-contamination (e.g. from contaminated foods or wipes used for cleaning) [81, 126]. Surfaces were involved in foodborne outbreaks [86, 127], and viruses have been detected on surfaces and utensils in food settings that were and were not involved in norovirus outbreaks [95, 96, 128-130]. Surfaces can play a role in virus transmission by introducing the virus to food or by cross-contaminating produce.

Food handlers

Outbreak investigations indicate that food handlers are the most likely source of noroviruses in ready-to-eat products [26, 131], and in the US, about 50% of norovirus outbreaks have been linked to infected food handlers [26, 132]. The term "food handler" typically refers to retail and kitchen workers but here includes workers at all stages of the farm-to-fork continuum. Not only the frequency, but also the size of outbreaks related to food handlers can be substantial, with up to several thousand infected individuals [127, 133]. Norovirus characteristics may explain why infected food handlers are more commonly implicated in norovirus outbreaks than in those caused by other foodborne pathogens [11]. Food handlers can contaminate produce during (i) primary production (e.g., at harvest), (ii) processing (e.g., while sorting and packaging produce) or (iii) food preparation (e.g., preparing desserts or salads). They can contribute to the spread of noroviruses either by introducing the virus onto food, via poor hand hygiene or vomit aerosols, or by cross-contamination while handling contaminated food. Norovirus loads are reportedly similar in food handlers with and without symptoms [68] and norovirus outbreaks have been linked to asymptomatic and post-symptomatic food handlers [72, 74, 134, 135].

Other potential sources

Zoonotic transmission of norovirus has not been proven, but during primary food production, the use of animal manure may play a role in the introduction of human noroviruses if animal and human excretions are collected in one septic tank before application to the fields [136, 137]. However, the application of raw manure to fresh produce is restricted [138]. Contaminated soil, dust, or insects may also introduce noroviruses to produce, as described for other pathogens that are shed in feces [139]. Whether and to what extent such sources contribute to the foodborne health risk of noroviruses is unknown.

Norovirus detection

Sensitive and specific virus detection methods are crucial to understand the role of noroviruses as a foodborne virus and as a food safety threat. The virus was first visualized in 1972 using electron microscopy (EM) in human stool samples [140]. EM is rapid, but not very sensitive, and once the norovirus genome had been sequenced [59], the more sensitive reverse-transcriptase-polymerase-chain-reaction (RT-PCR) assays became the reference method for detecting norovirus in fecal specimens, water, and food [141]. RT-PCRs typically target the ORF1/ORF2 region of the norovirus genome, which is highly conserved [142, 143] and thus allows, detection of a broad range of norovirus genotypes, with different primer sets used for NoV GI and GII. The European Committee of Standardization (CEN) formed a group (CEN/TAG 4) to harmonize and validate protocols for virus detection in foods (shellfish, soft berries, bottled water, salad vegetables and, food surfaces), which recently resulted in the CEN/ISO TS 15216-2. The protocols for fresh produce consist of (i) virus elution from the food, (ii) a virus concentration step, if needed, (iii) nucleic acid extraction, and (iv) detection of virus genomes by RT-PCR. The procedures are monitored with appropriate controls to examine their performance and to verify the obtained results. The protocols are currently undergoing the validation process. A certified ISO procedure is a first step to implement European legislation on viral food safety and to establish microbiological criteria. However, the value of molecular detection of viruses on foods is debatable, as viral genomes do not necessarily represent infectious virus particles, and norovirus genomes were and were not detected on fresh produce that was and was not associated with norovirus outbreaks [28-30, 93, 96]. Other difficulties that hamper the successful detection of viruses on foods are (i) virus concentration below the limit of detection, which may still pose a health risk [93, 144], (ii) the inhomogeneous distribution of virus particles in food batches [145], and (iii) the short shelf-life of fresh produce.

Norovirus surrogates

To date, human noroviruses cannot be cultured efficiently, and no small animal provides

a model in which to study norovirus infectivity. Thus, surrogate viruses are studied to infer the behavior of human noroviruses. Feline and canine caliciviruses (FCV, CaCV) were mainly used until discovery of a cultivable murine norovirus (MNV-1), which more closely resembles the properties of human noroviruses [146]. MNV-1 and human noroviruses share similar structural and genetic features such as size, shape, and genome structure; and both viruses are shed in feces and are transmitted via the fecal oral route [146]. The MNV-1 virus is, unlike FCV-1, resistant to low pH and highly stable in the environment [147]. However, MNV-1 does cause systemic disease and differs in viral receptor binding as compared to human noroviruses [146, 148-150]. Recently, Tulane virus, a novel rhesus calicivirus, was suggested as an improved surrogate, because it recognizes the type A and B histo-blood group antigens, as does human norovirus [151, 152]. However, MNV-1 remains favored as a surrogate for norovirus persistence because the Tulane virus lacks persistence at low pH and low temperature in water [153], two conditions in which human norovirus persists, as human challenge studies showed [154, 155]. Since human volunteer studies are expensive and not practicable for most researches, working with surrogates is the best method currently available to approach human norovirus infectivity. However, it must be kept in mind that MNV-1 does not fully reflect the behavior of human noroviruses. In environmental monitoring studies, human adenoviruses were identified as possible indicators for the presence of infectious noroviruses [157].

Mitigation strategies

Efficient mitigation strategies are required to lower the risk of foodborne norovirus disease. Mitigation can focus on measures to prevent norovirus contamination and on measures to reduce the number of norovirus particles on fresh produce. Preventive measures to avoid contamination are to be preferred [5, 158].

Prevention of norovirus contamination

Existing preventive measures for the primary production of fresh produce in the EU rely currently on principles of hygiene laid down in Regulation EC No 852/2004, Good Agricultural Practice, and Good Manufacturing Practice during processing [5]. These regulations are very general and not specific for viruses. In 2012, however, the Codex Alimentarius Commission (CAC) released "Guidelines on the application of general principles of food hygiene to the control of viruses in food", with an annex on fresh produce [158]. The guidelines give recommendations on the prevention of virus introduction in food chains from primary production through consumption and provide advice to governments on a framework for virus control. The described prevention measures aim at the main introduction sources of norovirus: water, food handlers, and surfaces. While virus-specific, the recommendations tend to be general, especially for water.

The documents states that "effort should be made to use only clean water for the production of food", and clean water is defined as "water that does not compromise food safety in the circumstances of its use" [158]. To select appropriate water sources, sanitary surveys are suggested [113] and, if needed "corrective actions" are advised to minimize the risk. In addition, water delivery techniques, such as drip irrigation instead of overhead sprinklers, are recommended. Among CAC guidelines to prevent contamination by food handlers, recommendations include hand washing with soap under running water and subsequent drying with disposable towels. They further suggest excluding any food handlers showing clinical symptoms from the workplace. Training of food handlers is recommended. Compliance of food handlers to hand washing recommendations is crucial to prevent introduction of noroviruses into food chains [159]. Yet food handlers reportedly wash their hands only about one-third of the time after performing activities for which hand washing is endorsed [160]. Vaccination of food handlers as a prevention measure in not feasible, as a vaccine against norovirus infection is not yet available [161]. To prevent potential food contamination by surfaces, disinfection with chlorine is recommended at a concentration of 200 to 1000 ppm free chlorine [158]. Wiping with or without disinfectant reduces the residual contamination of hard surfaces significantly, but not always to a level low enough to exclude continued norovirus transmission [62, 126].

Measures to reduce norovirus numbers on fresh produce

The literature indicates that current safety procedures in food processing cannot eliminate viral pathogens [162, 163]. A 3 $\log_{10}$-unit reduction of infectious pathogens is generally deemed to reduce pathogen contamination levels to an acceptable health risk [5, 158, 162]. To combine food safety of fresh produce with consumer demands for minimally processed products such as lettuce and soft berries is challenging, because of the perishability of the produce. When measures are currently applied in fresh produce chains, they include mostly washing with water, as frequently done for ready-to-eat lettuce. Depending on the legal regulations, washing can be performed with or without disinfectants, of which chlorine is the most in use [164]. Either way, washing of fresh produce is considered not suitable to eliminate noroviruses [158] as it only results in a 1 to 2 $\log_{10}$-unit reduction [165]. Modified atmosphere packaging, applied to prolong the shelf-life of e.g. pre-cut lettuce and to reduce growth of bacteria, is also unlikely to affect viruses [166]. To preserve fruit quality of raspberries, not even a washing step is applied before retail. For berries to be consumed fresh, possible contamination is generally challenged post-harvest only by the applied storage conditions, in which noroviruses may persist [167, 168]. Numerous recent studies have been published on the applicability and efficacy of novel non-thermal intervention measures to reduce the number of infectious noroviruses on fresh produce, such as: high pressure processing [163, 169, 170]; various types of irradiation [171-173]; disinfectant treatments with ozone [174-176],

chlorine dioxide [167], and hydrogen peroxide [177]; the addition of surfactants to washing waters with or without chlorine [178], or plant ingredients such as grapeseed extract and other natural compounds [179, 180]; ultrasound treatments [181, 182], and cold plasma [183]. Studies on particular treatment processes are mostly very few, and those focused on a given treatment differ in process conditions like the food matrix, virus type/strain, and applied temperature or pH, even though treatment efficacies depend heavily on such conditions [162]. Overall, a single treatment appears unlikely to reliably achieve a 3 $\log_{10}$-unit reduction of noroviruses for various fresh produce types without causing deterioration of fresh produce.

Microbiological risk assessment

The food legislation in the EU has to be based on risk analysis, a process consisting of risk assessment, risk management, and risk communication that has emerged in the last decade as a structured model for improving our food control systems [184, 185]. Microbiological risk assessment can be used to model the fate of pathogens along the food chain and facilitates decision making on microbiological hazard management [184]. It allows an a priori estimation on the (i) health risks associated with microbial contamination of foods (ii) efficacy of current and novel mitigations strategies in reducing health risk and (iii) relative contribution of various exposure pathways to the health risk [184, 186, 187]. Microbial risk assessment can be applied to identify and validate potential Critical Control Points (CCPs) in a process controlled by a HACCP system and to identify gaps where new data could improve the estimation of a given health risk [184]. Therewith risk assessment can assists governments to set national and international food safety standards and assist industry to develop safe foods for consumers [186]. The core elements of a risk assessment are hazard identification, exposure assessment, and hazard and risk characterization [184, 186, 188]. Depending on the research question and the available data, a risk assessment can be qualitative, semi-quantitative or quantitative. Preferably a probabilistic approach is used and if the data allows, model inputs of the exposure assessment should be described by probability distributions, with consideration of the variability inherent in the system as well as the uncertainty in the input parameters [189].

Risk assessments in food safety currently focus primarily on bacterial pathogens, and viral risk assessments are hampered by limited data on, for example virus contamination levels in food and other reservoirs, virus transfer proportions and natural persistence on foods [5, 190, 191]. For viruses on fresh produce, there exist only a few relevant quantitative risk assessment models on irrigation and transmission of viruses by food handlers at retail [114, 123, 159, 192, 193]. At present, no model is available that describes the whole farm-to-fork-continuum, which is needed to estimate the effect and relevance of introduction sources and intervention measures along the whole chain.

Scope of the thesis

The aim of this thesis was to improve the knowledge base for fresh produce safety with respect to noroviruses, by generating expertise on the (i) persistence of norovirus, (ii) contribution of norovirus introduction sources, and (iii) efficacy of possible mitigation strategies in fresh produce chains. Research was focused on soft berries and lettuce, as these food commodities are most frequently linked to norovirus outbreaks associated with fresh produce.

In **chapter 2** the persistence of noroviruses on soft berries during their shelf-life at commonly applied storage conditions was studied. Decay parameters were determined by fitting monophasic, biphasic, and Weibull models through the generated data points, and the impact of the food matrix on norovirus persistence was analyzed. The resulting data are essential to design control strategies and to estimate the health risk for consumers, as only infectious virus particles pose a health risk. To reduce norovirus transmission via food, a good understanding of the sources of norovirus contamination in food chains is needed. Therefore, in **chapter 3**, the contribution of food handlers to norovirus introduction on fresh produce was assessed. Quantitative data on virus transfer from hands to produce were determined by estimating transfer proportions from gloved fingertips to soft berries and lettuce, and vice versa. These transfer proportions were used to study the spread of noroviruses on fresh produce and to analyze associated health risk. To this end, we used an example of a food handler picking raspberries with contaminated fingertips. In **chapter 4**, the persistence of noroviruses in commonly used pesticide dilutions was studied to evaluate the potential of pesticide application in introducing noroviruses into fresh produce chains. In **chapter 5**, farm-to-fork risk assessment models for soft fruit and salad vegetable supply chains were developed. These quantitative models are designed to identify the contribution of contamination sources in introducing norovirus in food chains, and to estimate public health risks. The input for this quantitative risk assessment was based on data from previous chapters, data from the literature, and data gathered in the European FP7 project 'VITAL', which focused on an integrated approach to data collection and analysis for use in risk assessment and management in fresh produce chains. In **chapter 6**, the virucidal properties of wipes with a singlet oxygen generating immobilized photosensitizer coating were studied to analyze their potential as a mitigation strategy to prevent transmission of norovirus via surfaces. In **chapter 7**, the findings of the previous chapters are discussed as to how they may contribute to improve fresh produce safety with respect to noroviruses.

References

1. Hall, G., Kirk, M.D., Becker, N., Gregory, J.E., Unicomb, L., Millard, G., Stafford, R., Lalor, K., and OzFoodNet Working, G., *Estimating foodborne gastroenteritis, Australia.* Emerg Infect Dis, 2005. 11(8):1257-64.
2. Painter, J.A., Hoekstra, R.M., Ayers, T., Tauxe, R.V., Braden, C.R., Angulo, F.J., and Griffin, P.M., *Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998-2008.* Emerg Infect Dis, 2013. 19(3):407-15.
3. Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.A., Roy, S.L., Jones, J.L., and Griffin, P.M., *Foodborne illness acquired in the United States-major pathogens.* Emerg Infect Dis, 2011. 17(1):7-15.
4. Zuber, S., Butot, S., and Baert, L., *Effects of treatments used in food processing on viruses*, in *Foodborne viruses and prions and their significance for public health*, F.J.M. Smulders, B. Norrung, and H. Budka, Editors. 2013, Wageningen Academic Publishers: The Netherlands.
5. EFSA, *Scientific Opinion, An update on the present knowledge on the occurrence and control of foodborne viruses.* EFSA Journal, 2011. 9(7):2190-2286.
6. Koopmans, M. and Duizer, E., *Foodborne viruses: an emerging problem.* Int J Food Microbiol, 2004. 90(1):23-41.
7. O'Neill, H.J., McCaughey, C., Coyle, P.V., Wyatt, D.E., and Mitchell, F., *Clinical utility of nested multiplex RT-PCR for group F adenovirus, rotavirus and norwalk-like viruses in acute viral gastroenteritis in children and adults.* J Clin Virol, 2002. 25(3):335-43.
8. Glass, R.I., Parashar, U.D., and Estes, M.K., *Norovirus gastroenteritis.* N Engl J Med, 2009. 361(18):1776-85.
9. Tam, C.C., Rodrigues, L.C., Viviani, L., Dodds, J.P., Evans, M.R., Hunter, P.R., Gray, J.J., Letley, L.H., Rait, G., Tompkins, D.S., O'Brien, S.J., and Committee, I.I.D.S.E., *Longitudinal study of infectious intestinal disease in the UK (IID2 study): incidence in the community and presenting to general practice.* Gut, 2012. 61(1):69-77.
10. Hedlund, K.O., Rubilar-Abreu, E., and Svensson, L., *Epidemiology of calicivirus infections in Sweden, 1994-1998.* J Infect Dis, 2000. 181 Suppl 2:S275-80.
11. Lopman, B.A., Adak, G.K., Reacher, M.H., and Brown, D.W., *Two epidemiologic patterns of norovirus outbreaks: surveillance in England and wales, 1992-2000.* Emerg Infect Dis, 2003. 9(1):71-7.
12. van Duynhoven, Y.T., de Jager, C.M., Kortbeek, L.M., Vennema, H., Koopmans, M.P., van Leusden, F., van der Poel, W.H., van den Broek, M.J., and eXplosie Project, T., *A one-year intensified study of outbreaks of gastroenteritis in The Netherlands.* Epidemiol Infect, 2005. 133(1):9-21.
13. Verhoef, L., Boxman, I.L., Duizer, E., Rutjes, S.A., Vennema, H., Friesema, I.H., de Roda Husman, A.M., and Koopmans, M., *Multiple exposures during a norovirus outbreak on a river-cruise sailing through Europe, 2006.* Euro Surveill, 2008. 13(24).
14. Vivancos, R., Keenan, A., Sopwith, W., Smith, K., Quigley, C., Mutton, K., Dardamissis, E., Nichols, G., Harris, J., Gallimore, C., Verhoef, L., Syed, Q., and Reid, J., *Norovirus outbreak in a cruise ship sailing around the British Isles: investigation and multi-agency management of an international outbreak.* J Infect, 2010. 60(6):478-85.
15. Wikswo, M.E., Cortes, J., Hall, A.J., Vaughan, G., Howard, C., Gregoricus, N., and Cramer, E.H., *Disease transmission and passenger behaviors during a high morbidity Norovirus outbreak on a cruise ship, January 2009.* Clin Infect Dis, 2011. 52(9):1116-22.
16. Makary, P., Maunula, L., Niskanen, T., Kuusi, M., Virtanen, M., Pajunen, S., Ollgren, J., and Tran Minh, N.N., *Multiple norovirus outbreaks among workplace canteen users in Finland, July 2006.* Epidemiol Infect, 2009. 137(3):402-7.
17. Wadl, M., Scherer, K., Nielsen, S., Diedrich, S., Ellerbroek, L., Frank, C., Gatzer, R., Hoehne, M., Johne, R., Klein, G., Koch, J., Schulenburg, J., Thielbein, U., Stark, K., and Bernard, H., *Food-borne norovirus-outbreak at a military base, Germany, 2009.* BMC Infect Dis, 2010. 10:30.
18. Showell, D., Sundkvist, T., Reacher, M., and Gray, J., *Norovirus outbreak associated with canteen salad in Suffolk, United Kingdom.* Euro Surveill, 2007. 12(11):E071129 6.
19. de Wit, M.A., Koopmans, M.P., Kortbeek, L.M., Wannet, W.J., Vinje, J., van Leusden, F., Bartelds, A.I., and van Duynhoven, Y.T., *Sensor, a population-based cohort study on gastroenteritis in the Netherlands: incidence and etiology.* Am J Epidemiol, 2001. 154(7):666-74.

20. Verhoef, L., Koopmans, M., W, V.A.N.P., Duizer, E., Haagsma, J., Werber, D., L, V.A.N.A., and Havelaar, A., *The estimated disease burden of norovirus in The Netherlands.* Epidemiol Infect, 2012:1-11.
21. Havelaar, A.H., Galindo, A.V., Kurowicka, D., and Cooke, R.M., *Attribution of foodborne pathogens using structured expert elicitation.* Foodborne Pathog Dis, 2008. 5(5):649-59.
22. Havelaar, A.H., Haagsma, J.A., Mangen, M.J., Kemmeren, J.M., Verhoef, L.P., Vijgen, S.M., Wilson, M., Friesema, I.H., Kortbeek, L.M., van Duynhoven, Y.T., and van Pelt, W., *Disease burden of foodborne pathogens in the Netherlands, 2009.* Int J Food Microbiol, 2012. 156(3):231-8.
23. Batz, M.B., Hoffmann, S., and Morris, J.G., Jr., *Ranking the disease burden of 14 pathogens in food sources in the United States using attribution data from outbreak investigations and expert elicitation.* J Food Prot, 2012. 75(7):1278-91.
24. Davidson, V.J., Ravel, A., Nguyen, T.N., Fazil, A., and Ruzante, J.M., *Food-specific attribution of selected gastrointestinal illnesses: estimates from a Canadian expert elicitation survey.* Foodborne Pathog Dis, 2011. 8(9):983-95.
25. Bitler, E.J., Matthews, J.E., Dickey, B.W., Eisenberg, J.N., and Leon, J.S., *Norovirus outbreaks: a systematic review of commonly implicated transmission routes and vehicles.* Epidemiol Infect, 2013. 141(8):1563-71.
26. Hall, A.J., Eisenbart, V.G., Etingue, A.L., Gould, L.H., Lopman, B.A., and Parashar, U.D., *Epidemiology of foodborne norovirus outbreaks, United States, 2001-2008.* Emerg Infect Dis, 2012. 18(10):1566-73.
27. Mathijs, E., Stals, A., Baert, L., Botteldoorn, N., Denayer, S., Mauroy, A., Scipioni, A., Daube, G., Dierick, K., Herman, L., Van Coillie, E., Uyttendaele, M., and Thiry, E., *A review of known and hypothetical transmission routes for noroviruses.* Food Environ Virol, 2012. 4(4):131-52.
28. Falkenhorst, G., Krusell, L., Lisby, M., Madsen, S.B., Bottiger, B., and Molbak, K., *Imported frozen raspberries cause a series of norovirus outbreaks in Denmark, 2005.* Euro Surveill, 2005. 10(9):E050922 2.
29. Sarvikivi, E., Roivainen, M., Maunula, L., Niskanen, T., Korhonen, T., Lappalainen, M., and Kuusi, M., *Multiple norovirus outbreaks linked to imported frozen raspberries.* Epidemiol Infect, 2011:1-8.
30. Ethelberg, S., Lisby, M., Bottiger, B., Schultz, A.C., Villif, A., Jensen, T., Olsen, K.E., Scheutz, F., Kjelso, C., and Muller, L., *Outbreaks of gastroenteritis linked to lettuce, Denmark, January 2010.* Euro Surveill, 2010. 15(6).
31. Made, D., Trubner, K., Neubert, E., Hohne, M., and Johne, R., *Detection and Typing of Norovirus from Frozen Strawberries Involved in a Large-Scale Gastroenteritis Outbreak in Germany.* Food Environ Virol, 2013.
32. Ercsey-Ravasz, M., Toroczkai, Z., Lakner, Z., and Baranyi, J., *Complexity of the international agro-food trade network and its impact on food safety.* PLoS One, 2012. 7(5):1-7.
33. Verhoef, L., Kouyos, R.D., Vennema, H., Kroneman, A., Siebenga, J., van Pelt, W., Koopmans, M., and Foodborne Viruses in Europe, N., *An integrated approach to identifying international foodborne norovirus outbreaks.* Emerg Infect Dis, 2011. 17(3):412-8.
34. Siebenga, J.J., Lemey, P., Kosakovsky Pond, S.L., Rambaut, A., Vennema, H., and Koopmans, M., *Phylodynamic reconstruction reveals norovirus GII.4 epidemic expansions and their molecular determinants.* PLoS Pathog, 2010. 6(5):1-13.
35. Siebenga, J.J., Vennema, H., Zheng, D.P., Vinje, J., Lee, B.E., Pang, X.L., Ho, E.C., Lim, W., Choudekar, A., Broor, S., Halperin, T., Rasool, N.B., Hewitt, J., Greening, G.E., Jin, M., Duan, Z.J., Lucero, Y., O'Ryan, M., Hoehne, M., Schreier, E., Ratcliff, R.M., White, P.A., Iritani, N., Reuter, G., and Koopmans, M., *Norovirus illness is a global problem: emergence and spread of norovirus GII.4 variants, 2001-2007.* J Infect Dis, 2009. 200(5):802-12.
36. Kroneman A, V.L., Harris J, Vennema H, Duizer E, van Duynhoven Y, Gray J, Iturriza M, Böttiger B, Falkenhorst G, Johnsen C, von Bonsdorff CH, Maunula L, Kuusi M, Pothier P, Gallay A, Schreier E, Höhne M, Koch J, Szücs G, Reuter G, Krisztalovics K, Lynch M, McKeown P, Foley B, Coughlan S, Ruggeri FM, Di Bartolo I, Vainio K, Isakbaeva E, Poljsak-Prijatelj M, Grom AH, Mijovski JZ, Bosch A, Buesa J, Fauquier AS, Hernandéz-Pezzi G, Hedlund KO, Koopmans M., *Analysis of integrated virological and epidemiological reports of norovirus outbreaks collected within the Foodborne Viruses in Europe network from 1 July 2001 to 30 June 2006.* Journal of Clinical Microbiology, 2008. 46(9):2959-2965.
37. Vega, E., Barclay, L., Gregoricus, N., Shirley, S.H., Lee, D., and Vinje, J., *Genotypic and Epidemiologic Trends of Norovirus Outbreaks in the United States, 2009-2013.* J Clin Microbiol, 2013. 52(1):147-55.

38. Verhoef, L., Vennema, H., van Pelt, W., Lees, D., Boshuizen, H., Henshilwood, K., and Koopmans, M., *Use of norovirus genotype profiles to differentiate origins of foodborne outbreaks.* Emerg Infect Dis, 2010. 16(4):617-24.

39. Gallimore, C.I., Pipkin, C., Shrimpton, H., Green, A.D., Pickford, Y., McCartney, C., Sutherland, G., Brown, D.W., and Gray, J.J., *Detection of multiple enteric virus strains within a foodborne outbreak of gastroenteritis: an indication of the source of contamination.* Epidemiol Infect, 2005. 133(1):41-7.

40. Sasaki, Y., Kai, A., Hayashi, Y., Shinkai, T., Noguchi, Y., Hasegawa, M., Sadamasu, K., Mori, K., Tabei, Y., Nagashima, M., Morozumi, S., and Yamamoto, T., *Multiple viral infections and genomic divergence among noroviruses during an outbreak of acute gastroenteritis.* J Clin Microbiol, 2006. 44(3):790-7.

41. Lopman, B.A., Reacher, M.H., Vipond, I.B., Sarangi, J., and Brown, D.W., *Clinical manifestation of norovirus gastroenteritis in health care settings.* Clin Infect Dis, 2004. 39(3):318-24.

42. Siebenga, J.J., Beersma, M.F., Vennema, H., van Biezen, P., Hartwig, N.J., and Koopmans, M., *High prevalence of prolonged norovirus shedding and illness among hospitalized patients: a model for in vivo molecular evolution.* J Infect Dis, 2008. 198(7):994-1001.

43. Harris, J.P., Edmunds, W.J., Pebody, R., Brown, D.W., and Lopman, B.A., *Deaths from norovirus among the elderly, England and Wales.* Emerg Infect Dis, 2008. 14(10):1546-52.

44. Patel, M.M., Widdowson, M.A., Glass, R.I., Akazawa, K., Vinje, J., and Parashar, U.D., *Systematic literature review of role of noroviruses in sporadic gastroenteritis.* Emerg Infect Dis, 2008. 14(8):1224-31.

45. Mattner, F., Sohr, D., Heim, A., Gastmeier, P., Vennema, H., and Koopmans, M., *Risk groups for clinical complications of norovirus infections: an outbreak investigation.* Clin Microbiol Infect, 2006. 12(1):69-74.

46. van Asten, L., Siebenga, J., van den Wijngaard, C., Verheij, R., van Vliet, H., Kretzschmar, M., Boshuizen, H., van Pelt, W., and Koopmans, M., *Unspecified gastroenteritis illness and deaths in the elderly associated with norovirus epidemics.* Epidemiology, 2011. 22(3):336-43.

47. Huang, P., Farkas, T., Marionneau, S., Zhong, W., Ruvoen-Clouet, N., Morrow, A.L., Altaye, M., Pickering, L.K., Newburg, D.S., LePendu, J., and Jiang, X., *Noroviruses bind to human ABO, Lewis, and secretor histo-blood group antigens: identification of 4 distinct strain-specific patterns.* J Infect Dis, 2003. 188(1):19-31.

48. Huang, P., Farkas, T., Zhong, W., Tan, M., Thornton, S., Morrow, A.L., and Jiang, X., *Norovirus and histo-blood group antigens: demonstration of a wide spectrum of strain specificities and classification of two major binding groups among multiple binding patterns.* J Virol, 2005. 79(11):6714-22.

49. Lindesmith, L., Moe, C., Marionneau, S., Ruvoen, N., Jiang, X., Lindblad, L., Stewart, P., LePendu, J., and Baric, R., *Human susceptibility and resistance to Norwalk virus infection.* Nat Med, 2003. 9(5):548-53.

50. Lindesmith, L.C., Donaldson, E.F., Lobue, A.D., Cannon, J.L., Zheng, D.P., Vinje, J., and Baric, R.S., *Mechanisms of GII.4 norovirus persistence in human populations.* PLoS Med, 2008. 5(2):e31.

51. Larsson, M.M., Rydell, G.E., Grahn, A., Rodriguez-Diaz, J., Akerlind, B., Hutson, A.M., Estes, M.K., Larson, G., and Svensson, L., *Antibody prevalence and titer to norovirus (genogroup II) correlate with secretor (FUT2) but not with ABO phenotype or Lewis (FUT3) genotype.* J Infect Dis, 2006. 194(10):1422-7.

52. Hutson, A.M., Airaud, F., LePendu, J., Estes, M.K., and Atmar, R.L., *Norwalk virus infection associates with secretor status genotyped from sera.* J Med Virol, 2005. 77(1):116-20.

53. Tan, M., Jin, M., Xie, H., Duan, Z., Jiang, X., and Fang, Z., *Outbreak studies of a GII-3 and a GII-4 norovirus revealed an association between HBGA phenotypes and viral infection.* J Med Virol, 2008. 80(7):1296-301.

54. Bucardo, F., Kindberg, E., Paniagua, M., Vildevall, M., and Svensson, L., *Genetic Susceptibility to Symptomatic Norovirus Infection in Nicaragua.* Journal of Medical Virology, 2009. 81(4):728-735.

55. Kindberg, E., Akerlind, B., Johnsen, C., Knudsen, J.D., Heltberg, O., Larson, G., Bottiger, B., and Svensson, L., *Host genetic resistance to symptomatic norovirus (GGII.4) infections in Denmark.* Journal of Clinical Microbiology, 2007. 45(8):2720-2722.

56. Wyatt, R.G., Dolin, R., Blacklow, N.R., DuPont, H.L., Buscho, R.F., Thornhill, T.S., Kapikian, A.Z., and Chanock, R.M., *Comparison of three agents of acute infectious nonbacterial gastroenteritis by cross-challenge in volunteers.* J Infect Dis, 1974. 129(6):709-14.

57. Johnson, P.C., Mathewson, J.J., DuPont, H.L., and Greenberg, H.B., *Multiple-challenge study of host susceptibility to Norwalk gastroenteritis in US adults.* J Infect Dis, 1990. 161(1):18-21.
58. Parrino, T.A., Schreiber, D.S., Trier, J.S., Kapikian, A.Z., and Blacklow, N.R., *Clinical immunity in acute gastroenteritis caused by Norwalk agent.* N Engl J Med, 1977. 297(2):86-9.
59. Xi, J.N., Graham, D.Y., Wang, K.N., and Estes, M.K., *Norwalk virus genome cloning and characterization.* Science, 1990. 250(4987):1580-3.
60. Jiang, X., Wang, M., Wang, K., and Estes, M.K., *Sequence and genomic organization of Norwalk virus.* Virology, 1993. 195(1):51-61.
61. Howie, R., Alfa, M.J., and Coombs, K., *Survival of enveloped and non-enveloped viruses on surfaces compared with other micro-organisms and impact of suboptimal disinfectant exposure.* J Hosp Infect, 2008. 69(4):368-76.
62. Tuladhar, E., Hazeleger, W.C., Koopmans, M., Zwietering, M.H., Beumer, R.R., and Duizer, E., *Residual viral and bacterial contamination of surfaces after cleaning and disinfection.* Appl Environ Microbiol, 2012. 78(21):7769-75.
63. Tuladhar, E., Terpstra, P., Koopmans, M., and Duizer, E., *Virucidal efficacy of hydrogen peroxide vapour disinfection.* J Hosp Infect, 2012. 80(2):110-5.
64. Butot, S., Putallaz, T., Amoroso, R., and Sanchez, G., *Inactivation of enteric viruses in minimally processed berries and herbs.* Appl Environ Microbiol, 2009. 75(12):4155-61.
65. Hewitt, J., Rivera-Aban, M., and Greening, G.E., *Evaluation of murine norovirus as a surrogate for human norovirus and hepatitis A virus in heat inactivation studies.* J Appl Microbiol, 2009. 107(1):65-71.
66. Atmar, R.L., Opekun, A.R., Gilger, M.A., Estes, M.K., Crawford, S.E., Neill, F.H., and Graham, D.Y., *Norwalk virus shedding after experimental human infection.* Emerg Infect Dis, 2008. 14(10):1553-7.
67. Lee, N., Chan, M.C., Wong, B., Choi, K.W., Sin, W., Lui, G., Chan, P.K., Lai, R.W., Cockram, C.S., Sung, J.J., and Leung, W.K., *Fecal viral concentration and diarrhea in norovirus gastroenteritis.* Emerg Infect Dis, 2007. 13(9):1399-401.
68. Ozawa, K., Oka, T., Takeda, N., and Hansman, G.S., *Norovirus infections in symptomatic and asymptomatic food handlers in Japan.* J Clin Microbiol, 2007. 45(12):3996-4005.
69. Teunis, P.F., Moe, C.L., Liu, P., Miller, S.E., Lindesmith, L., Baric, R.S., Le Pendu, J., and Calderon, R.L., *Norwalk virus: how infectious is it?* J Med Virol, 2008. 80(8):1468-76.
70. Thebault, A., Teunis, P.F., Le Pendu, J., Le Guyader, F.S., and Denis, J.B., *Infectivity of GI and GII noroviruses established from oyster related outbreaks.* Epidemics, 2013. 5(2):98-110.
71. Atmar, R.L., Opekun, A.R., Gilger, M.A., Estes, M.K., Crawford, S.E., Neill, F.H., Ramani, S., Hill, H., Ferreira, J., and Graham, D.Y., *Determination of the 50% Human Infectious Dose for Norwalk Virus.* J Infect Dis, 2013.
72. Thornley, C.N., Hewitt, J., Perumal, L., Van Gessel, S.M., Wong, J., David, S.A., Rapana, J.P., Li, S., Marshall, J.C., and Greening, G.E., *Multiple outbreaks of a novel norovirus GII.4 linked to an infected post-symptomatic food handler.* Epidemiol Infect, 2013. 141(8):1585-97.
73. Jeong, A.Y., Jeong, H.S., Lee, J.S., Park, Y.C., Lee, S.H., Hwang, I.G., Kim, Y.J., Kim, Y.J., Jo, M.Y., Jung, S., Kim, K., and Cheon, D.S., *Occurrence of norovirus infections in asymptomatic food handlers in South Korea.* J Clin Microbiol, 2013. 51(2):598-600.
74. Nicolay, N., McDermott, R., Kelly, M., Gorby, M., Prendergast, T., Tuite, G., Coughlan, S., McKeown, P., and Sayers, G., *Potential role of asymptomatic kitchen food handlers during a food-borne outbreak of norovirus infection, Dublin, Ireland, March 2009.* Euro Surveill, 2011. 16(30).
75. Rockx, B., De Wit, M., Vennema, H., Vinje, J., De Bruin, E., Van Duynhoven, Y., and Koopmans, M., *Natural history of human calicivirus infection: a prospective cohort study.* Clin Infect Dis, 2002. 35(3):246-53.
76. Milbrath, M.O., Spicknall, I.H., Zelner, J.L., Moe, C.L., and Eisenberg, J.N., *Heterogeneity in norovirus shedding duration affects community risk.* Epidemiol Infect, 2013. 141(8):1572-84.
77. Zelner, J.L., Lopman, B.A., Hall, A.J., Ballesteros, S., and Grenfell, B.T., *Linking time-varying symptomatology and intensity of infectiousness to patterns of norovirus transmission.* PLoS One, 2013. 8(7):e68413.

78. Greig, J.D. and Lee, M.B., *Enteric outbreaks in long-term care facilities and recommendations for prevention: a review.* Epidemiol Infect, 2009. 137(2):145-55.

79. Barker, J., Stevens, D., and Bloomield, S., *Spread and prevention of some common viral infections in community facilities and domestic homes.* Journal of Applied Microbiology, 2001. 91:7-21.

80. Boone, S.A. and Gerba, C.P., *Significance of fomites in the spread of respiratory and enteric viral disease.* Appl Environ Microbiol, 2007. 73(6):1687-96.

81. Lopman, B., Gastanaduy, P., Park, G.W., Hall, A.J., Parashar, U.D., and Vinje, J., *Environmental transmission of norovirus gastroenteritis.* Curr Opin Virol, 2012. 2(1):96-102.

82. Boxman, I.L., Dijkman, R., te Loeke, N.A., Hagele, G., Tilburg, J.J., Vennema, H., and Koopmans, M., *Environmental swabs as a tool in norovirus outbreak investigation, including outbreaks on cruise ships.* J Food Prot, 2009. 72(1):111-9.

83. Repp, K.K. and Keene, W.E., *A point-source norovirus outbreak caused by exposure to fomites.* J Infect Dis, 2012. 205(11):1639-41.

84. Cheesbrough, J.S., Green, J., Gallimore, C.I., Wright, P.A., and Brown, D.W., *Widespread environmental contamination with Norwalk-like viruses (NLV) detected in a prolonged hotel outbreak of gastroenteritis.* Epidemiol Infect, 2000. 125(1):93-8.

85. Marks, P.J., Vipond, I.B., Carlisle, D., Deakin, D., Fey, R.E., and Caul, E.O., *Evidence for airborne transmission of Norwalk-like virus (NLV) in a hotel restaurant.* Epidemiol Infect, 2000. 124(3):481-7.

86. Patterson, W., Haswell, P., Fryers, P.T., and Green, J., *Outbreak of small round structured virus gastroenteritis arose after kitchen assistant vomited.* Commun Dis Rep CDR Rev, 1997. 7(7):101-3.

87. Thornley, C.N., Emslie, N.A., Sprott, T.W., Greening, G.E., and Rapana, J.P., *Recurring norovirus transmission on an airplane.* Clinical Infectious Disease, 2011. 53(6):515-520.

88. Evans, M.R., Meldrum, R., Lane, W., Gardner, D., Ribeiro, C.D., Gallimore, C.I., and Westmoreland, D., *An outbreak of viral gastroenteritis following environmental contamination at a concert hall.* Epidemiol Infect, 2002. 129(2):355-60.

89. Isakbaeva, E.T., Widdowson, M.A., Beard, R.S., Bulens, S.N., Mullins, J., Monroe, S.S., Bresee, J., Sassano, P., Cramer, E.H., and Glass, R.I., *Norovirus transmission on cruise ship.* Emerg Infect Dis, 2005. 11(1):154-8.

90. Otter, J.A., Yezli, S., and French, G.L., *The role played by contaminated surfaces in the transmission of nosocomial pathogens.* Infect Control Hosp Epidemiol, 2011. 32(7):687-99.

91. Ragaerta, P., Verbeke, W., Devlieghere, F., and Debevere, J., *Consumer perception and choice of minimally processed vegetables and packaged fruits.* Food Quality and Preference 2004. 15(259-270).

92. Berger, C.N., Sodha, S.V., Shaw, R.K., Griffin, P.M., Pink, D., Hand, P., and Frankel, G., *Fresh fruit and vegetables as vehicles for the transmission of human pathogens.* Environ Microbiol, 2010. 12(9):2385-97.

93. Baert, L., Mattison, K., Loisy-Hamon, F., Harlow, J., Martyres, A., Lebeau, B., Stals, A., Van Coillie, E., Herman, L., and Uyttendaele, M., *Review: norovirus prevalence in Belgian, Canadian and French fresh produce: a threat to human health?* Int J Food Microbiol, 2011. 151(3):261-9.

94. El-Senousy, W.M., Costafreda, M.I., Pinto, R.M., and Bosch, A., *Method validation for norovirus detection in naturally contaminated irrigation water and fresh produce.* Int J Food Microbiol, 2013. 167(1):74-9.

95. Kokkinos, P., Kozyra, I., Lazic, S., Bouwknegt, M., Rutjes, S., Willems, K., Moloney, R., de Roda Husman, A.M., Kaupke, A., Legaki, E., D'Agostino, M., Cook, N., Rzezutka, A., Petrovic, T., and Vantarakis, A., *Harmonised investigation of the occurrence of human enteric viruses in the leafy green vegetable supply chain in three European countries.* Food Environ Virol, 2012. 4(4):179-91.

96. Maunula, L., Kaupke, A., Vasickova, P., Soderberg, K., Kozyra, I., Lazic, S., van der Poel, W.H., Bouwknegt, M., Rutjes, S., Willems, K.A., Moloney, R., D'Agostino, M., de Roda Husman, A.M., von Bonsdorff, C.H., Rzezutka, A., Pavlik, I., Petrovic, T., and Cook, N., *Tracing enteric viruses in the European berry fruit supply chain.* Int J Food Microbiol, 2013. 167(2):177-85.

97. Brassard, J., Gagne, M.J., Genereux, M., and Cote, C., *Detection of human food-borne and zoonotic viruses on irrigated, field-grown strawberries.* Appl Environ Microbiol, 2012. 78(10):3763-6.

98. Verhoef, L., Kouyos, R.D., Vennema, H., Kroneman, A., Siebenga, J., van Pelt, W., and Koopmans, M., *An integrated approach to identifying international foodborne norovirus outbreaks.* Emerg Infect Dis, 2011. 17(3):412-8.
99. CAC, *Code of hygienic practice for fresh fruits and vegetables. Adopted 2003. Revision 2010 (new Annex III for Fresh Leafy Vegetables).* 2010, Codex Alimentarius Commission.
100. Knox, J.W., Tyrrel, S.F., Daccache, A., and Weatherhead, E.K., *A geospatial approach to assessing microbiological water quality risks associated with irrigation abstraction.* Water and Environment Journal, 2011. 125:282-289.
101. Steele, M. and Odumeru, J., *Irrigation water as source of foodborne pathogens on fruit and vegetables.* J Food Prot, 2004. 67(12):2839-49.
102. Dijkstra, A.F. and de Roda Husman, A.M., *Bottled and Drinking Water*, in *Food Safety Management: A Practical Guide for the Food Industry*, Y. Motarjemi and H. Lelieveld, Editors. 2013, Elsevier: US.
103. Borchardt, M.A., Bertz, P.D., Spencer, S.K., and Battigelli, D.A., *Incidence of enteric viruses in groundwater from household wells in Wisconsin.* Appl Environ Microbiol, 2003. 69(2):1172-80.
104. Fout, G.S., Martinson, B.C., Moyer, M.W., and Dahling, D.R., *A multiplex reverse transcription-PCR method for detection of human enteric viruses in groundwater.* Appl Environ Microbiol, 2003. 69(6):3158-64.
105. Gabrieli, R., Maccari, F., Ruta, A., Pana, A., Divizia, M., , *Norovirus Detection in Groundwater.* Food and Environmental Virology, 2009. 1:92-96.
106. Haramoto, E., Katayama, H., Oguma, K., and Ohgaki, S., *Application of cation-coated filter method to detection of noroviruses, enteroviruses, adenoviruses, and torque teno viruses in the Tamagawa River in Japan.* Appl Environ Microbiol, 2005. 71(5):2403-11.
107. Horman, A., Rimhanen-Finne, R., Maunula, L., von Bonsdorff, C.H., Torvela, N., Heikinheimo, A., and Hanninen, M.L., *Campylobacter spp., Giardia spp., Cryptosporidium spp., noroviruses, and indicator organisms in surface water in southwestern Finland, 2000-2001.* Appl Environ Microbiol, 2004. 70(1):87-95.
108. Lodder, W.J. and de Roda Husman, A.M., *Presence of noroviruses and other enteric viruses in sewage and surface waters in The Netherlands.* Appl Environ Microbiol, 2005. 71(3):1453-61.
109. Lodder, W.J., van den Berg, H.H., Rutjes, S.A., and de Roda Husman, A.M., *Presence of enteric viruses in source waters for drinking water production in The Netherlands.* Appl Environ Microbiol, 2010. 76(17):5965-71.
110. Lee, B.R., Lee, S.G., Park, J.H., Kim, K.Y., Ryu, S.R., Rhee, O.J., Park, J.W., Lee, J.S., and Paik, S.Y., *Norovirus contamination levels in ground water treatment systems used for food-catering facilities in South Korea.* Viruses, 2013. 5(7):1646-54.
111. Gerba, C.P. and Choi, C.Y., *Role of Irrigation Water in Crop Contamination by Viruses* in *Viruses in Foods*, M.S. Goyal, Editor. 2006, Springer: New York. p. 257-263.
112. Rodriguez-Lazaro, D., Cook, N., Ruggeri, F.M., Sellwood, J., Nasser, A., Nascimento, M.S., D'Agostino, M., Santos, R., Saiz, J.C., Rzezutka, A., Bosch, A., Girones, R., Carducci, A., Muscillo, M., Kovac, K., Diez-Valcarce, M., Vantarakis, A., von Bonsdorff, C.H., de Roda Husman, A.M., Hernandez, M., and van der Poel, W.H., *Virus hazards from food, water and other contaminated environments.* FEMS Microbiol Rev, 2012. 36(4):786-814.
113. WHO, *Guidelines for the safe use of wastewater, excreta and greywater.* Wastewater use in agriculture, 2006. 2: http://whqlibdoc.who.int/publications/2006/9241546832_eng.pdf
114. Hamilton, A.J., Stagnitti, F., Premier, R., Boland, A.M., and Hale, G., *Quantitative microbial risk assessment models for consumption of raw vegetables irrigated with reclaimed water.* Appl Environ Microbiol, 2006. 72(5):3284-90.
115. Wei, J., Jin, Y., Sims, T., and Kniel, K.E., *Manure- and biosolids-resident murine norovirus 1 attachment to and internalization by Romaine lettuce.* Appl Environ Microbiol, 2010. 76(2):578-83.
116. Gandhi, K.M., Mandrell, R.E., and Tian, P., *Binding of virus-like particles of Norwalk virus to romaine lettuce veins.* Appl Environ Microbiol, 2010. 76(24):7997-8003.
117. Dicaprio, E., Ma, Y., Purgianto, A., Hughes, J., and Li, J., *Internalization and dissemination of human norovirus and animal caliciviruses in hydroponically grown romaine lettuce.* Appl Environ Microbiol, 2012. 78(17):6143-52.
118. Li, J., Predmore, A., Divers, E., and Lou, F., *New interventions against human norovirus: progress, opportunities, and challenges.* Annu Rev Food Sci Technol, 2012. 3:331-52.

119. Esseili, M.A., Wang, Q., and Saif, L.J., *Binding of human GII.4 norovirus virus-like particles to carbohydrates of romaine lettuce leaf cell wall materials.* Appl Environ Microbiol, 2012. 78(3):786-94.

120. Urbanucci, A., Myrmel, M., Berg, I., von Bonsdorff, C.H., and Maunula, L., *Potential internalisation of caliciviruses in lettuce.* Int J Food Microbiol, 2009. 135(2):175-8.

121. Wei, J., Jin, Y., Sims, T., and Kniel, K.E., *Internalization of murine norovirus 1 by Lactuca sativa during irrigation.* Appl Environ Microbiol, 2011. 77(7):2508-12.

122. Newell, D.G., Koopmans, M., Verhoef, L., Duizer, E., Aidara-Kane, A., Sprong, H., Opsteegh, M., Langelaar, M., Threfall, J., Scheutz, F., van der Giessen, J., and Kruse, H., *Food-borne diseases - the challenges of 20 years ago still persist while new ones continue to emerge.* Int J Food Microbiol, 2010. 139:3-15.

123. Stine, S.W., Song, I., Choi, C.Y., and Gerba, C.P., *Application of microbial risk assessment to the development of standards for enteric pathogens in water used to irrigate fresh produce.* J Food Prot, 2005. 68(5):913-8.

124. Cheong, S., Lee, C., Song, S.W., Choi, W.C., Lee, C.H., and Kim, S.J., *Enteric viruses in raw vegetables and groundwater used for irrigation in South Korea.* Appl Environ Microbiol, 2009. 75(24):7745-51.

125. Kayed, D., *Microbial quality of irrigation water used in the production of fresh produce in Arizona*, in *Departement of Microbiology and Immunology*. 2004, University of Arizona: Tucson, AZ.

126. Barker, J., Vipond, I.B., and Bloomfield, S.F., *Effects of cleaning and disinfection in reducing the spread of Norovirus contamination via environmental surfaces.* J Hosp Infect, 2004. 58(1):42-9.

127. de Wit, M.A., Widdowson, M.A., Vennema, H., de Bruin, E., Fernandes, T., and Koopmans, M., *Large outbreak of norovirus: the baker who should have known better.* Journal of Infection, 2007. 55(2):188–193.

128. Boxman, I., Dijkman, R., Verhoef, L., Maat, A., van Dijk, G., Vennema, H., and Koopmans, M., *Norovirus on swabs taken from hands illustrate route of transmission: a case study.* J Food Prot, 2009. 72(8):1753-5.

129. Boxman, I.L., Verhoef, L., Dijkman, R., Hagele, G., Te Loeke, N.A., and Koopmans, M., *Year-round prevalence of norovirus in the environment of catering companies without a recently reported outbreak of gastroenteritis.* Appl Environ Microbiol, 2011. 77(9):2968-74.

130. Ronnqvist, M., Ratto, M., Tuominen, P., Salo, S., and Maunula, L., *Swabs as a tool for monitoring the presence of norovirus on environmental surfaces in the food industry.* J Food Prot, 2013. 76(8):1421-8.

131. Baert, L., Uyttendaele, M., Stals, A., van Coillie, E., Dierick, K., Debevere, J., and Botteldoorn, N., *Reported foodborne outbreaks due to noroviruses in Belgium: the link between food and patient investigations in an international context.* Epidemiol Infect, 2009. 137(3):316-25.

132. Widdowson, M.A., Sulka, A., Bulens, S.N., Beard, R.S., Chaves, S.S., Hammond, R., Salehi, E.D., Swanson, E., Totaro, J., Woron, R., Mead, P.S., Bresee, J.S., Monroe, S.S., and Glass, R.I., *Norovirus and foodborne disease, United States, 1991-2000.* Emerg Infect Dis, 2005. 11(1):95-102.

133. Friedman, D.S., Heisey-Grove, D., Argyros, F., Berl, E., Nsubuga, J., Stiles, T., Fontana, J., Beard, R.S., Monroe, S., McGrath, M.E., Sutherby, H., Dicker, R.C., DeMaria, A., and Matyas, B.T., *An outbreak of norovirus gastroenteritis associated with wedding cakes.* Epidemiol Infect, 2005. 133(6):1057-63.

134. Barrabeig, I., Rovira, A., Buesa, J., Bartolome, R., Pinto, R., Prellezo, H., and Dominguez, A., *Foodborne norovirus outbreak: the role of an asymptomatic food handler.* BMC Infect Dis, 2010. 10:269.

135. Parashar, U.D., Dow, L., Fankhauser, R.L., Humphrey, C.D., Miller, J., Ando, T., Williams, K.S., Eddy, C.R., Noel, J.S., Ingram, T., Bresee, J.S., Monroe, S.S., and Glass, R.I., *An outbreak of viral gastroenteritis associated with consumption of sandwiches: implications for the control of transmission by food handlers.* Epidemiol Infect, 1998. 121(3):615-21.

136. Deng, M.Y. and Cliver, D.O., *Persistence of inoculated hepatitis A virus in mixed human and animal wastes.* Appl Environ Microbiol, 1995. 61(1):87-91.

137. Wei, J., Jin, Y., Sims, T., and Kniel, K.E., *Survival of murine norovirus and hepatitis A virus in different types of manure and biosolids.* Foodborne Pathog Dis, 2010. 7(8):901-6.

138. FDA, *Guidance for Industry Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables.* U. S. Food and Drug Administration, 1998.

139. Beuchat, L.R. and Ryu, J.H., *Produce handling and processing practices.* Emerg Infect Dis, 1997. 3(4):459-65.

140. Kapikian, A.Z., Wyatt, R.G., Dolin, R., Thornhill, T.S., Kalica, A.R., and Chanock, R.M., *Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis.* J Virol, 1972. 10(5):1075-81.

141. Atmar, R.L. and Estes, M.K., *Diagnosis of noncultivatable gastroenteritis viruses, the human caliciviruses.* Clin Microbiol Rev, 2001. 14(1):15-37.

142. Jothikumar, N., Lowther, J.A., Henshilwood, K., Lees, D.N., Hill, V.R., and Vinje, J., *Rapid and sensitive detection of noroviruses by using TaqMan-based one-step reverse transcription-PCR assays and application to naturally contaminated shellfish samples.* Appl Environ Microbiol, 2005. 71(4):1870-5.

143. Kageyama, T., Kojima, S., Shinohara, M., Uchida, K., Fukushi, S., Hoshino, F.B., Takeda, N., and Katayama, K., *Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR.* J Clin Microbiol, 2003. 41(4):1548-57.

144. Stals, A., Baert, L., Jasson, V., Van Coillie, E., and Uyttendaele, M., *Screening of fruit products for norovirus and the difficulty of interpreting positive PCR results.* J Food Prot, 2011. 74(3):425-31.

145. Rutjes, S.A., Lodder-Verschoor, F., van der Poel, W.H., van Duijnhoven, Y.T., and de Roda Husman, A.M., *Detection of noroviruses in foods: a study on virus extraction procedures in foods implicated in outbreaks of human gastroenteritis.* J Food Prot, 2006. 69(8):1949-56.

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

147. Cannon, J.L., Papafragkou, E., Park, G.W., Osborne, J., Jaykus, L.A., and Vinje, J., *Surrogates for the study of norovirus stability and inactivation in the environment: aA comparison of murine norovirus and feline calicivirus.* J Food Prot, 2006. 69(11):2761-5.

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

149. Taube, S., Perry, J.W., Yetming, K., Patel, S.P., Auble, H., Shu, L., Nawar, H.F., Lee, C.H., Connell, T.D., Shayman, J.A., and Wobus, C.E., *Ganglioside-linked terminal sialic acid moieties on murine macrophages function as attachment receptors for murine noroviruses.* J Virol, 2009. 83(9):4092-101.

150. Tan, M. and Jiang, X., *Norovirus gastroenteritis, carbohydrate receptors, and animal models.* PLoS Pathog, 2010. 6(8):1.5.

151. Farkas, T., Cross, R.W., Hargitt, E., 3rd, Lerche, N.W., Morrow, A.L., and Sestak, K., *Genetic diversity and histo-blood group antigen interactions of rhesus enteric caliciviruses.* J Virol, 2010. 84(17):8617-25.

152. Farkas, T., Sestak, K., Wei, C., and Jiang, X., *Characterization of a rhesus monkey calicivirus representing a new genus of Caliciviridae.* J Virol, 2008. 82(11):5408-16.

153. Hirneisen, K.A. and Kniel, K.E., *Comparing human norovirus surrogates: murine norovirus and Tulane virus.* J Food Prot, 2013. 76(1):139-43.

154. Dolin, R., Blacklow, N.R., DuPont, H., Buscho, R.F., Wyatt, R.G., Kasel, J.A., Hornick, R., and Chanock, R.M., *Biological properties of Norwalk agent of acute infectious nonbacterial gastroenteritis.* Proc Soc Exp Biol Med, 1972. 140(2):578-83.

155. Seitz, S.R., Leon, J.S., Schwab, K.J., Lyon, G.M., Dowd, M., McDaniels, M., Abdulhafid, G., Fernandez, M.L., Lindesmith, L.C., Baric, R.S., and Moe, C.L., *Norovirus infectivity in humans and persistence in water.* Appl Environ Microbiol, 2011. 77(19):6884-8.

156. Hundesa, A., Maluquer de Motes, C., Bofill-Mas, S., Albinana-Gimenez, N., and Girones, R., *Identification of human and animal adenoviruses and polyomaviruses for determination of sources of fecal contamination in the environment.* Appl Environ Microbiol, 2006. 72(12):7886-93.

157. Wyn-Jones, A.P., Carducci, A., Cook, N., D'Agostino, M., Divizia, M., Fleischer, J., Gantzer, C., Gawler, A., Girones, R., Holler, C., de Roda Husman, A.M., Kay, D., Kozyra, I., Lopez-Pila, J., Muscillo, M., Nascimento, M.S., Papageorgiou, G., Rutjes, S., Sellwood, J., Szewzyk, R., and Wyer, M., Surveillance of adenoviruses and noroviruses in European recreational waters. Water Res, 2011. 45(3):1025-38.

158. CAC, *Guidlines on the application of general principles of food hygiene to the control of viruses in food*. 2012, Codex Alimentarius Commission.

159. Mokhtari, A. and Jaykus, L.A., *Quantitative exposure model for the transmission of norovirus in retail food preparation.* Int J Food Microbiol, 2009. 133(1-2):38-47.

160. Green, L.R., Selman, C.A., Radke, V., Ripley, D., Mack, J.C., Reimann, D.W., Stigger, T., Motsinger, M., and Bushnell, L., *Food worker hand washing practices: an observation study.* J Food Prot, 2006. 69(10):2417-23.

161. Richardson, C., Bargatze, R.F., Goodwin, R., and Mendelman, P.M., *Norovirus virus-like particle vaccines for the prevention of acute gastroenteritis.* Expert Rev Vaccines, 2013. 12(2):155-67.

162. Baert, L., Debevere, J., and Uyttendaele, M., *The efficacy of preservation methods to inactivate foodborne viruses.* Int J Food Microbiol, 2009. 131(2-3):83-94.

163. Lou, F., Neetoo, H., Chen, H., and Li, J., *Inactivation of a human norovirus surrogate by high-pressure processing: effectiveness, mechanism, and potential application in the fresh produce industry.* Appl Environ Microbiol, 2011. 77(5):1862-71.

164. Betts, G. and Everis, L., *Alternatives to hypochlorite washing systems for the decontamination of fresh fruit and vegetables*. Improving the safety of fresh fruit and vegetables, ed. W. Jongen. 2005, Cambridge: Woodhead Publishing in Food Science and Technology.

165. Baert, L., Vandekinderen, I., Devlieghere, F., Van Coillie, E., Debevere, J., and Uyttendaele, M., *Inactivation of murine norovirus 1 and Bacteroides fragilis infecting phage B40-8 by the use of sodium hypochlorite and peroxyacetic acid as decontaminating agents for shredded iceberg lettuce.* Commun Agric Appl Biol Sci, 2008. 73(1):97-101.

166. Bidawid, S., Farber, J.M., and Sattar, S.A., *Survival of hepatitis A virus on modified atmosphere-packaged (MAP) lettuce.* Food Microbiology, 2001. 18(1):95-102.

167. Butot, S., Putallaz, T., and Sanchez, G., *Effects of sanitation, freezing and frozen storage on enteric viruses in berries and herbs.* Int J Food Microbiol, 2008. 126(1-2):30-5.

168. Fallahi, S. and Mattison, K., *Evaluation of murine norovirus persistence in environments relevant to food production and processing.* J Food Prot, 2011. 74(11):1847-51.

169. Kingsley, D.H., Holliman, D.R., Calci, K.R., Chen, H., and Flick, G.J., *Inactivation of a norovirus by high-pressure processing.* Appl Environ Microbiol, 2007. 73(2):581-5.

170. Kovac, K., Diez-Valcarce, M., Raspor, P., Hernandez, M., and Rodriguez-Lazaro, D., *Effect of high hydrostatic pressure processing on norovirus infectivity and genome stability in strawberry puree and mineral water.* Int J Food Microbiol, 2012. 152(1-2):35-9.

171. Bidawid, S., Farber, J.M., and Sattar, S.A., *Inactivation of hepatitis A virus (HAV) in fruits and vegetables by gamma irradiation.* International Journal of Food Microbiology, 2000. 57(1-2):91-97.

172. Feng, K., Divers, E., Ma, Y., and Li, J., *Inactivation of a human norovirus surrogate, human norovirus virus-like particles, and vesicular stomatitis virus by gamma irradiation.* Appl Environ Microbiol, 2011. 77(10):3507-17.

173. Fino, V.R. and Kniel, K.E., *UV light inactivation of hepatitis A virus, Aichi virus, and feline calicivirus on strawberries, green onions, and lettuce.* J Food Prot, 2008. 71(5):908-13.

174. Hirneisen, K.A. and Kniel, K.E., *Inactivation of internalized and surface contaminated enteric viruses in green onions.* Int J Food Microbiol, 2013. 166(2):201-6.

175. Hirneisen, K.A., Markland, S.M., and Kniel, K.E., *Ozone inactivation of norovirus surrogates on fresh produce.* J Food Prot, 2011. 74(5):836-9.

176. Kim, J.G., Yousef, A.E., and Khadre, M.A., *Ozone and its current and future application in the food industry.* Adv Food Nutr Res, 2003. 45:167-218.

177. Li, D., Baert, L., De Jonghe, M., Van Coillie, E., Ryckeboer, J., Devlieghere, F., and Uyttendaele, M., *Inactivation of murine norovirus 1, coliphage phiX174, and Bacteroides [corrected] fragilis phage B40-8 on surfaces and fresh-cut iceberg lettuce by hydrogen peroxide and UV light.* Appl Environ Microbiol, 2011. 77(4):1399-404.

178. Predmore, A. and Li, J., *Enhanced removal of a human norovirus surrogate from fresh vegetables and fruits by a combination of surfactants and sanitizers.* Appl Environ Microbiol, 2011. 77(14):4829-38.

179. Li, D., Baert, L., Zhang, D., Xia, M., Zhong, W., Van Coillie, E., Jiang, X., and Uyttendaele, M., *Effect of grape seed extract on human norovirus GII.4 and murine norovirus 1 in viral suspensions, on stainless steel discs, and in lettuce wash water.* Appl Environ Microbiol, 2012. 78(21):7572-8.

180. Su, X. and D'Souza, D.H., *Grape seed extract for foodborne virus reduction on produce.* Food Microbiol, 2013. 34(1):1-6.

181. Schultz, A.C., Uhrbrand, K., Norrung, B., and Dalsgaard, A., *Inactivation of norovirus surrogates on surfaces and raspberries by steam-ultrasound treatment.* J Food Prot, 2012. 75(2):376-81.

182. Su, X., Zivanovic, S., and D'Souza, D.H., *Inactivation of human enteric virus surrogates by high-intensity ultrasound.* Foodborne Pathog Dis, 2010. 7(9):1055-61.

183. Fernandez, A., Noriega, E., and Thompson, A., *Inactivation of Salmonella enterica serovar Typhimurium on fresh produce by cold atmospheric gas plasma technology.* Food Microbiol, 2013. 33(1):24-9.

184. FAO/WHO, *Exposure assessment of microbiological hazards in food : Guidelines*, in *Microbial Risk Assessment Series 7*. 2008: Rom. p. 92.

185. Romero-Barrios, P., Hempen, M., Messens, W., Stella, P., and Hugas, M., *Quantitative microbiological risk assessment (QMRA) of food-borne zoonoses at the European level.* Food Control 2013. 29:343-349.

186. Bassett, J., Nauta, M., Lindqvist, R., and Zwietering, M., *Tools for Microbiological Risk Assessment* in *ILSI Europe Risk Analysis in Food Microbiology Task Force, ILSI Europe Report Series*. 2012, International Life Sciences Institute.

187. Havelaar, A.H., Evers, E.G., and Nauta, M.J., *Challenges of quantitative microbial risk assessment at EU level.* Food Science & Technology 2008. 19:26-33.

188. Codex-Alimentarius-Commission, *Codex principles and guidelines for the conduct of microbiological risk assessment, Document CAC/GL-30 (1999)*. 1999.

189. Nauta, M.J., *Separation of uncertainty and variability in quantitative microbial risk assessment models.* International Journal of Food Microbiology 2010. 57:9–18.

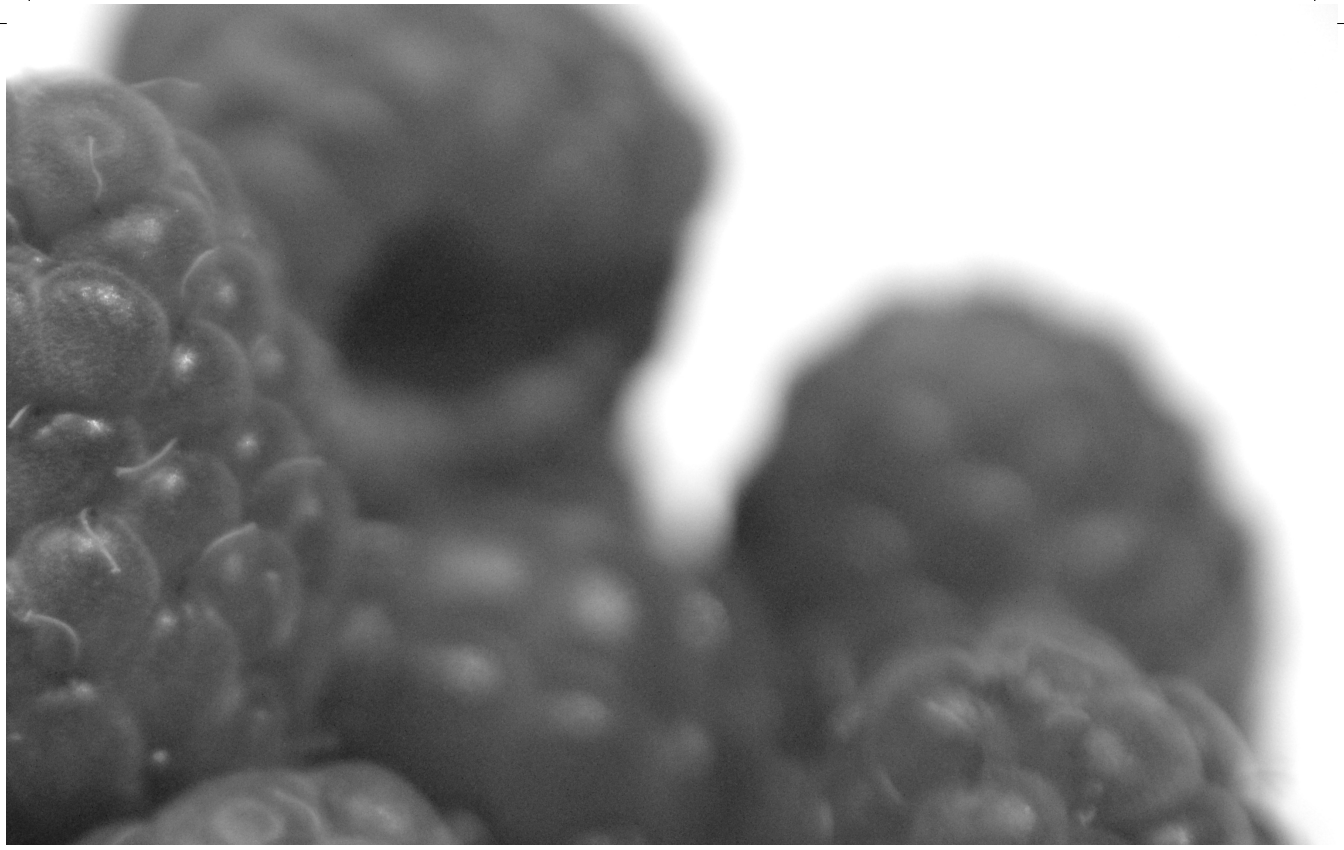
190. Havelaar, A.H., Rutjes, S.A., *Risk Assessment of Viruses in Food: Opportunities and Challenges* Food-Borne Viruses Progress and Challenges ed. D.O.C. M.P.G. Koopman, A. Bosch. 2008, Washington, DC: ASM Press.

191. De Roda Husman, A.M. and Bouwknegt, M., *Quantitative risk assessment for food- and waterborne viruses*. Viruses in food and water: Risks, surveillance and control, ed. N. Cook. Vol. 1. 2013, Cambridge, UK: Woodhead Publishing Limited.

192. Petterson, S.R. and Ashbolt, N.J., *Viral risks associated with wastewater reuse: modeling virus persistence on wastewater irrigated salad crops.* Water Sci Technol, 2001. 43(12):23-6.

193. Petterson, S.R., Ashbolt, N.J., and Sharma, A., *Microbial risks from wastewater irrigation of salad crops: a screening-level risk assessment.* Water Environ Res, 2001. 73(6):667-72.

2



Persistence of human norovirus GII.4 and GI.4, murine norovirus, and human adenovirus on soft berries as compared with PBS at commonly applied storage conditions

Katharina Verhaelen[a,b], Martijn Bouwknegt[a], Froukje Lodder-Verschoor[a], Saskia Rutjes[a], Ana Maria de Roda Husman[a,b]

[a]Laboratory for Zoonoses and Environmental Microbiology, Centre for Infectious Disease Control Netherlands, National Institute for Public Health and the Environment (RIVM), P.O. Box 1, NL-3720 BA Bilthoven, The Netherlands
[b]Faculty of Veterinary Medicine, Institute for Risk Assessment Sciences (IRAS), Utrecht University, P.O. Box 80.178, NL-3505 TD Utrecht, The Netherlands

International Journal of Food Microbiology 160 (2012) 137-144

Abstract

Human noroviruses (hNoV) have been detected on soft fruits. Especially raspberries have been found to be associated with outbreaks of gastroenteritis suggesting persistence of hNoV on these fruits. Therefore, the persistence of hNoV GII.4 and GI.4, murine norovirus (MNV-1, a culturable surrogate for hNoV), and human adenovirus (hAdV, an indicator for human fecal contamination), on raspberries, strawberries and in phosphate buffered saline (PBS) at 4 °C, 10 °C and 21 °C, mimicking commonly applied storage conditions was studied by molecular and cell culture techniques. Monophasic, biphasic and Weibull models were fitted to virus counts with maximum likelihood estimation. The tested viruses were persistent (≤0.5 $\log_{10}$-unit reduction in viral titer) under all studied conditions in PBS, at 4 °C and 10 °C on raspberries, and at 4 °C on strawberries. The difference in viral persistence on raspberries and strawberries was most pronounced at 21 °C. Here, infectious MNV-1 and hAdV particles decayed rapidly on strawberries with TFL-values (time for the first $\log_{10}$-unit reduction) of only 1 day (95% CI of 0.6–1 and 0.8–1 days, respectively). On raspberries, however, the TFL-value of infectious MNV-1 was found to likely exceed the shelf life of the berries with 3 days (95% CI of 2.8–3.1 days); hAdV remained infectious with only 0.3 $\log_{10}$-unit reduction (95% CI of 0.2–0.4) in viral titer. For hNoV GI, a TFL-value of 2 days (95% CI 1–4 days) was determined based on the targeted genome fragment, whereas the TFL-value of hNoV GII exceeded the shelf life of strawberries at 21 °C. The greater viral persistence on raspberries as compared to strawberries, especially at 21 °C, may at least in part explain why raspberries are more frequently associated with hNoV outbreaks than strawberries. Moreover, our results show that due to the high persistence of the virus already low contamination levels of the highly infectious hNoV may be associated with an infection risk of humans after consumption of raspberries. The estimated decay parameters and uncertainties of this study serve as important input requirements in the quantitative assessment of public health risks from the consumption of soft fruits.

Introduction

Human norovirus (hNoV), a single stranded non-enveloped RNA virus of the family Caliciviridae, is a major cause of viral gastroenteritis worldwide [1]. The high rate of evolution within the virus capsid allows the virus to escape herd immunity [2], and new emerging strains are posing a continuing threat to public health. HNoV disease is described as mild and self-limiting gastroenteritis, characterized by non-bloody diarrhea and vomiting [3]. However, severe illness can occur particularly in the young, elderly and immuno-compromised population, as was shown recently in The Netherlands; hNoV causes several hundreds of hospitalizations of children and elderly and approximately 20 deaths per year [4, 5]. HNoV transmission occurs through oral exposure to the viruses in feces and/or aerosolized vomitus either by direct contact with infected individuals or indirectly by exposure to virus contaminated food, water, fomites and surfaces [1]. Food plays an important role in hNoV transmission [6, 7], and an estimated 20% of hNoV outbreaks in Europe are foodborne [8]. Food becomes contaminated with hNoV by fecal contamination by contact with water, soil, and surfaces, by cross-contamination, and/or by an infected food-handler. The high probability of infection per ingested hNoV particle [9] combined with high numbers of shed virus [10] contribute to the spread of the virus by food consumption. Moreover, the virus is resistant to several processes and conditions found in the food industry such as low pH [11, 12], treatment with UV-light [13], low temperatures [12, 14-16], and washing with and without disinfectants [15, 17]. Even though hNoV genogroup II (hNoV GII) is responsible for most hNoV outbreaks [18] hNoV genogroup I (hNoV GI) is relatively more frequently detected in foodborne outbreaks [8]. This may be explained by a greater persistence of hNoV GI compared to hNoV GII as shown by Butot et al. (2009), da Silva et al. (2007), and Hewitt et al. (2009).

Fresh produce plays an important role in the transmission of hNoV via food. HNoV accounts for more than 40% of fresh produce associated outbreaks [22, 23] with numerous outbreaks associated with raspberries [24-32]. However, few hNoV outbreaks related to strawberries have been reported to date even though strawberry production exceeds raspberry production about ten times [33] and strawberries are more prone to hNoV contamination than raspberries for the following reasons. Firstly, strawberries grow closer to the ground and are thus more susceptible to contamination with irrigation water and soil; and secondly, unlike raspberries, strawberries cannot be harvested mechanically resulting in more contact of the fruit with food handlers. The attribution of hNoV illness to food is, however, challenging, due to e.g. difficulties in detecting hNoV [29, 34, 35], the absence of a systematic surveillance for foodborne viral disease [36], and the fact that secondary person to person transmission may mask foodborne transmission of hNoV [8, 37]. As a consequence, the contribution of food matrices in

causing foodborne hNoV outbreaks is not clear and the significance of foodborne hNoV disease to public health may be estimated incorrectly.

Quantitative microbial risk assessment (QMRA) can be used to aid the estimation of the risk of foodborne hNoV illness and to elucidate the role of different food commodities in the transmission of hNoV [38]. To be able to perform a QMRA, data on the risk of exposure are needed. The exposure of a consumer is determined by the amount of a certain food item consumed, and the prevalence and load of infectious virus particles on the food commodity. The infectious viral load is in turn determined by the level of virus contamination and the persistence of the specific virus. HNoV persistence on fresh raspberries post-harvest is challenged by the applied storage conditions such as temperature and relative humidity, the duration of storage (shelf life), and the fruit matrix itself. Specific storage temperatures are not legally required for intact fresh raspberries. Abrupt cooling after harvest is recommended and fresh produce is commonly stored during retail in a fridge (≤4 °C), in open self service fridges (≤10 °C) or at room temperature (about 21 °C). Besides a washing step that may be applied for strawberries, which is characterized by a low removal of hNoV [15], post-harvest conditions for raspberries and strawberries are similar. Data on norovirus persistence on raspberries and strawberries at the mentioned storage conditions are not yet available.

Therefore, the aim of this study was to determine the decay rates of hNoV on raspberries and strawberries under commonly applied storage conditions for soft berries at 4 °C, 10 °C and 21 °C. Persistence was studied on raspberries and strawberries, and in phosphate buffered saline (PBS) which was used as a reference point for viral decay. Since hNoV cannot be cultured, PCR based assays were used for hNoV GI.4 and GII.4. Moreover, both murine norovirus (MNV), a cultivable surrogate of hNoV, and human adenovirus (hAdV), suggested as an indicator for human fecal pollution [39] were included in the study and detected by both molecular and cell culture techniques. Mengovirus (vMC_0) was used as a process control [19, 40] in the persistence experiments.

Materials and Methods

Viruses and cells

HNoV GI.4 and hNoV GII.4 strains from clinical stool samples were kindly provided by Dr. Erwin Duizer (Laboratory for Infectious Diseases and Perinatal Screening (RIVM, The Netherlands). MNV-1 (kindly provided by Dr. Herbert W. Virgin, Washington University, St. Louis, USA) and hAdV-2 were made available, under agreement, by the group of Dr. Franco M. Ruggeri (Istituto Superiore de Sanitá, Rome, Italy). Mengovirus

(vMC_0) (Martin and Palmenberg, 1996), kindly provided by Dr. David Lees, (CEFAS, Dorset, UK) was used as a process control to monitor virus recovery. Virus stocks were obtained by three freeze–thaw cycles of infected cells and subsequent centrifugation at 1500 g for 15 min. RAW-264.7 cells (ATCC-TIB-71), used to propagate MNV-1, were grown in 1×DMEM (Invitrogen, The Netherlands) supplemented with 10% fetal calf serum (PAA, Germany), 1% penicillin/streptomycin (Invitrogen), 1% HEPES (Lonza, The Netherlands) and 1% Glutamine (Lonza). A549 cells (ATTC-CCL-185), used to propagate hAdV, were grown in 1×MEM (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen), 1% penicillin/ streptomycin and 1% non essential amino acids (Invitrogen).

Virus spiking

Raspberry batches (harvested the same day) of 25 g were prepared. The amount of strawberries varied between 20 and 30 g and 1.7 mL of PBS was used. 3×100 µL of inoculum, consisting of hAdV-2, MNV-1, and 100 times in PBS diluted hNoV GI.4 and GII.4 stock, were spiked in small droplets onto the fruit surface. Each batch of each matrix was spiked with about 4×10^5 and 1×10^6 virus particles of MNV-1 and hAdV, and 8×10^6 and 2×10^6 genomic copies of hNoV GI and GII, respectively.

Storage conditions and sampling

The spiked batches were stored overnight at 4 °C and the next day for 4 h at 21 °C to allow the inoculum to dry. Subsequently, samples were stored at 4 °C and 10 °C in an incubator and at room temperature (21 °C) at a relative humidity of 70%, 58% and 36%, respectively. Samples were analyzed daily for 3 days and batches stored at 4 °C and 10 °C were also analyzed at days 5 and 7. The storage period was chosen based on the shelf-life of a raspberry at the given temperatures. The experiment was performed in duplicate, only the reference point T_0 taken after the drying of inoculum was conducted in triplicate. Each sampling point included a negative control where no virus was spiked in/on the matrix.

Virus elution from fruits

Virus elution from fruits was performed based on Dubois et al. 2006. Briefly, the berries were added to 40 mL of elution buffer (1.21% Tris base, 0.38% glycine and 1% beef extract, (pH 9.5)) containing 6500 U pectinase (Sigma, The Netherlands). Additionally, 20 µL of the process control virus vMC_0 was spiked into the buffer (C_p ~31). The tubes were agitated at room temperature at 40 rpm for 20 min. The pH of the buffer was controlled every 10 min and adjusted to 9.5 with 1 M sodium hydroxide if it decreased below 9.0, extending the period of agitation by 10 min. Thereafter the samples were centrifuged for 5 min at 10,000 g at 4 °C, the supernatant was transferred

to a new centrifugation tube and centrifuged for 30 min at 10,000 g at 4 °C. The pH of the supernatant was adjusted to 7.2 using 1 N HCL. To precipitate the virus, tubes were agitated at 20 rpm for 1 h at 4 °C after addition of 0.25 volumes of a PEG/NaCl solution (50% PEG 8000 (Promega, The Netherlands), 1.5% NaCl (Merck, The Netherlands)). Next, the tubes were centrifuged for 30 min at 10,000 g at 4 °C. The supernatant was discarded, the tubes were centrifuged for another 5 min at the same speed to compact the pellet, and subsequently the pellet was dissolved in 1 mL PBS and split into equal volumes. A chloroform: butanol extraction was performed on the subsample used for the nucleic acid extraction. The other subsample used to determine virus infectivity was diluted 10 times in Hanks' balanced salt solution (Invitrogen, The Netherlands and 50 g/L peptone (Oxoid, United Kingdom) and sequentially filtered through 0.8 µm and 0.22 µm syringe driven filters, pretreated with PBS containing 10% fetal calf serum. No virus elution for the control experiment with PBS was necessary. Samples were stored at –80 °C.

Determination of MNV-1 titer

To determine the titer of infective MNV-1 particles endpoint dilutions were performed. 96 well plates (Corning, The Netherlands) were seeded with 100 µL RAW-264.7 cells in growth medium containing 2% fetal calf serum at a concentration of 4×10^5 cells/mL. Cells were allowed to settle and attach for about 4 h and 100 µL serial 0.5 $\log_{10}$-unit sample dilutions in growth medium without FCS were added. For each sample seven dilutions were tested in tenfold. Plates were incubated for 7 days at 37 °C with 5% CO_2 and observed for cytopathic effect.

Determination of hAdV-2 titer

Infectivity of hAdV-2 was determined using a suspension plaque assay [42]. For this purpose, 5 mL of A549 cells in growth medium at a concentration of 6×10^5 cells/mL was seeded into 25 cm2 vented cell culture flasks (Corning). Samples were diluted in PBS and 100 µL of sample was added to the cell suspension. Samples were analyzed in different dilutions, at least in quadruplicate. After an incubation period of 4 h at 37 °C with 5% CO_2, 5 mL of overlying medium ((10% 10× MEM (Invitrogen), 2% FCS (Invitrogen), 1% L-glutamine 29 g/L (Lonza), 2.5% $NaHCO_3$ (7.5%, Invitrogen), 2% Pen/Strep 5000 unit/mL (Invitrogen), 50% carboxymethylcellulose (3%, Sigma), and 32.5% sterile deionized water) was added and the flasks were incubated for 7 days at 37 °C with 5% CO_2. Subsequently, cells were fixed by adding 5 mL of 10% formalin (Merck). After at least 1 h incubation the formalin was discarded, the flasks were rinsed with water and 10 mL of 0.1% crystal violet (Sigma) was added for about 10 min to stain the cells.

Molecular detection

Nucleic acids were extracted using the NucliSens miniMag magnetic extraction kit (bioMérieux, The Netherlands) following the manufacturer's instructions with minor modifications. In each extraction cycle, a negative control containing no target was included. The previously included vMC_0 served as a positive control. A Lightcycler 480 (Roche Diagnostics) was used for real time PCR, using TaqMan hydrolysis probes. The used primers and probes are listed in Table 1. To control for PCR inhibition, a competitive internal amplification control (IAC) (Yorkshire Bioscience Ltd., UK), specific for each target primer set [43], was added to each reaction, at a concentration previously found not to influence the detection of the target signal. Instead of FAM, VIC was used as a reporter dye of the IAC (Table 1).

Norovirus GI, GII and MNV-1 were amplified using the UltraSense One-Step Quantitative RT-PCR System (Invitrogen). Briefly, 10 µL of nucleic acid extract was added to 10 µL of master mix containing 1× RNA ultrasense reaction mix, 500 nM forward primer, 900 nM reverse primer, 250 nM Probe, 100 nM IAC probe, 1× Rox reference dye, 1 µL RNA ultrasense enzyme mix and 0.6 µL IAC for hNoV GI and GII detection. For detection of MNV-1 the used probe and primer concentrations were 200 nM. Reverse transcription was performed at 50 °C for 15 min, followed by 2 min of preheating at 95 °C and 45 cycles of amplification consisting of denaturation at 95 °C for 15 s and annealing and elongation at 60 °C for 1 min. Mengovirus was amplified using the Platinum Quantitative RT-PCR ThermoScript One-Step System (Invitrogen). 5 µL of nucleic acid extract was added to 20 µL of master mix containing a final concentration of 1.25× ThermoScript reaction mix, 625 nM forward primer, 1125 nM reverse primer, 313 nM Probe, 1.25× Rox reference dye, 0.5 µL ThermoScript Platinum Taq enzyme-mix and 3.5 µL molecular grade water. Cycling parameters are as mentioned above, but the

Table 1. Primers and probes

Virus	Primer/Probe	Sequence	Reference
hNoV GI	QNIF4	CGC TGG ATG CGN TTC CAT	[20]
	NV1LCR	CCT TAG ACG CCA TCA TCA TTT AC	[44]
	NV1LCPR	(FAM)-TGG ACA GGA GAY CGC RAT CT-(BHQ1)	[44]
hNoV GII	QNIF2	ATG TTC AGR TGG ATG AGR TTC TCW GA	[45]
	COG2R	TCG ACG CCA TCT TCA TTC ACA	[46]
	QNIFS	(FAM)-AGC ACG TGG GAG GGC GAT CG-(BHQ1)	[45]
MNV-1	FwORF1/ORF2	CAC GCC ACC GAT CTG TTC TG	[47]
	RvORF1/ORF2	GCG CTG CGC CAT CAC TC	[47]
	MGBORF1/ORF2	(FAM)-CGC TTT GGA ACA ATG-(BHQ1)	[47]
vMC_0	Mengo 110	GCG GGT CCT GCC GAA AGT	[40]
	Mengo 209	GAA GTA ACA TAT AGA CAG ACG CAC AC	[40]
	Mengo 147	(FAM)-ATC ACA TTA CTG GCC GAA GC-(BHQ1)	[40]
hAdV-2	AdF	CWT ACA TGC ACA TCK CSG G	[48]
	AdR	CRC GGG CRA AYT GCA CCA G	[48]

reverse transcription was performed for 1 h and 55 min. HAdV-2 was amplified using the TaqMan Universal PCR Master Mix (Applied Biosystems), see Bofill-Mas et al. (2010).

Genome quantification

Viral genomes were quantified using standard curves. For the construction of the hNoV GI, hNoV GII and MNV standard, the plasmid pCR 2.1-TOPO (Invitrogen) was used containing synthetic DNA molecules of the three target sequences of the qRT-PCR.[50] Plasmids were kindly provided by David Rodriguez- Lázaro (Instituto Tecnológico Agrario de Castilla y León (ITACyL), Spain). The plasmid was linearized using the BamH1 restriction enzyme (Promega) and subsequently purified using the Qiaprep Spin Midiprep Kit (Qiagen). Transcription of the plasmid was performed using T7 RNA polymerase Riboprobe (Promega) according to the manufacturer›s protocol. Remaining DNA was digested using DNase and RNA was purified stepwise with Trizol, chloroform, isopropanol and ethanol. For the construction of the hAdV standard the plasmid pBR322 containing the hAdV 41 hexon sequence, kindly provided by Rosina Girones (Department of Microbiology, Faculty of Biology, University of Barcelona, Spain) was used. The plasmid was linearized and purified following Bofill-Mas et al. (2010). The standard was quantified by UV-absorbance at 260 nm and a standard curve was generated using 10 fold serial dilutions of the plasmid. Tenfold dilution of the standards were included in each PCR run, diluted in the sample matrix to account for a possible impact of the sample matrix on the efficiency of the PCR.

Data analysis

Virus decay was modeled as monophasic exponential, biphasic exponential and Weibull decay to test hypothesis regarding a constant decay rate, two different decay rates and a time-dependent decay rate, respectively. The likelihood ratio test was used to select the best fitting models among the exponential models and among the monophasic exponential model and Weibull model. Decay curves with an upward concavity were fitted to the Weibull model since the biphasic model failed to converge.

Exponential decay was modeled according to

$$\log_{10}[C_t] = \log_{10}\left[C_0\left(w\,\mathrm{Exp}(-\lambda_1 t) + (1-w)\,\mathrm{Exp}(-\lambda_2 t)\right)\right] \quad (1)$$

with c_0 being the initial concentration (n per g or ml) at time zero, λ_1 and λ_2 decay rates per min, t the time in min, and w the mixing parameter [51,52]. If w equals 1, then the model describes monophasic exponential decay; otherwise the decay is biphasic.

Weibull decay was modeled according to

$$\log_{10}[c_t] = \log_{10}[c_0] - \left(\frac{t}{\delta}\right)^p \quad (2)$$

with δ being the scale parameter and p the shape parameter [53].

Model parameters were estimated by maximum likelihood, where infectivity counts were included using the presence/absence profile for MNV-1 and the plaque assay counts for hAdV assuming homogeneous ('Poisson') mixing. For genome counts based on RT-PCR, the intercept and slope parameter of the standard curve were estimated simultaneously with the decay rate parameters as joint likelihood. Genomic copies were considered to be log normally distributed. Uncertainty in parameter estimates was assessed by adaptive rejection Markov Chain Monte Carlo (MCMC) sampling from the likelihood using the Metropolis–Hastings algorithm [54,55], in Mathematica 8.0 (Wolfram, 2011).

The time required for the first 1 $\log_{10}$-unit reduction in virus titer (TFL) was predicted by solving t from equations 1 or 2 for c_t/c_0=0.1 using the most likely parameter values. The associated 95% interval was assessed by solving t for parameter values from the MCMC posterior. Unlike conventional D-values, TFL-values that are not based on monophasic decay models cannot be extrapolated, meaning that the time needed for an additional $\log_{10}$-unit reduction is not necessarily equal to the time for the first $\log_{10}$-unit reduction. To develop a general model for the effect of temperature on the estimated decay rates on soft fruit, average daily $\log_{10}$ reductions (ADRs) were calculated by dividing the total estimated reduction at the end of the experiment by the number of observation days (either three or seven). Estimated ADRs were $\log_{10}$ transformed and fitted to the linear model

$$\log_{10}[\mathrm{ADR}] = \beta_0 + \beta_1 T + \beta_2 F + \beta_3 M + \beta_4 V + \varepsilon \quad (3)$$

with β being the regression parameters, T the temperature (°C), F the fruit (strawberry (F=0) or raspberry (F=1)), M the detection method (cell culture (M=0) or PCR (M=1)) and V the virus-type. The latter variable was included as class variable (MNV-1, hNoV GI, hNoV GII or hAdV) or as dichotomous variable (V=0 for RNA viruses and V=1 for the DNA virus). The $\log_{10}$ ADRs were considered to be normally distributed. Observations with an estimated ADR of zero were included as censored observation in the likelihood estimation, taking the lowest estimated ADR as cut-off for its likelihood contribution.

This cut-off was chosen, because the lowest ADR would have been observable given the experimental approach, whereas we lack data supporting the detection of any smaller ADR. The likelihood ratio test was used to test whether β-values differed significantly from zero at an α-level of 0.05.

Results

In this study, the impact of ambient conditions and food matrix on the persistence of hNoV GII, hNoV GI, MNV-1 and hAdV genomes and infectious MNV-1 and hAdV particles was studied. Viral persistence was expressed as the observed reduction in viral titer over the studied period (Table 2) and TFL-values were determined for those data sets for which a 1 $\log_{10}$-unit reduction was achieved within the studied period (Table 3). Viral decay was illustrated in Figs. 1–4 whereby differences in the initial virus levels on raspberries and strawberries as compared with PBS are a consequence of the different recoveries. The modeling parameters required for the QMRA (parameters of Eqs. (1) and (2)) are not given, but will be provided on request.

Model fitting

As shown in Figs. 1–4 most data sets followed exponential decay models, with the monophasic decay model fitting the data in most cases. Biphasic viral decay was mostly observed on strawberries. Out of the 30 data sets analyzed, the Weibull model fitted best in only three cases: for MNV-1 decay on strawberries at 10 °C and on raspberries and in PBS at 21 °C. Since all studied viruses were persistent at 4 °C on/in all studied matrices (see Table 2), the corresponding figures are not presented.

Effect of the matrix and ambient conditions on viral persistence

The results show that viral persistence was challenged by a combination of ambient conditions (temperature, relative humidity (inversely dependent on the ambient temperature)) and food matrix. Viral decay was lowest in PBS, followed by raspberries and then strawberries. In PBS, viruses were stable at all tested temperatures (≤ 0.5 $\log_{10}$-unit reduction), whereas on raspberries and strawberries hNoV GII, hNoV GI, MNV-1 and hAdV were more persistent at 4 °C followed by 10 °C and 21 °C with a relative humidity of 70%, 58%, and 36%, respectively. Especially on strawberries, viruses decayed rapidly at 21 °C, with a TFL-value of only one day for MNV-1 and hAdV by cell culture and 2 days for hNoV GI based on the number of detected genome fragments (Table 3). The observed decay was biphasic and after a rapid initial reduction hNoV GI, and MNV-1 remained relatively stable with a final reduction of 1.1 $\log_{10}$-unit and 1.4 $\log_{10}$-unit after three days of storage (Table 2). For hNoV GII, the TFL-value exceeded the studied period. On raspberries, decay at 21 °C was less pronounced with a TFL-value

Table 2. Mean $\log_{10}$-unit reductions and the corresponding 95 % confidence intervals (CI) of hNoV GII.4, hNoV GI.4, MNV-1 and hAdV on raspberries, strawberries and PBS after 7 days of storage at 4°C and 10 °C and after 3 days at 21 °C are given. Values for infectious virus particles are given, determined by cell culture (CC), and for genomic copies, determined by PCR. C_t represents the viral titer after storage of 7 days at 4 °C and 10 °C and after 3 days at 21 °C, and C_0 is the viral titer at time point T_0.

			Raspberry		Strawberry		PBS	
Temp °C	Method	Virus	$Log(C_0/C_t)$	95% CI	$Log(C_0/C_t)$	95% CI	$Log(C_0/C_t)$	95% CI
4	PCR	hNoV GII	0	–	0	–	0	–
	PCR	hNoV GI	0	–	0	–	0.1	0.04 – 0.2
	PCR	MNV	0	–	0	–	0	–
	CC	MNV	0	–	0	–	0	–
	PCR	hAdV	0.1	0.01 – 0.3	0.6	0.3 – 0.8	0	–
	CC	hAdV	0.4	0.2 – 0.5	0.2	0.2 – 0.3	0	–
10	PCR	hNoV GII	0.3	0.1 – 0.5	0.4	0.1 – 0.6	0	–
	PCR	hNoV GI	0.4	0.1– 0.6	0.5	0.03 – 0.8	0.1	0.1 – 0.2
	PCR	MNV	0	–	0.3	0.03 – 0.4	0	–
	CC	MNV	0.5	0.3 – 0.6	0.9	0.7 – 1.0	0	–
	PCR	hAdV	0.2	0.1 – 0.4	0	–	0	–
	CC	hAdV	0.2	0.1 – 0.3	1.2	1.0 – 1.3	0	–
21	PCR	hNoV GII	0.2	0.003 – 0.2	0.5	0.4 – 0.7	0	–
	PCR	hNoV GI	0.3	0.03 – 0.4	1.2	0.3 – 1.2	0.2	0.08– 0.2
	PCR	MNV	0.5	0.3 – 0.8	0.9	0.6 – 1.2	0	–
	CC	MNV	1.1	0.8 – 1.4	1.4	1.2 – 1.5	0.4	0.2 – 0.6
	PCR	hAdV	0.5	0.4 – 0.6	0	–	0.1	0.06 – 0.1
	CC	hAdV	0.3	0.2 – 0.4	1.9	1.8 – 2.2	0	–

Table 3. The mean TFL-values (time for the first 1log_{10}-unit reduction) and the 95% confidence intervals (CI) are given for infectious virus particles determined by cell culture (CC) or for genomic copies determined by PCR. Only those TFL-values are stated which did not exceed the studied period.

Virus	Method	Matrix	Temperature (°C)	TFL-value (day(s))	95 % CI (day(s))
MNV	CC	Raspberry	21	3	2.8 – 3.1
MNV	CC	Strawberry	10	7	7 – 8
hAdV	CC	Strawberry	10	6	5 – 7
MNV	CC	Strawberry	21	1	0.6 – 1
MNV	PCR	Strawberry	21	3	2 – 6
hNoV GI	PCR	Strawberry	21	2	1 – 4
hAdV	CC	Strawberry	21	1	0.8 – 1

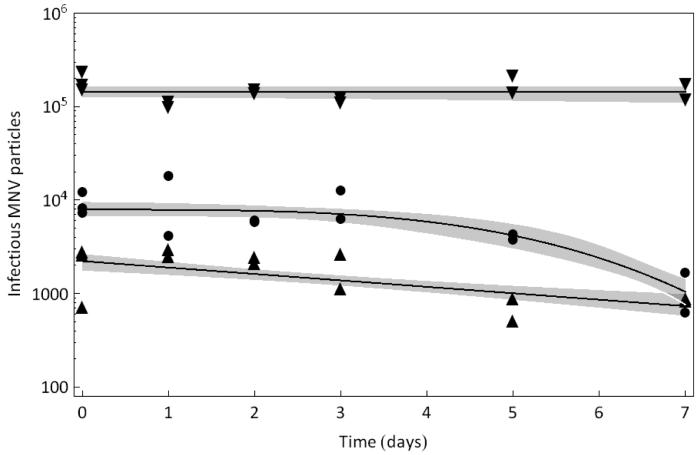


Figure 1. MNV persistence at 10 °C (▼ PBS, ● strawberries, ▲ raspberries) based on infectivity data. Each plot marker represents a data point. The grey area represents the 95 % confidence interval.

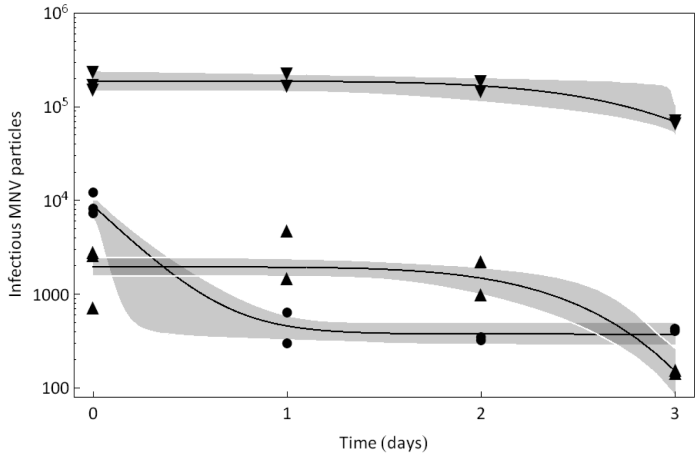


Figure 2. MNV persistence at 21 °C (▼ PBS, ● strawberries, ▲ raspberries) based on infectivity data. Each plot marker represents a data point. The grey area represents the 95 % confidence interval.

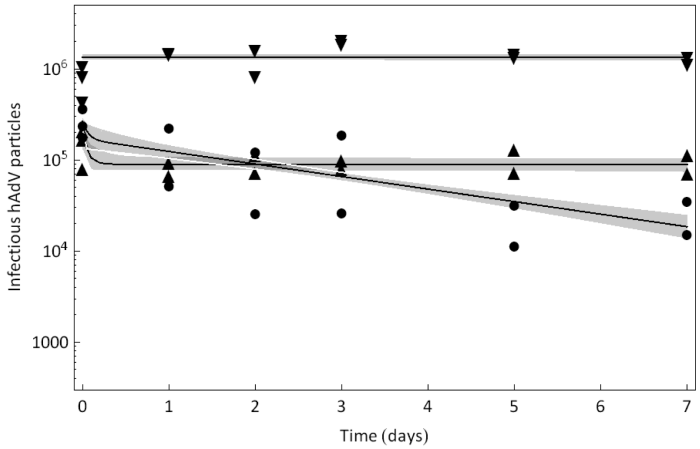


Figure 3. HAdV persistence at 10 °C (▼ PBS, ● strawberries, ▲ raspberries) based on infectivity data. Each plot marker represents a data point. The grey area represents the 95 % confidence interval.

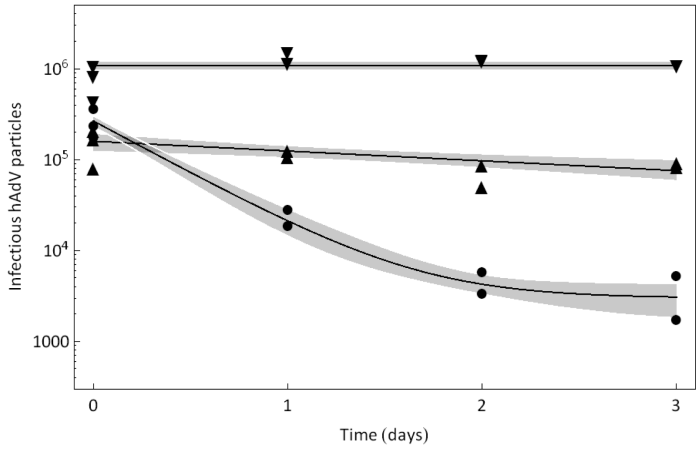


Figure 4: HAdV persistence at 21 °C (▼ PBS, ● strawberries, ▲ raspberries) based on infectivity data. Each plot marker represents a data point. The grey area represents the 95 % confidence interval.

of 3 days for infectious MNV-1 and decay in hAdV particles of 0.3 $\log_{10}$-units within the studied period. Even at 10 °C infectious MNV-1 and hAdV decayed noticeably on strawberries with a TFL-value of 7 days, whereas viruses were stable on raspberries (≤0.5 $\log_{10}$-unit reduction). All other TFL-values exceeded the studied period i.e. the shelf-life of raspberries and strawberries.

Effect of virus type on viral persistence

Overall, HNoV GII, GI and MNV RNA fragments showed similar persistence patterns. Only at 21 °C on strawberries a pronounced difference in the persistence of hNoV GII,

GI and MNV-1was found, with hNoV GII being more persistent than hNoV GI and MNV (Tables 2 and 3). Infectious MNV-1 and hAdV particles were distinguished by an overall similar persistence. Whereas infectious hAdV was slightly more stable on raspberries and in PBS, MNV-1 occurred to be slightly more stable on strawberries.

Virus detection by PCR compared to cell culture

The number of infectious hAdV and MNV-1 particles was found to decrease rapidly on strawberries at 21 °C by cell culture. This decay was reflected in a decrease in genomic copies for MNV-1 with a TFL-value of 3 days (Table 3), however, no decrease in genomic hAdV copies was observed (Table 2). In all other experiments, a higher persistence was observed at lower temperatures in PBS followed by raspberries and strawberries by both cell culture and by PCR.

General virus decay model

The effect of temperature on ADRs is shown in Fig. 5. There was no statistically significant difference among the different RNA viruses (p=0.24). Therefore the virus type was included dichotomously as either RNA or DNA virus. Variables not improving the model fit were all two-way interaction terms (p>0.13), the matrix (p=0.40) and virus type (p=0.61). The type of detection (PCR or cell culture) and temperature were significantly associated with ADRs (p=0.02 and p<0.0001, respectively). The $\log_{10}$ ADR

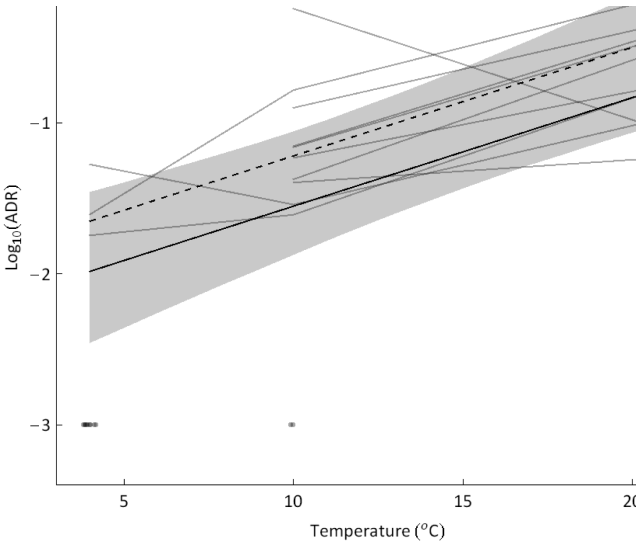


Figure 5. Generic model for temperature dependence of virus decay. The grey lines represent virus-matrix specific $\log_{10}$ ADR profiles for increasing temperatures. The solid line represents the fitted generic model for PCR data, the dashed line for cell culture. The grey area represents the total 95% interval for the regression lines. The dots represented at the $\log_{10}$ ADR-level of -3 are censored observations and displayed at this level for identification.

was estimated to increase by 0.077 (95% interval: 0.055–0.103) per °C temperature increase. The $\log_{10}$ ADR value at 0 °C (the intercept) based on PCR was -2.36 (-2.00 to -2.87), based on cell culture -2.02 (-1.71 to -2.34). The estimated average reduction for an infectious virus at 15 °C, for example, would mount to $10^{-2.02+15\times0.077} = 0.14$ $\log_{10}$ units per day, for virus genomes $10^{2.36+15\times0.077} = 0.062$ $\log_{10}$ units. The estimated average D-values are approximately 1/0.14=7 days and 1/0.062=16 days, respectively, at 15 °C. Note that these predicted D-values lie outside the actual observation time in the experiment.

Discussion

The current study demonstrated that noroviruses were less persistent on strawberries as compared with raspberries. The difference in viral persistence was most pronounced at 21 °C. MNV-1 infectivity dropped about 1.5 $\log_{10}$-unit on strawberries after just one day of storage at room temperature, whereas no virus decay was observed on raspberries during this period. Only after 3 days a 1 $\log_{10}$-unit decrease in MNV-1 infectivity occurred at room temperature. Yet, in practice raspberries are rarely stored for more than 2 days at room temperature due to the perishability of the product. Assuming a similar persistence for MNV-1 and hNoV, hNoV is therefore likely to stay infectious on raspberries during retail at all tested conditions and is also more likely to persist on raspberries in the fields compared with strawberries. The results of our study are in agreement with Kurdziel et al. (2001), who studied the persistence of poliovirus on raspberries over 10 days at 4 °C and found no viral decay for this small, enteric virus. Mattison et al. (2007) studied the persistence of feline Calicivirus (FCV), a hNoV surrogate used before the discovery of MNV-1, on strawberries. They showed that also FCV is reduced rapidly on strawberries with a sharp initial decrease at room temperature, which was not found if strawberries were stored at 4 °C. The established greater persistence of noroviruses on raspberries as compared to strawberries may in part explain why hNoV outbreaks are more often associated with raspberries than with strawberries.

HAdV was included in the study since the virus is suggested as an index virus for the presence of human fecal contamination [39]. Moreover, hAdV is also a possible foodborne pathogen [6]. Unlike noroviruses which are small (28–35 nm) ss-RNA viruses, hAdV is a medium-sized virus of 70– 90 nm composed of a ds-DNA genome. Nevertheless, comparing viral decay of hAdV and hNoV GI and GII showed similar decay rates and decay kinetics for the viruses, except for hAdV genomes at 21 °C on strawberries, which appeared to be more stable. Yet, the type of virus did not significantly improve the fit of the regression model on the average daily $\log_{10}$-unit reduction, indicating that the type of studied viruses had overall no significant impact on the observed decay found on soft berries. This suggested that hAdV is a suitable index virus for hNoV in terms of persistence, based on molecular detection. Unlike for MNV-1, no correlation between

the observed reduction in infectious particles on strawberries and the number of genomic copies was found for hAdV at room temperature. These findings suggest that the target of inactivation is the viral capsid and not the genome. Due to the lower stability of RNA as compared with that of DNA [37], decay of genomic copies may be found more rapidly for RNA viruses such as noroviruses than for DNA viruses such as hAdV. However, due to the small fraction of the genome targeted by PCR it cannot be ruled out, that the target of inactivation is yet the genome.

Overall we found a similar persistence of hNoV GII and hNoV GI RNA, whereby decay of hNoV GI was more pronounced on strawberries as compared with hNoV GII. These findings are not in accordance with other studies. Butot et al. (2009) described that the resistance of hNoV GI to freeze drying was significantly higher compared to hNoV GII, and Hewitt et al. (2009) described that hNoV GI was more resistant to heat inactivation in water and milk. Therefore, our results do not support the hypothesis that hNoV GI is more often associated with food-borne hNoV outbreaks due to a higher persistence of the virus compared to hNoV GII. However, the obtained decay rates of hNoV GII and GI are based on molecular data and do not necessarily relate to hNoV infectivity. In the absence of a hNoV cell culture assay, molecular methods potentially allowing to distinguish between infectious and noninfectious hNoV particles, such as the use of an intercalating dye propidium monoazide PCR assay [58], or a sample treatment with proteinase K and RNase have been described [47]. However, their application for other noroviruses under commonly applied storage conditions need further study.

The obtained results showed that viral decay is determined, besides by viral characteristics, by a combination of ambient conditions and food matrix. But what mechanism could explain the observed difference in viral persistence on raspberries and strawberries? Possible factors that influenced the infectivity of virus particles under the tested conditions were the temperature, the relative humidity, and the fruit matrix itself. The fruit matrix may influence virus infectivity by its (i) chemical composition (antiviral compounds), (ii) enzymatic activity or by (iii) physiological parameters such as the respiration rate. It may be that strawberries contain certain antiviral compounds on their surface and their antiviral effect may become more apparent at higher temperatures. The presence of enzymes, e.g. proteases, on the surface of strawberries may be another explanation for the differing viral persistence on raspberries and strawberries. Proteases can originate from the fruit itself or from bacteria or fungi present on the fruit surface. Enzyme activity is temperature dependent, which would explain why viral inactivation increases with higher temperatures. And lastly the respiration rate could be an explanation. Raspberries are characterized by a higher (aerobic) respiration rate

(162–264 mg CO_2/kg/h) [59] compared to strawberries (102–196 mg CO_2/kg/h,) [60] at 20 °C, leading to a higher humidity in the microclimate of raspberries. Viruses prone to desiccation, such as MNV-1 [11], may thus be protected from desiccation on raspberries at room temperature but not on strawberries. The surface structure could be another interesting factor that affects viral persistence on berries. It is reported that for instance the efficacy of washing treatments in removing virus particles on fresh produce could be influenced by the presence of hydrophobic pockets [61]. However, to date no data have been reported that suggest an impact of the surface structure of berries on the persistence of viruses in a temperature dependent manner.

Summarizing, we can conclude that due to the high persistence of hNoV on raspberries at commonly applied storage conditions, already low contamination levels of the highly infectious hNoV may be associated with an increased infection risk in humans after consumption. In contrast, due to the lower viral persistence, strawberries are less likely to transmit the virus as compared with raspberries, which, together with a possible applied washing step, may explain why hNoV outbreaks are frequently associated with raspberries and not with strawberries. The difference in viral persistence on strawberries and raspberries further showed that it is difficult to make any predictions on viral persistence even for seemingly similar food matrices such as raspberries and strawberries. Yet, reliable viral decay rates are crucial to estimate the risk of infection from consuming certain food products. The investigation of viral persistence at all possible storage conditions and matrixes is, however, very laborious. As a first step, we provided a generic model for predicting average daily $\log_{10}$ reductions as a function of temperature, allowing risk assessors to apply this model to any temperature in the studied range. Besides getting a better understanding on viral characteristics that influence the persistence, it is vital to study the food characteristics that affect the persistence of viruses on foods, allowing us to group food items based on these criteria into different risk categories for the transmission of certain viruses.

Acknowledgements

This work was supported by the EU FP7 project 'Integrated monitoring and control of foodborne viruses in European food supply chains (VITAL)', contract no. 213178 and was conducted in part for the account of the National Institute of Public Health and the Environment (RIVM) for the Strategic RIVM Research project "Norovirus inactivation". Moreover, we would like to acknowledge Mohammed Alsady for his great contribution on the experimental set up and Prof. Dr. Arie Havelaar, Dr. Erwin Duizer, and the VITAL partners for the critical review of the paper.

References

1. Glass, R.I., Parashar, U.D., and Estes, M.K., *Norovirus gastroenteritis.* N Engl J Med, 2009. 361(18):1776-85.
2. Bull, R.A. and White, P.A., *Mechanisms of GII.4 norovirus evolution.* Trends Microbiol, 2011. 19(5):233-40.
3. Rockx, B., De Wit, M., Vennema, H., Vinje, J., De Bruin, E., Van Duynhoven, Y., and Koopmans, M., *Natural history of human calicivirus infection: a prospective cohort study.* Clin Infect Dis, 2002. 35(3):246-53.
4. Mattner, F., Sohr, D., Heim, A., Gastmeier, P., Vennema, H., and Koopmans, M., *Risk groups for clinical complications of norovirus infections: an outbreak investigation.* Clin Microbiol Infect, 2006. 12(1):69-74.
5. van Asten, L., Siebenga, J., van den Wijngaard, C., Verheij, R., van Vliet, H., Kretzschmar, M., Boshuizen, H., van Pelt, W., and Koopmans, M., *Unspecified gastroenteritis illness and deaths in the elderly associated with norovirus epidemics.* Epidemiology, 2011. 22(3):336-43.
6. Koopmans, M. and Duizer, E., *Foodborne viruses: an emerging problem.* Int J Food Microbiol, 2004. 90(1):23-41.
7. Mead, P.S., Slutsker, L., Griffin, P.M., and Tauxe, R.V., *Food-related illness and death in the united states reply to dr. hedberg.* Emerg Infect Dis, 1999. 5(6):841-2.
8. Verhoef, L., Vennema, H., van Pelt, W., Lees, D., Boshuizen, H., Henshilwood, K., and Koopmans, M., *Use of norovirus genotype profiles to differentiate origins of foodborne outbreaks.* Emerg Infect Dis, 2010. 16(4):617-24.
9. Teunis, P.F., Moe, C.L., Liu, P., Miller, S.E., Lindesmith, L., Baric, R.S., Le Pendu, J., and Calderon, R.L., *Norwalk virus: how infectious is it?* J Med Virol, 2008. 80(8):1468-76.
10. Chan, M.C., Sung, J.J., Lam, R.K., Chan, P.K., Lee, N.L., Lai, R.W., and Leung, W.K., *Fecal viral load and norovirus-associated gastroenteritis.* Emerg Infect Dis, 2006. 12(8):1278-80.
11. Cannon, J.L., Papafragkou, E., Park, G.W., Osborne, J., Jaykus, L.A., and Vinje, J., *Surrogates for the study of norovirus stability and inactivation in the environment: aA comparison of murine norovirus and feline calicivirus.* J Food Prot, 2006. 69(11):2761-5.
12. Mormann, S., Dabisch, M., and Becker, B., *Effects of technological processes on the tenacity and inactivation of norovirus genogroup II in experimentally contaminated foods.* Appl Environ Microbiol, 2010. 76(2):536-45.
13. De Roda Husman, A.M., Bijkerk, P., Lodder, W., Van Den Berg, H., Pribil, W., Cabaj, A., Gehringer, P., Sommer, R., and Duizer, E., *Calicivirus inactivation by nonionizing (253.7-nanometer-wavelength [UV]) and ionizing (gamma) radiation.* Appl Environ Microbiol, 2004. 70(9):5089-93.
14. Baert, L., Uyttendaele, M., Vermeersch, M., Van Coillie, E., and Debevere, J., *Survival and transfer of murine norovirus 1, a surrogate for human noroviruses, during the production process of deep-frozen onions and spinach.* J Food Prot, 2008. 71(8):1590-7.
15. Butot, S., Putallaz, T., and Sanchez, G., *Effects of sanitation, freezing and frozen storage on enteric viruses in berries and herbs.* Int J Food Microbiol, 2008. 126(1-2):30-5.
16. Dawson, D.J., Paish, A., Staffell, L.M., Seymour, I.J., and Appleton, H., *Survival of viruses on fresh produce, using MS2 as a surrogate for norovirus.* J Appl Microbiol, 2005. 98(1):203-9.
17. Baert, L., Vandekinderen, I., Devlieghere, F., Van Coillie, E., Debevere, J., and Uyttendaele, M., *Efficacy of sodium hypochlorite and peroxyacetic acid to reduce murine norovirus 1, B40-8, Listeria monocytogenes, and Escherichia coli O157:H7 on shredded iceberg lettuce and in residual wash water.* J Food Prot, 2009. 72(5):1047-54.
18. Kroneman A, V.L., Harris J, Vennema H, Duizer E, van Duynhoven Y, Gray J, Iturriza M, Böttiger B, Falkenhorst G, Johnsen C, von Bonsdorff CH, Maunula L, Kuusi M, Pothier P, Gallay A, Schreier E, Höhne M, Koch J, Szücs G, Reuter G, Krisztalovics K, Lynch M, McKeown P, Foley B, Coughlan S, Ruggeri FM, Di Bartolo I, Vainio K, Isakbaeva E, Poljsak-Prijatelj M, Grom AH, Mijovski JZ, Bosch A, Buesa J, Fauquier AS, Hernandéz-Pezzi G, Hedlund KO, Koopmans M., *Analysis of integrated virological and epidemiological reports of norovirus outbreaks collected within the Foodborne Viruses in Europe network from 1 July 2001 to 30 June 2006.* Journal of Clinical Microbiology, 2008. 46(9):2959-2965.
19. Butot, S., Putallaz, T., Amoroso, R., and Sanchez, G., *Inactivation of enteric viruses in minimally processed berries and herbs.* Appl Environ Microbiol, 2009. 75(12):4155-61.

20. da Silva, A.K., Le Saux, J.C., Parnaudeau, S., Pommepuy, M., Elimelech, M., and Le Guyader, F.S., *Evaluation of removal of noroviruses during wastewater treatment, using real-time reverse transcription-PCR: different behaviors of genogroups I and II.* Appl Environ Microbiol, 2007. 73(24):7891-7.
21. Hewitt, J., Rivera-Aban, M., and Greening, G.E., *Evaluation of murine norovirus as a surrogate for human norovirus and hepatitis A virus in heat inactivation studies.* J Appl Microbiol, 2009. 107(1):65-71.
22. Berger, C.N., Sodha, S.V., Shaw, R.K., Griffin, P.M., Pink, D., Hand, P., and Frankel, G., *Fresh fruit and vegetables as vehicles for the transmission of human pathogens.* Environ Microbiol, 2010. 12(9):2385-97.
23. Doyle, M.P. and Erickson, M.C., *Summer meeting 2007 - the problems with fresh produce: an overview.* J Appl Microbiol, 2008. 105(2):317-30.
24. Cotterelle, B., Drougard, C., Rolland, J., Becamel, M., Boudon, M., Pinede, S., Traore, O., Balay, K., Pothier, P., and Espie, E., *Outbreak of norovirus infection associated with the consumption of frozen raspberries, France, March 2005.* Euro Surveill, 2005. 10(4):E050428 1.
25. Falkenhorst, G., Krusell, L., Lisby, M., Madsen, S.B., Bottiger, B., and Molbak, K., *Imported frozen raspberries cause a series of norovirus outbreaks in Denmark, 2005.* Euro Surveill, 2005. 10(9):E050922 2.
26. Gaulin, C.D., Ramsay, D., Cardinal, P., and D'Halevyn, M.A., *[Epidemic of gastroenteritis of viral origin associated with eating imported raspberries].* Can J Public Health, 1999. 90(1):37-40.
27. Hjertqvist, M., Johansson, A., Svensson, N., Abom, P.E., Magnusson, C., Olsson, M., Hedlund, K.O., and Andersson, Y., *Four outbreaks of norovirus gastroenteritis after consuming raspberries, Sweden, June-August 2006.* Euro Surveill, 2006. 11(9):E060907 1.
28. Korsager, B., Hede, S., Boggild, H., Bottiger, B.E., and Molbak, K., *Two outbreaks of norovirus infections associated with the consumption of imported frozen raspberries, Denmark, May-June 2005.* Euro Surveill, 2005. 10(6):E050623 1.
29. Le Guyader, F.S., Mittelholzer, C., Haugarreau, L., Hedlund, K.O., Alsterlund, R., Pommepuy, M., and Svensson, L., *Detection of noroviruses in raspberries associated with a gastroenteritis outbreak.* Int J Food Microbiol, 2004. 97(2):179-86.
30. Maunula, L., Roivainen, M., Keranen, M., Makela, S., Soderberg, K., Summa, M., von Bonsdorff, C.H., Lappalainen, M., Korhonen, T., Kuusi, M., and Niskanen, T., *Detection of human norovirus from frozen raspberries in a cluster of gastroenteritis outbreaks.* Euro Surveill, 2009. 14(49).
31. Ponka, A., Maunula, L., von Bonsdorff, C.H., and Lyytikainen, O., *An outbreak of calicivirus associated with consumption of frozen raspberries.* Epidemiol Infect, 1999. 123(3):469-74.
32. Sarvikivi, E., Roivainen, M., Maunula, L., Niskanen, T., Korhonen, T., Lappalainen, M., and Kuusi, M., *Multiple norovirus outbreaks linked to imported frozen raspberries.* Epidemiol Infect, 2011:1-8.
33. FAO, *Food and agriculture organization of the united nations, FAOSTAT.* http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor, 2009.
34. Rutjes, S.A., Lodder-Verschoor, F., van der Poel, W.H., van Duijnhoven, Y.T., and de Roda Husman, A.M., *Detection of noroviruses in foods: a study on virus extraction procedures in foods implicated in outbreaks of human gastroenteritis.* J Food Prot, 2006. 69(8):1949-56.
35. Stals, A., Baert, L., Jasson, V., Van Coillie, E., and Uyttendaele, M., *Screening of fruit products for norovirus and the difficulty of interpreting positive PCR results.* J Food Prot, 2011. 74(3):425-31.
36. Newell, D.G., Koopmans, M., Verhoef, L., Duizer, E., Aidara-Kane, A., Sprong, H., Opsteegh, M., Langelaar, M., Threfall, J., Scheutz, F., van der Giessen, J., and Kruse, H., *Food-borne diseases - the challenges of 20 years ago still persist while new ones continue to emerge.* Int J Food Microbiol, 2010. 139:3-15.
37. Carter, M.J., *Enterically infecting viruses: pathogenicity, transmission and significance for food and waterborne infection.* J Appl Microbiol, 2005. 98(6):1354-80.
38. Havelaar, A.H., Rutjes, S.A., *Risk Assessment of Viruses in Food: Opportunities and Challenges* Food-Borne Viruses Progress and Challenges ed. D.O.C. M.P.G. Koopman, A. Bosch. 2008, Washington, DC: ASM Press.
39. Hundesa, A., Maluquer de Motes, C., Bofill-Mas, S., Albinana-Gimenez, N., and Girones, R., *Identification of human and animal adenoviruses and polyomaviruses for determination of sources of fecal contamination in the environment.* Appl Environ Microbiol, 2006. 72(12):7886-93.

40. Costafreda, M.I., Bosch, A., and Pinto, R.M., *Development, evaluation, and standardization of a real-time TaqMan reverse transcription-PCR assay for quantification of hepatitis A virus in clinical and shellfish samples.* Appl Environ Microbiol, 2006. 72(6):3846-55.
41. Dubois, E., Hennechart, C., Deboosere, N., Merle, G., Legeay, O., Burger, C., Le Calve, M., Lombard, B., Ferre, V., and Traore, O., *Intra-laboratory validation of a concentration method adapted for the enumeration of infectious F-specific RNA coliphage, enterovirus, and hepatitis A virus from inoculated leaves of salad vegetables.* Int J Food Microbiol, 2006. 108(2):164-71.
42. Wyn-Jones, A.P., Carducci, A., Cook, N., D'Agostino, M., Divizia, M., Fleischer, J., Gantzer, C., Gawler, A., Girones, R., Holler, C., de Roda Husman, A.M., Kay, D., Kozyra, I., Lopez-Pila, J., Muscillo, M., Nascimento, M.S., Papageorgiou, G., Rutjes, S., Sellwood, J., Szewzyk, R., and Wyer, M., *Surveillance of adenoviruses and noroviruses in European recreational waters.* Water Res, 2011. 45(3):1025-38.
43. Diez-Valcarce, M., Kovač, K., Cook, N., Rodríguez-Lázaro, D., Hernández, M., *Construction and Analytical Application of Internal Amplification Controls (IAC) for Detection of Food Supply Chain-Relevant Viruses by Real-Time PCR-Based Assays.* Food Analytical Methods 2011. 4(3):437-445.
44. Svraka, S., Duizer, E., Vennema, H., de Bruin, E., van der Veer, B., Dorresteijn, B., and Koopmans, M., *Etiological role of viruses in outbreaks of acute gastroenteritis in The Netherlands from 1994 through 2005.* J Clin Microbiol, 2007. 45(5):1389-94.
45. Loisy, F., Atmar, R.L., Guillon, P., Le Cann, P., Pommepuy, M., and Le Guyader, F.S., *Real-time RT-PCR for norovirus screening in shellfish.* J Virol Methods, 2005. 123(1):1-7.
46. Kageyama, T., Kojima, S., Shinohara, M., Uchida, K., Fukushi, S., Hoshino, F.B., Takeda, N., and Katayama, K., *Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR.* J Clin Microbiol, 2003. 41(4):1548-57.
47. Baert, L., Wobus, C.E., Van Coillie, E., Thackray, L.B., Debevere, J., and Uyttendaele, M., *Detection of murine norovirus 1 by using plaque assay, transfection assay, and real-time reverse transcription-PCR before and after heat exposure.* Appl Environ Microbiol, 2008. 74(2):543-6.
48. Hernroth, B.E., Conden-Hansson, A.C., Rehnstam-Holm, A.S., Girones, R., and Allard, A.K., *Environmental factors influencing human viral pathogens and their potential indicator organisms in the blue mussel, Mytilus edulis: the first Scandinavian report.* Appl Environ Microbiol, 2002. 68(9):4523-33.
49. Bofill-Mas, S., Calgua, B., Clemente-Casares, P., La Rosa, G., Laconelli M., Muscillo, M., Rutjes, S., Roda Husman, A.M., Grunert A., Gräber, I., Verani, M., Carducci, A., Calvo, M., Wyn-Jones, P., Girones,R., *Quantification of Human Adenoviruses in European Recreational Waters* Food and Environmental Virology 2010. 2(1):101–109.
50. Martinez-Martinez, M., Diez-Valcarce, M., Hernandez, M., and Rodriguez-Lazaro, D., *Design and Application of Nucleic Acid Standards for Quantitative Detection of Enteric Viruses by Real-Time PCR.* Food Environ Virol, 2011. 3(2):92-98.
51. de Roda Husman, A.M., Lodder, W.J., Rutjes, S.A., Schijven, J.F., and Teunis, P.F., *Long-term inactivation study of three enteroviruses in artificial surface and groundwaters, using PCR and cell culture.* Appl Environ Microbiol, 2009. 75(4):1050-7.
52. Cerf, O., *Tailing of survival curves of bacterial spores.* J Appl Bacteriol, 1977. 42(1):1-19.
53. Coroller, L., Leguerinel, I., Mettler, E., Savy, N., and Mafart, P., *General model, based on two mixed weibull distributions of bacterial resistance, for describing various shapes of inactivation curves.* Appl Environ Microbiol, 2006. 72(10):6493-502.
54. Gilks, W.R., Richardson, S., and Spiegelhalter, D.J., *Markov Chain Monte Carlo in Practice*. 1996, London: Chapman&Hall.
55. Teunis, P., Takumi, K., and Shinagawa, K., *Dose response for infection by Escherichia coli O157:H7 from outbreak data.* Risk Anal, 2004. 24(2):401-7.
56. Kurdziel, A.S., Wilkinson, N., Langton, S., and Cook, N., *Survival of poliovirus on soft fruit and salad vegetables.* J Food Prot, 2001. 64(5):706-9.

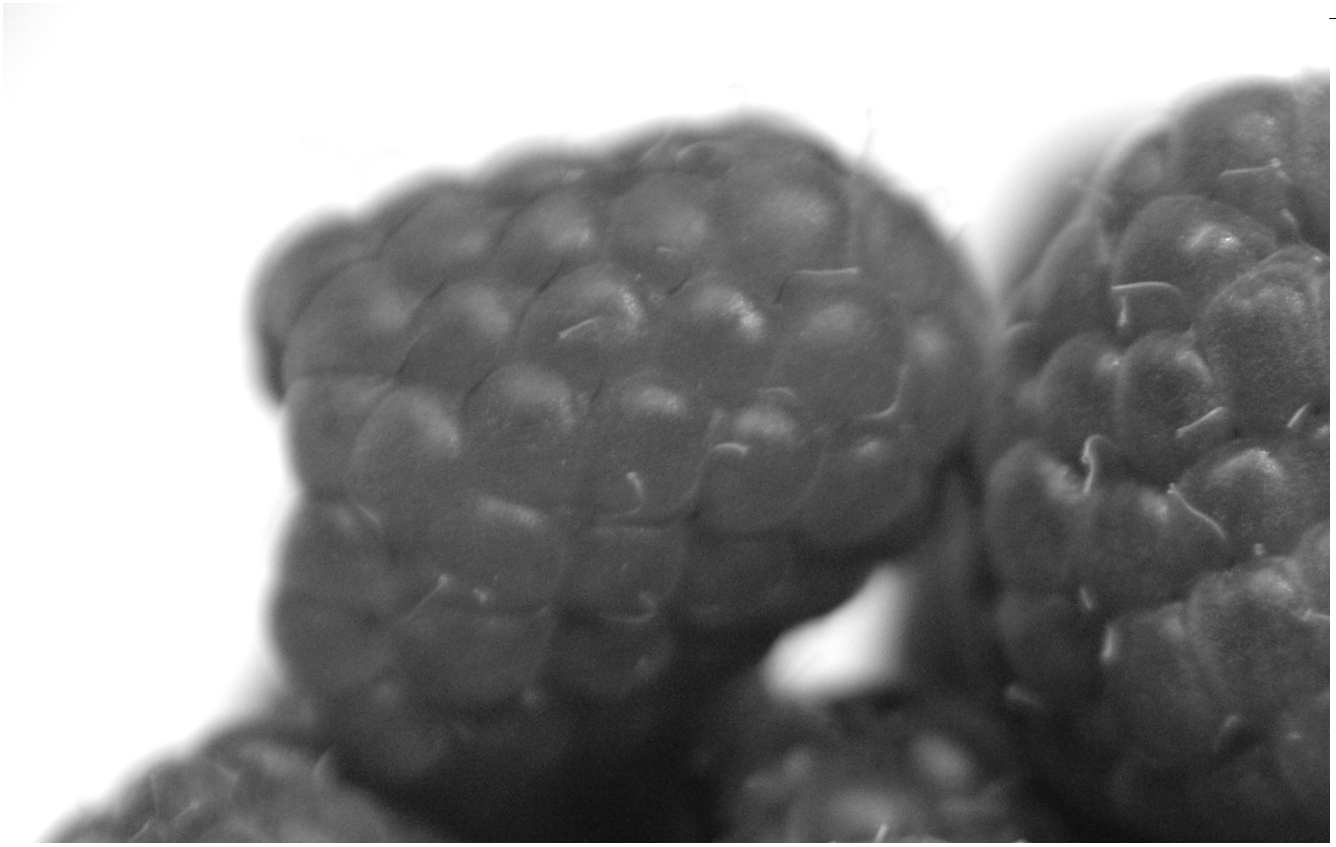
57. Mattison, K., Karthikeyan, K., Abebe, M., Malik, N., Sattar, S.A., Farber, J.M., and Bidawid, S., *Survival of calicivirus in foods and on surfaces: experiments with feline calicivirus as a surrogate for norovirus.* J Food Prot, 2007. 70(2):500-3.

58. Parshionikar, S., Laseke, I., and Fout, G.S., *Use of propidium monoazide in reverse transcriptase PCR to distinguish between infectious and noninfectious enteric viruses in water samples.* Appl Environ Microbiol, 2010. 76(13):4318-26.

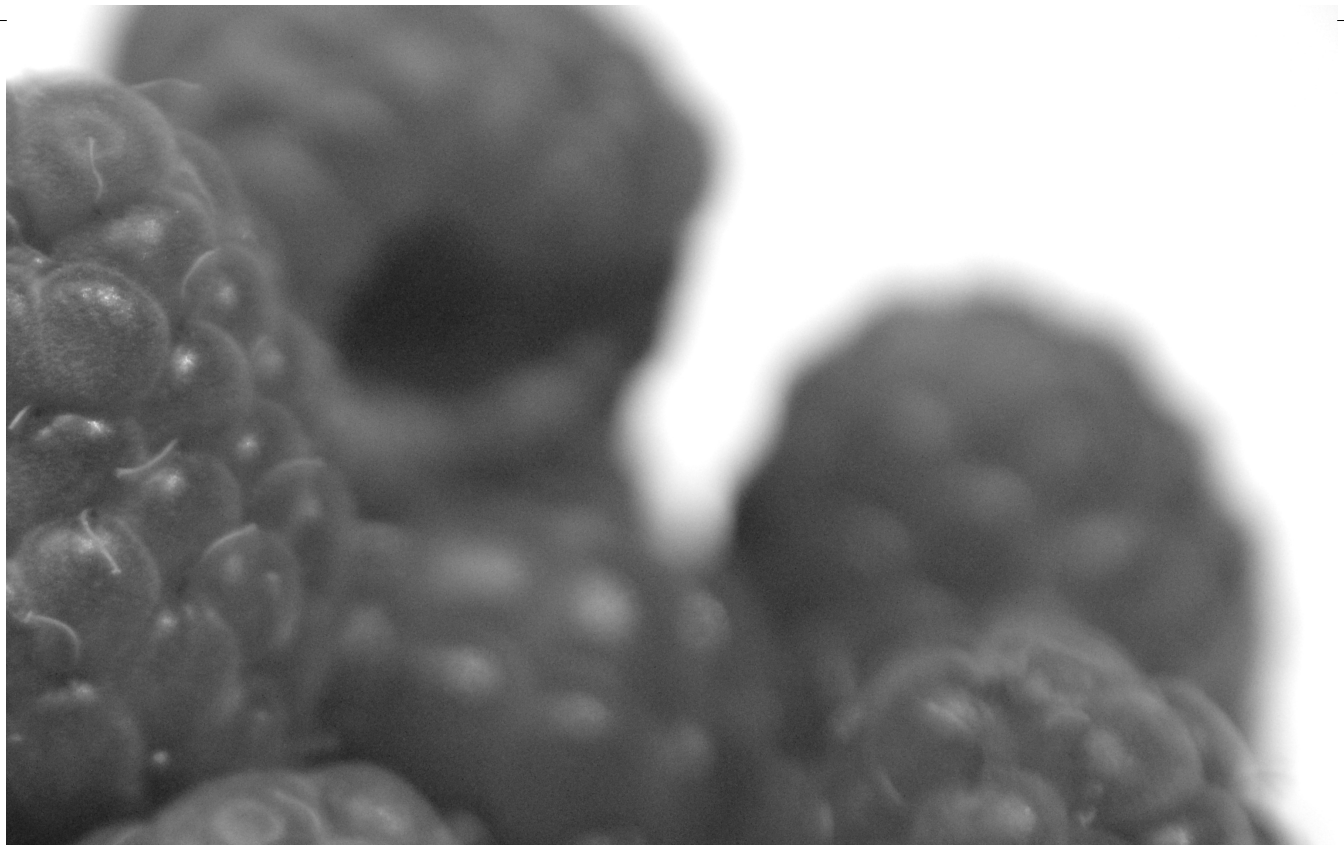
59. Robbins, J., Moore, P.P., and Patterson, M., *Fruit Respiration Rates and Firmness of red Raspberry and related Rubus Genotypes* Acta Horticulturae (ISHS), 1989. 262:311-318.

60. Sy, K.V., McWatters, K.H., and Beuchat, L.R., *Efficacy of gaseous chlorine dioxide as a sanitizer for killing Salmonella, yeasts, and molds on blueberries, strawberries, and raspberries.* J Food Prot, 2005. 68(6):1165-75.

61. Adams, M.R., Hartley, A.D., and Cox, L.J., *Factors affecting the efficacy of washing procedures used in the production of prepared salads.* Food Microbiology, 1989. 6:69–77.



3



Virus transfer proportions between gloved fingertips, soft berries, and lettuce, and associated health risks

Katharina Verhaelen[a,b], Martijn Bouwknegt[a], Anna Carratalà[c,d], Froukje Lodder-Verschoor[a], Marta Diez-Valcarce[e,f], David Rodríguez-Lázaro[e,f], Ana Maria de Roda Husman[a,b], Saskia A. Rutjes[a]

[a]Laboratory for Zoonoses and Environmental Microbiology, Centre for Infectious Disease Control Netherlands, National Institute for Public Health and the Environment (RIVM), P.O. Box 1, NL-3720 BA Bilthoven, The Netherlands
[b]Faculty of Veterinary Medicine, Institute for Risk Assessment Sciences (IRAS), Utrecht University, P.O. Box 80.178, NL-3508 TD Utrecht, The Netherlands.
[c]Department of Microbiology, Faculty of Biology, University of Barcelona, Av. Diagonal 643, 08028 Barcelona, Spain.
[d]Environmental Chemistry Laboratory, École Polytechnique Fédérale de Lausanne, 1015 Lausanne, Switzerland (current affiliation).
[e]Instituto Tecnológico Agrario de Castilla y León (ITACyL), Valladolid, Spain.
[f]Faculty of Science-University of Burgos, Pza. Misael Bañuelos s/n 09001 – Burgos, Spain (current affiliation)

International Journal of Food Microbiology 166 (2013) 419-425

Abstract

Multiple outbreaks of human norovirus (hNoV) have been associated with fresh produce, such as soft berries and lettuce. Even though food handlers are considered an important source for the introduction of hNoV into food chains, their contribution to public health risks associated with hNoV remains unknown. To assess to which extent food handlers contribute to the introduction and spread of hNoV in fresh produce chains quantitative virus transfer data are needed. We estimated transfer proportions of hNoV GI.4, GII.4, murine norovirus (MNV-1), a culturable surrogate of hNoV, and human adenovirus (hAdV-2), a human pathogen proposed as an indicator for human faecal pollution, between gloved fingertips and raspberries, strawberries, and lettuce, by quantitative RT-PCR and cell culture if applicable. Virus transfer proportions were corrected for virus–matrix specific recoveries, and variability and uncertainty of the parameters were estimated. Virus transfer from gloves to soft berries was generally lower as compared to lettuce, with mean transfer proportions ranging between 0.1 to 2.3% and 9 to 10% for infectious MNV-1 and hAdV-2, respectively. Transfer from produce to glove was mostly greater than transfer from glove to produce, adding to the likelihood of virus transfer due to cross contamination from contaminated produce via food handlers. HNoV GI.4 and hNoV GII.4 showed no significant difference between their mean transfer proportions. Using the estimated transfer proportions, we studied the impact of low and high transfer proportions on the public health risk, based on a scenario in which a food handler picked raspberries with contaminated fingertips. Given the made assumptions, we could show that for a pathogen as infectious as hNoV, low transfer proportions may pose a greater public health risk than high transfer proportions, due to a greater viral spread. We demonstrated the potential of food handlers in spreading hNoV in food chains, showing that prevention of virus contamination on food handlers' hands is crucial for food safety. Nevertheless, complete prevention of virus contamination on fresh produce cannot be achieved in reality, and reliable and effective intervention measures are consequently required. We estimated that, especially for low transfer proportions, a robust one $\log_{10}$-unit reduction of infectious hNoV on contaminated produce, and on food handlers' hands, could lower the public health risk substantially. Using the obtained data in quantitative risk assessment will aid in elucidating the contribution of food handlers in hNoV transmission.

Introduction

Human norovirus (hNoV) is the most important foodborne pathogen in terms of disease outbreaks, with an estimated 58% of foodborne outbreaks being associated with this virus in the US [1]. Fresh produce, such as lettuce and soft berries are common vehicles for hNoV transmission [2, 3]. Food handlers are assumed to be the most likely source of hNoV for ready to eat products [3, 4] and in the US, about 50% of hNoV outbreaks have been linked to ill food handlers [5]. Not only the frequency, but also the reported size of outbreaks related to food handlers can be substantial, with up to several thousand infected individuals [6].

The characteristics of hNoV facilitate the spread of the virus via food handlers. HNoV is the most infectious virus described with an estimated average probability of infection for a single specific Norwalk virus particle to be close to 50% [7]. At the same time hNoV is shed in extremely high numbers at a median of 9.5×10^{10} genomic copies/g faeces as measured by quantitative RT-PCR [8]. This means that the transfer of minuscule amounts of faeces can result in hNoV infection upon exposure. In addition, viral shedding preceding the onset of illness in up to 30% of infected persons [9] and a high incidence of asymptomatic infections [10, 11] make it virtually impossible to exclude food handlers shedding viruses from food production processes. These hNoV characteristics may explain why infected food handlers were more commonly identified in food-related hNoV outbreaks than in those caused by other pathogens [12]. To date, hNoV cannot be cultured efficiently and infectivity has to be studied using surrogate viruses. Previously feline and canine caliciviruses were used, but currently a cultivable norovirus, murine norovirus MNV-1 is used, which resembles more closely the properties of hNoV [13]. Food handlers can contaminate produce in the (i) primary production phase e.g. at harvest, in the (ii) processing phase e.g. while sorting and packaging produce or in the (iii) food preparation phase e.g. preparing desserts or salads. They can contribute to the spread of hNoV either by introducing the virus onto food via poor hand hygiene, or by cross-contamination while handling contaminated food. The contribution of food handlers in the introduction and spread of hNoV in fresh produce chains and the associated health risk are difficult to assess. To determine a possible link between hNoV illness and a specific food item is intricate; attributing additionally the contamination source of this food item to the outbreak is a major challenge.

Quantitative microbial risk assessment (QMRA) can be used to prioritize the contribution of pathogen contamination sources to the overall food contamination and [14] can be applied to elucidate the role of food handlers in hNoV transmission. For this purpose, quantitative data on transfer coefficients of hNoV from finger pads to produce are needed and additionally from produce to finger pads to study the effects of possible

cross-contamination. Only two recent studies have described the transfer of noroviruses from gloved fingertips to fresh produce [15, 16]. However, besides studying different virus–matrix combinations, we also estimated the uncertainty and variability of the determined transfer proportions, crucial aspects for an accurate description of a public health risk by QMRA [17].

We quantified the transfer proportions of hNoV GI.4, hNoV GII.4, murine norovirus (MNV-1) and human adenovirus (hAdV-2) between gloved fingertips and raspberries, strawberries and lettuce and between raspberries and lettuce and gloved fingertips. Human adenovirus was included, because the virus is suggested as an indicator for viral contamination [18] and is furthermore a potential foodborne pathogen. Virus numbers were determined by molecular techniques and cell culture if possible. The obtained data on transfer proportions and their variations were used to estimate the number of produce a single food handler can contaminate and the corresponding contamination levels of viruses per food item, comparing a low and a high transfer proportion. Relating these data to dose–response models further allowed us to determine associated health risks.

Materials and methods

Viruses and cells

Two human norovirus strains, one belonging to genogroup I (hNoV GI.4) and one to genogroup II (hNoV GII.4), originating from clinical stool samples were kindly provided by Dr. Erwin Duizer (Laboratory for Infectious Diseases and Perinatal Screening RIVM, The Netherlands). Murine norovirus (MNV-1) (kindly provided by Dr. Herbert W. Virgin, Washington University, St. Louis, USA) and human adenovirus (hAdV-2) were made available, under agreement by the group of Dr. Franco M. Ruggeri (Istituto Superiore de Sanitá, Rome, Italy). Recombinant mengovirus (vMC_0) [19], kindly provided by Dr. David Lees (CEFAS, Dorset, UK) was used as a process control virus to monitor the efficiency of virus recovery.MNV-1was propagated in RAW-264.7 cells (ATCC-TIB-71) and hAdV-2 in A549 cells (ATTC-CCL-185) (for details see Verhaelen et al., 2012).

Virus spiking

Virus transfer from finger pads to fresh produce was studied using gloves (VWR, Nitrile), because gloves are commonly used by food handlers in food environments and because spiking unprotected fingertips with human pathogenic viruses entails a health risk. In total 50 μL of a virus suspension, consisting of hNoV GI.4, hNoV GII.4 (10% faecal suspension) and MNV-1, hAdV-2 (neat stock suspension), were spiked on the middle finger, index finger and thumb of a glove in a marked area of about 1 cm2. The spike contained about 2×10^8, 4×10^8, 7×10^8 and 2×10^8 genomic copies of hAdV-2,

MNV-1, hNoV GI and GII, respectively. The number of genomic copies of hAdV-2 and MNV- 1 related to 5×10^6 infectious hAdV-2 particles and 5×10^4 infectious MNV particles, respectively. The inoculum was left to dry at room temperature for 2 h, until visibly dry. To study virus transfer from produce to gloves, raspberries were sliced into half and the outer surfaces were evenly spiked with 150 µL of the same virus suspension aiming for a homogenous distribution of virus particles on the raspberry surfaces. Halving the raspberry allowed a more consistent spiking of the berry. Transfer from lettuce to gloves was studied by spiking a 4 cm^2 piece of iceberg lettuce in the same manner. The inoculum was left to dry for 4 h, until visibly dry.

Virus transfer

Here, we describe the methodology of virus transfer from gloved finger tips to produce and vice versa. Raspberries and strawberries were picked up with gloved fingers using the middle finger, index finger and thumb and were transferred into 20 mL of tris-glycine beef extract buffer (TGBE) (1.21% Tris base, 0.38% glycine and 1% beef extract, (pH 9.5) after holding it for 5 s. Lettuce was treated in the same manner for comparability of the transfer proportions. The gloves were carefully pulled off the hand and the spiked area on the three finger tips was cut out with a sterile scalpel and transferred into a 50 mL Greiner tube containing 5 mL of TGBE buffer. The experiment was successively repeated ten times for each transfer route on the same day. Similarly, in experiments on virus transfer from produce to glove, the spiked produce was touched successively with the index finger, middle finger and thumb for 5 s. The three glove pieces and the three raspberry halves or iceberg lettuce pieces were pooled in 5 and 20 mL of TGBE, respectively. The experiment was successively repeated ten times for each transfer route on the same day. For strawberries, only transfer from glove to strawberry was studied to elaborate whether a difference in virus transfer from food handlers to strawberries and raspberries may explain why outbreaks of hNoV are more frequently linked to raspberries.

Measurement of the donor and recipient surface

For transfer of viruses from glove to produce, the complete spiked area of the glove was in touch with the produce. However, for transfer from produce to gloves, the area touched by the glove was smaller than the spiked area of the produce, leading to an underestimation of the transfer rate. Therefore, the surface fraction of the produce covered by the glove was determined to correct the number of spiked viruses, assuming a homogenous distribution of virus particles. To determine the surface of a raspberry, the surface of in total nine raspberries was measured by incising the berry and flattening it gently on graph paper to establish the covered area. A linear relation of raspberry surface and weight was determined allowing the measurement of the surface of a raspberry by its weight. For lettuce a 4 cm^2 leaf was cut out as a defined lettuce surface.

To measure the area of a finger in touch with a raspberry, the berry was inked and touched with a glove. The tip of the glove (with a stain representing the touched area) was cut off, and a picture was taken containing a reference point with a known surface. Thereafter the pictures were exported into Matlab (Matlab R2012B, TheMathWorks), pixels were binarized based on the colour contrast, and the average surface area of the stain on the glove was calculated based on ten measurements. Unlike raspberries, lettuce has a smooth surface. The surface of a fingertip in touch with a piece of lettuce could thus be simply determined by pressing the thumb, indicator and middle finger on graph paper and establishing the covered area (performed in sextuplicate).

Virus elution

Elution of hNoV GI.4, hNoV GII.4, MNV-1 and hAdV-2 from the spiked produce was performed as previously described [20]. Briefly, the viruses were eluted by rotation at 45 rpm in 20 mL TGBE buffer. As a process control, 20 µl (C_q(quantification cycle) of about 31) of vMC_0 was added to the TGBE. Consequently, virus particles were concentrated with polyethyleneglycol at a pH of 7.2. The final concentrates were split into two, and the subsample used for cell culture was filter sterilized using 0.22 µm syringe driven filters and a chloroform:butanol extraction was performed on the subsample used for molecular detection. To elute the viruses from gloves, gloves were rotated for 30 min at 45 rpm in 5 mL TGBE, containing 20 µL of the process control virus. Thereafter the tubes were centrifuged for 5 min at 2000 g to spin down the liquid. The liquid was then transferred into a new tube and the pH of the sample was adjusted to about 7 using HCl. Samples were stored at -80 °C.

Estimation of MNV-1 titer

Endpoint dilutions were performed to determine the titer of infective MNV-1 particles (see Verhaelen et al., 2012). Briefly, 96 well plates (Corning, The Netherlands) were seeded with 100 µL RAW- 265.7 cells in maintenance medium containing 2% fetal calf serum (FCS) at a concentration of 4×10^5 cells/mL. Eight serial 0.5 $\log_{10}$-unit dilutions of each sample were prepared in the medium without FCS. Each dilution was analysed in 10 fold adding 100 µL of the sample dilution per well. Plates were incubated for 7 days at 37 °C with 5% CO_2 and inspected for cytopathic effect.

Estimation of hAdV-2 titer

Infectivity of hAdV-2 was determined using a suspension plaque assay [21]. In short, 5 mL of A549 cells at a concentration of 6×10^5 cells/mL was seeded into 25 cm^2 vented cell culture flasks (Corning). Samples were diluted in PBS and 100 µL of sample was added to the cell suspension. Samples were analysed in different dilutions in at least quadruplicate. After an incubation period of 4 h at 37 °C with 5% CO_2, 5 mL of over-

laying medium (including 2% FCS (Gibco), 1.5% carboxymethylcellulose (Sigma)) was added and the flasks were incubated for 7 days at 37 °C with 5% CO_2. Subsequently, cells were fixed with formalin and afterwards stained with crystal violet (Sigma) to visualise the plaques.

Molecular detection

Nucleic acids were extracted using the NucliSens miniMag magnetic extraction kit (bioMérieux, TheNetherlands) following the manufacturer's instructions with minor modifications. In each extraction cycle, a negative control without target was included. The previously included vMC_0 served as a positive control. A Lightcycler 480 (Roche Diagnostics, Almere, The Netherlands) was used for real time PCR, using TaqMan hydrolysis probes. The primers and probes used, and PCR conditions were as previously described [20]. For detection of norovirus RNA the UltraSense One-Step Quantitative RT-PCR System (Invitrogen) and for hAdV-2 the TaqMan Universal PCR Master Mix (Applied Biosystems) were used. To control for PCR inhibition, a competitive internal amplification control (IAC) (Yorkshire Bioscience Ltd., UK), was added to each reaction at a concentration that was previously tested to not influence the detection of the target signal [22]. Virus quantification was performed using standard curves. For the construction of the hNoV GI, hNoV GII and MNV standard, the plasmid pCR 2.1-TOPO (Invitrogen) was used containing synthetic DNA molecules of the three target sequences of the qRT-PCR [23]. Plasmids were kindly provided by David Rodriguez-Lázaro (Instituto Tecnológico Agrario de Castilla y León (ITACyL), Spain). Tenfold dilution of the standards were included in each PCR run. For further details read Verhaelen et al. (2012).

Statistical analysis

Numbers of infectious virus particles and genomes were estimated by maximum likelihood. Virus counts were included using the presence/absence profile for MNV-1 and the plaque assay counts for hAdV-2 assuming homogeneous ('Poisson') mixing. For genome counts based on RT-PCR, the intercept and slope parameter of the standard curve were estimated simultaneously with the genome count using a joint likelihood function. Genomic copies were considered to be lognormally distributed. Virus transfer was expressed as the proportion of viruses (f) transferred from a recipient surface (N_R) relative to the sum of viruses on the donor surface (N_D) [24, 25] adjusting for the different virus recoveries from the surfaces:

$$f = \frac{N_R}{N_R + N_D} \tag{1}$$

The donor surface refers to the surface spiked with viruses and the recipient surface refers to the surface touched by the donor surface. The virus recovery was determined for each virus–matrix combination. To this end, the number of viruses that was detected by PCR or infectivity assays after drying of the virus on a certain matrix was divided by the number of virus particles initially spiked on the particular matrix determined in the spike suspension. Raspberries and strawberries were previously found to have similar recoveries [26] and therefore only the recovery of raspberries was determined and used for both berries.

The transfer proportions and recoveries were fitted to three beta distributions with a joint likelihood function to assess their variability. The parameters α and β of the beta distribution were re-parameterized to represent the mean transferred proportion of viruses, μ, and the associated precision, φ, where $\alpha = \mu\varphi$ and $\beta = (1 - \mu)\varphi$. The parameter φ describes the variability in transferred proportion, with a larger value indicating less variability (i.e., a less wide beta-distribution) and vice versa. The uncertainty of the transfer proportion was assessed by adaptive rejection Markov Chain Monte Carlo (MCMC) sampling from the likelihood function using the Metropolis–Hästings algorithm [27, 28]. The data analysis was performed using Mathematica 8.0 (Wolfram, Champaign, IL, USA).

Statistical differences among the proportion of viruses transferred between different donor and recipient surfaces were assessed using beta regression, using the re-parameterization of α and β as described above [29, 30]. The response variable was the transferred proportion, and covariates were the virus type and the transfer route. The parameters μ and φ were modelled as linear functions of the predictor variables and related to the response variable through a logit- and log-link function, respectively. The contribution of model terms to the overall model fit and the inclusion of φ as single parameter or as function of explanatory variables were assessed using the likelihood ratio test with $\alpha = 0.05$, using backward elimination of variables. Interaction terms were tested for the remaining variables. These analyses were done in R (version 2.15) with the betareg-package. Three separate models were analysed. The first model considered differences between transferred proportion for hAdV-2 and MNV-1 determined by cell culture data, including 'virus' and 'transfer route' as explanatory variables. The second model considered the transferred proportion as determined by PCR data and included 'virus' and 'transfer route' as explanatory variables. The third model was a general norovirus model on all RT-PCR data forMNV-1, hNoV GI and hNoV GII. This model was included, because it can be used by risk assessors to perform a general norovirus risk assessment, necessary because mostly data are missing to perform genotype specific risk assessments, even though relevant due to the many differences in e.g. virus pathogenicity. The average estimated transferred fraction (μ) between the three noroviruses was not significantly different ($p = 0.12$), which is why the data was pooled.

Quantifying viral contamination levels and related health risks

The impact of transfer proportion on the number of contaminated produce, the corresponding contamination levels on the produce, and the related public health risk were studied comparing a low and a high transfer proportion. The estimations were based on a scenario in which a food handler harvested raspberries with contaminated fingertips, by sequentially picking the fruits. The number of viruses on the recipient surface N_R and on the donor surface N_D is described by the following equations:

$$N_R = f \cdot (1-f)^{i-1} N_0 \tag{2}$$

$$N_D = (1-f)^i N_0 \tag{3}$$

with N_0 being the initial number of viruses on the donor surface, f being the transfer probability and i being the number of transfer events. In the studied scenario, we chose raspberries as an example; however, the obtained results are not specific for raspberries and hold as well for other food items. As initial virus concentration N_0 1000 virus particles were chosen, which is based on the high virus load per gram of faeces shed by an infected human, relatively low. As transfer proportion the lowest and the highest estimated transfer proportion for transfer from gloves to produce of the general norovirus model were used. The transfer proportions were considered independent of the virus concentration. Furthermore, viruses were assumed to not be aggregated and cross-contamination between raspberries was ignored. The effect on public health of low and high transfer proportions following the consumption of contaminated berries was estimated by Monte Carlo simulation using the variability distribution of the transfer proportions. A 1000 simulations with each 1000 simulated sequentially picked raspberries were completed for initial hNoV concentrations ranging from 50 to 1,000,000 particles per hand. Raspberries were assumed to be consumed independently, and the probability of infection per raspberry was estimated using the hNoV dose–response model [7]. The total number of expected infections was calculated by summing the probabilities of infection per simulation (thus yielding a variability distribution with a 1000 estimated numbers of infections). Viruses on the berries were assumed to remain infectious until consumption, a plausible assumption if one infers from the results of previous studies on the persistence of noroviruses on raspberries [20, 31]. Moreover, we assumed that one infectious virus particle is equal to one dose-unit as used in the dose–response model of Teunis et al. (2008).

Results

We estimated transfer proportions of hNoV GII.4, hNoV GI.4, MNV-1 and hAdV-2 from gloved fingertips to soft berries and lettuce and vice versa, while accounting for the corresponding virus–matrix specific recoveries. The recoveries are presented in Table 1 and the mean viral transfer proportions and their confidence intervals are presented in Tables 2 and 3. Moreover, the possible virus distribution on raspberries due to contamination by a food handler based on the estimated transfer proportions was determined using 'high' (9.8%) and 'low' (0.2%) transfer proportions (Fig. 1). The estimated contamination levels were correlated to a dose–response model to determine the effect of transfer proportions on a potential health risk (Fig. 2).

Virus recoveries

The estimated recoveries differed depending on the type of virus, the detection method and the matrices (Table 1). Relatively small 95% intervals suggest a good reproducibility of the used elution procedure for the four different viruses from lettuce, berries, and gloves as detected by PCR or culture, except for hAdV-2 genomes from gloves. Overall, virus recovery from gloves and lettuce was more efficient as compared with soft berries, although this difference was less pronounced for the recovery of viral genomes as compared to infectious viruses. Recovery of hAdV-2 was higher as compared to the different noroviruses tested by either detection method, except for recovery from lettuce by PCR. MNV-1, hNoV GI.4 and hNoV GII.4 were recovered in similar percentages of 20 to 30% from gloves. However, recoveries of noroviruses from lettuce and soft berries were more diverse, whereby hNoV GI.4 behaved similar to MNV-1. HNoV GII.4 recovery was lower as compared to MNV-1 and hNoV GI.4, especially from lettuce.

Table 1. Virus recoveries in percentage and the corresponding 95 % variability intervals based on n = 10 repetitions for fresh produce and n = 30 for gloves.

Matrix	Virus	Recovery PCR [%]	Recovery cell culture [%]
Lettuce	hAdV-2	0.4 (0.2 – 0.7)	37 (22 – 54)
	MNV-1	12 (8 – 17)	6 (2.8 – 11)
	hNoV GI.4	29 (22 – 37)	n.a.*
	hNoV GII.4	2 (0.5 – 4.6)	n.a.
Raspberry	hAdV-2	19 (7 – 35)	2.5 (1.6 – 3.5)
	MNV-1	12 (5 – 21)	0.9 (0.1 – 2.8)
	hNoV GI.4	8.3 (3.5 – 15)	n.a.
	hNoV GII.4	1.2 (0.1 – 3.3)	n.a.
Glove	hAdV-2	68 (1 – 100)	32 (16 – 51)
	MNV-1	20 (4 – 45)	5.6 (1.0 – 14)
	hNoV GI.4	29 (10 – 54)	n.a.
	hNoV GII.4	20 (7.4 – 36)	n.a.

* n.a. = not applicable

Table 2. Estimated mean percentages (μ) of transferred viruses, the associated 95% confidence interval, and the variability parameter φ for infectivity data.

Virus	Transfer route									
	Glove to raspberry		Glove to strawberry		Glove to lettuce		Raspberry to glove		Lettuce to glove	
	μ [%]	φ	μ [%]	φ	μ [%]	φ	μ [%]	φ	μ [%]	φ
hAdV-2	0.1 (0.03 – 0.1)	2759	0.2 (0.07 – 0.3)	860	13 (9 – 18)	204	10 (6.0 – 15)	26	6.0 (3.9 – 9.0)	88
MNV-1	2.3 (0.1 – 9.7)	7.6	0.1 (0.02 – 0.3)	1087	8 (1.5 – 14)	26	6.1 (<0.001 – 9)	25	4.8 (2.5 – 8.0)	60

Table 3. Estimated mean percentages (μ) of transferred viruses, the associated 95% confidence interval, and the variability parameter φ for PCR data.

Virus	Transfer route									
	Glove to raspberry		Glove to strawberry		Glove to lettuce		Raspberry to glove		Lettuce to glove	
	μ [%]	φ	μ [%]	φ	μ [%]	φ	μ [%]	φ	μ [%]	φ
hAdV-2	0.01 (0.004 – 0.04)	2484	0.1 (0.04 – 0.14)	5898	25 (14 – 35)	28	27 (17 – 40)	10	3.0 (1.5 – 4.5)	19584
MNV-1	0.1 (0.02 – 0.15)	1568	0.5 (0.2 – 1)	554	18 (11 – 28)	15	11 (6.6 – 16)	73	23 (17 – 30)	39
hNoV GI	0.2 (0.02 – 0.5)	408	0.4 (0.1 – 0.5)	2948	4 (2.6 – 5.7)	104	16 (9.0 – 23)	18	23 (18 – 30)	38
hNoV GII	0.15 (< 0.001 – 1.0)*	215	2.6 (0.5 – 6.4)*	102	5.7 (0.9 – 15)*	40	15 (3.0 – 28)	15	27 (17 – 39)	23
General NoV model**	0.2 (<0.001 – 0.8)	339	1.3 (0.1 – 4.1)	112	9.8 (0.5 – 31)	13	18 (3.6 – 39)	15	26 (12 – 44)	27

* The joint likelihood function did not converge for these transfer routes and therefore the estimated transfer proportions were based on a model without variability of the recovery included.
**Model forced through data for noroviruses with 'transfer route' as explanatory variable.

Transfer proportions

C_q values of mengovirus, included to monitor the elution procedure and the nucleic acid extractions were constant, showing the consistency of the used procedures (data not shown). Table 2 lists the estimated transfer proportions of infectious virus particles and Table 3 of viral genomes and the general norovirus model. Virus transfer was overall greater from glove to lettuce as compared to transfer from glove to soft berries. Transfer from gloves to raspberries and strawberries as detected by PCR or cell culture was characterised mostly by transfer proportions of less than 1%. Mean transfer proportions of hAdV-2, MNV-1, hNoV GI.4 and hNoV GII.4 genomes from gloves to lettuce varied between 4 and 25% and between 8 and 13% for infectious MNV-1 and hAdV-2. Whereas transfer of infectious MNV-1 and hAdV-2 from gloves to raspberries was considerably lower as compared to transfer from raspberries to glove, transfer of these viruses from glove to lettuce was slightly higher as compared to transfer from lettuce to glove. By PCR, transfer from produce to glove was overall higher as compared to transfer from glove to produce. Transfer of viral genomes was mostly similar or higher as compared with transfer of infectious viruses. The regression model for hAdV-2 and MNV-1 data indicated that the transferred proportion of infectious hAdV-2 did not differ significantly from that of infectious MNV-1, but did differ between transfer routes ($p < 0.001$). The precision parameter differed significantly among transfer routes for infectivity data. The regression model for PCR data showed that transferred proportions differed significantly between viruses and transfer route ($p < 0.001$). Also their interaction term contributed significantly to the model fit ($p < 0.001$), indicating that the difference in transferred proportion among the viruses was not constant over the transfer routes (e.g., the difference in transfer proportions between hAdV-2 and MNV-1 for glove to lettuce may be different in magnitude than the difference for transfer from lettuce to glove). The precision parameter differed significantly among transfer routes and between the viruses for PCR data.

Quantifying viral contamination levels and related health risks

Based on the described scenario, the virus number varied between 1 and 623 virus particles per raspberry for the high transfer rate (9.8%) and an initial concentration of 1000 hNoV particles per hand (Fig. 1). On average 46 raspberries or approximately 200 g (weight of a raspberry approximately 4 g) were contaminated (Fig. 1) and were estimated to lead to approximately 20 infections on average (Fig. 2). Of these raspberries on average 27 (18–37) (about 60%) were contaminated with ≤10 virus particles. For a low transfer rate, the virus concentration per raspberry varied between 1 and 27 virus particles for the initial concentration of 1000 NoV particles per hand (Fig. 1). On average 366 raspberries, or approximately 1.5 kg, were contaminated, of which on average 364 (341–387) berries (about 99%) were contaminated with ≤10 virus particles.

The contaminations were estimated to lead to approximately 125 infections (Fig. 2). An intervention measure achieving a 1 $\log_{10}$-unit reduction of infectious virus particles on food handlers' hands resulted in an estimated reduction of about 10 and 100 infected individuals for a high and a low transfer rate, respectively, for initial virus concentrations of 1000 up to 10^6 particles (Fig. 2).

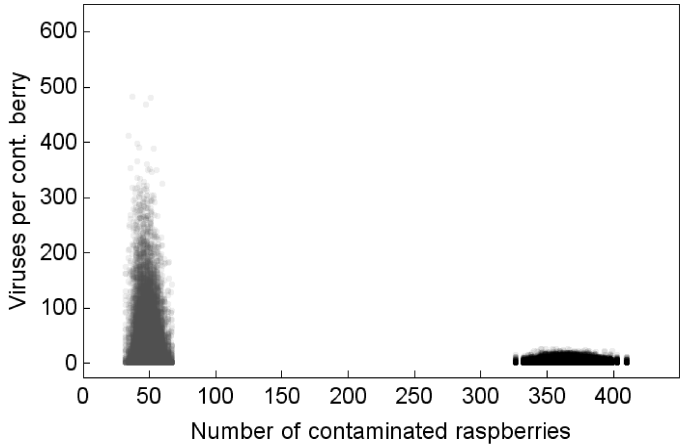


Figure 1. Number of estimated contaminated raspberries and the corresponding virus level based on a transfer proportion of 0.2% (black) and 9.8% (grey). The colour intensity of the plot markers reflects the frequency of the occurrence of certain virus concentrations. Infrequent concentrations are thus not visible. The whole range of contamination levels is stated it the Results section.

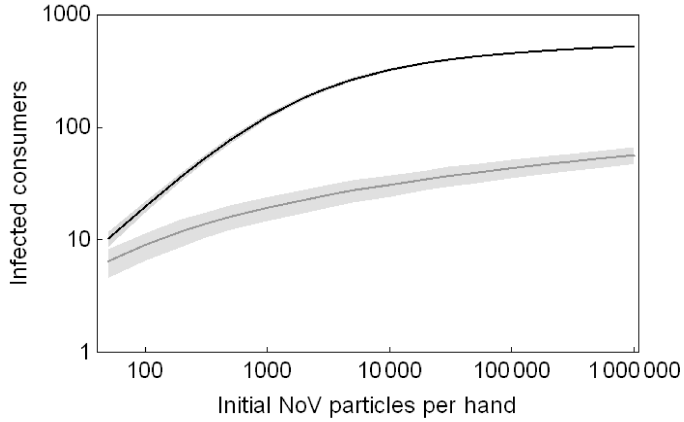


Figure 2. Number of infected individuals at low (0.2%; black) and high (9.8%; grey) transfer proportions depending on the initial hNoV concentration on the food handlers' hands.

Discussion

Contamination sources of hNoV in food chains are poorly studied, although knowledge on the relevance of these is crucial to implement effective prevention and intervention measures by risk managers. Food handlers are believed to play a significant role in hNoV introduction, but to which extent is yet unclear. Based on the estimated transfer proportions, we showed the potential of food handlers in spreading hNoV in food chains and consequent health risks, illustrating the need to focus food safety concepts on food handlers.

We demonstrated that viruses were transferred at different levels from gloved fingertips to produce, whereby viruses were more readily transferred between fingertips and lettuce as compared to fingertips and soft berries. A reason for this may be the difference in the applied pressure, as pressure is described to have a significant impact on transfer proportions [24]. Soft berries are very perishable and the applied pressure to pick a raspberry is considerably lower as compared to lettuce. As in other studies, the type of virus and the route of transfer were found to affect the transferred virus proportions [15, 24, 25, 32-34]. However, in our study there was overall no clear trend observable between the type of virus and the ease of virus transfer, and the transfer proportions were less affected by the virus type as compared to the transfer route. Unlike noroviruses which are small (28–35 nm) ss-RNA viruses, hAdV-2 is a medium sized virus of 70–90 nm composed of a ds-DNA genome and a capsid that possesses special fibre proteins. Nevertheless, comparing viral transfer of infectious hAdV-2 and MNV-1 showed no significant difference in transferred virus proportions.

Comparing our data to the two other publications on transfer proportions of hNoV from fingertips to fresh produce, similar transfer proportions were found. No other published study describes the transfer of infectious virus particles to soft berries, but Sharps et al. (2012) studied the transfer of hNoV GI.3b, hNoVGII.4 andMNV-1 genomes from gloved fingertips to raspberries and observed as well small transfer proportions with little, statistically not significant, differences between the viruses. Stals et al. (2013) studied the transfer of infectious MNV-1 and hNoV GII.4 genomes from gloved fingertips to lettuce and vice versa. The obtained results are generally within the estimated confidence intervals of our study, albeit that transfer of hNoV GII.4 from gloves to lettuce is markedly lower in their study. None of the published studies on virus transfer, including those performed on transfer of other viruses than hNoV between fingertips and fresh produce [32, 35], included the variability of virus–matrix specific recoveries and the variability and uncertainty of the estimated transfer proportions, as done in our study. However, Nauta (2010) states explicitly the necessity of doing so, and showed that a separation of uncertainty and variability of model parameters is crucial for an accurate

determination of the public health risk by QMRA, and illustrated that a major outbreak may be overlooked if the distinction between uncertainty and variability is neglected.

Using the estimated transfer proportions of viruses to produce, we illustrated their impact on the public health risk. Intuitively one may expect that a high transfer proportion results in a greater public health risk, because more viruses are introduced onto the food. However, that also means that fewer viruses remain on the food handlers' hands that can potentially be transmitted to the next food item. Low transfer proportions are characterised by a larger number of contaminated produce at lower contamination levels as compared to a high transfer proportion. The impact of hNoV transfer at low levels due to food handlers on public health is highlighted in a study of de Wit et al. (2007) where an infected baker contaminated rolls, which were served on a buffet lunch of a department resulting in 231 people to be ill out of 800 to 900 employees attending the lunch. In this study, a clear association of illness with the number of consumed rolls was shown, indicating that viruses were transferred in low numbers per roll to a large number of rolls. In the present study, the impact of transfer proportions on public health risk was described by a scenario in which a food handler picked raspberries with contaminated fingertips, transferring a low and a high virus proportion. Based on an initial hNoV count of 1000 virus particles on the food handlers' hands and the made assumptions, we estimated that the amount of raspberries contaminated by a food handler, was nearly eight times higher for the low transfer proportion (0.2%) as compared to the high transfer proportion (9.8%), resulting in a greater exposure of the consumer. For pathogens such as bacteria, which are generally characterised by a low probability of infection per cell, a greater spread may not necessarily be related to a higher public health risk. However, for a pathogen as infectious as hNoV causing infection at very low quantities [7], we showed that a low transfer proportion increased the estimated public health risk substantially as compared to a high transfer proportion, with about seven times more infected individuals, given the associated assumptions. One assumption is the transfer of single virus particles, which is unlikely to occur, because virus particles from aggregates [7]. This assumption may lead to an overestimation of the number of contaminated produce. Another assumption relates to a transfer proportion that is independent of the initial virus concentration. Stals et al. (2013) suggested a possible effect of virus concentration on transfer proportions. In contrast, the study of Julian et al. (2010) concluded that transfer proportions are not influenced by the virus concentration. Although the absolute value of the estimated risks might change when using concentration-dependent transfer proportions, the conclusion that low transfer proportions lead to a larger number of contaminated produce and number of infections holds.

Whereas low transfer proportions from hands to produce are likely of greater importance to public health, the opposite holds for cross contamination from a contaminated produce item to an uncontaminated item via hands. The higher the transfer, the more viruses are transferred to the food handlers' hands on a single contact event that can subsequently be distributed over several potentially uncontaminated food items. Our observation of the combination of a generally higher transfer proportion for NoV from produce to hands, and a lower transfer proportion from hands to produce (except for lettuce) add to the likelihood of virus transfer due to cross contamination.

Moreover, our results give an indication of the contamination levels that can be expected if food handlers are the source of viral contamination. Data on virus levels on fresh produce are essential to get a better idea on the efficacy of intervention measures necessary to reduce the virus load on produce to an acceptable level. Yet, information on virus levels on food items is scarce and low contamination levels, even though likely to cause disease, may not be detected given the relatively low recoveries of the present virus detection protocols and the possible heterogeneous distribution of virus particles in food batches. Based on the estimated distribution of virus particles on produce, we can infer that an intervention measure reducing the load of infectious virus particles on contaminated produce by 1 $\log_{10}$-unit can reduce the exposure of consumers considerably, especially in case of low transfer proportions. In addition, we estimated that under the given assumptions of the studied scenario, a 1 $\log_{10}$-unit reduction of infectious virus particles on food handlers' hands, achieved by e.g. hand washing, decreased the health risk of consumers. However, the compliance of hand washing is a challenge in practice [37]. The exclusion of food handlers shedding hNoV would be certainly the most efficient prevention measure to reduce the introduction of hNoV into food chains by food handlers. However, this approach seems not feasible, because infections may be asymptomatic and food handlers cannot be excluded from work for the whole period in which viruses may be shed [38].

Summarising, this study provides data for quantitative microbial risk assessment on transfer proportions of viruses from fingertips to estimates. We showed that for pathogens as infectious as hNoV, low transfer proportions could pose a greater public health risk as compared with high transfer proportions. This may be one reason, why especially soft berries are frequently associated with outbreaks of hNoV disease. We demonstrated that food handlers can spread hNoV to a large number of food items, making prevention of virus contamination on food handlers' hands crucial for food safety. Nevertheless, complete prevention is unlikely to be achieved in practice, giving the need of reliable and effective intervention measures. Here we showed that especially in case of low transfer proportions as expected for soft berries, interventions achieving a 1 $\log_{10}$-unit

reduction of infectious hNoV on the food handlers' hands or on contaminated produce, may lower the public health risk substantially.

Acknowledgements

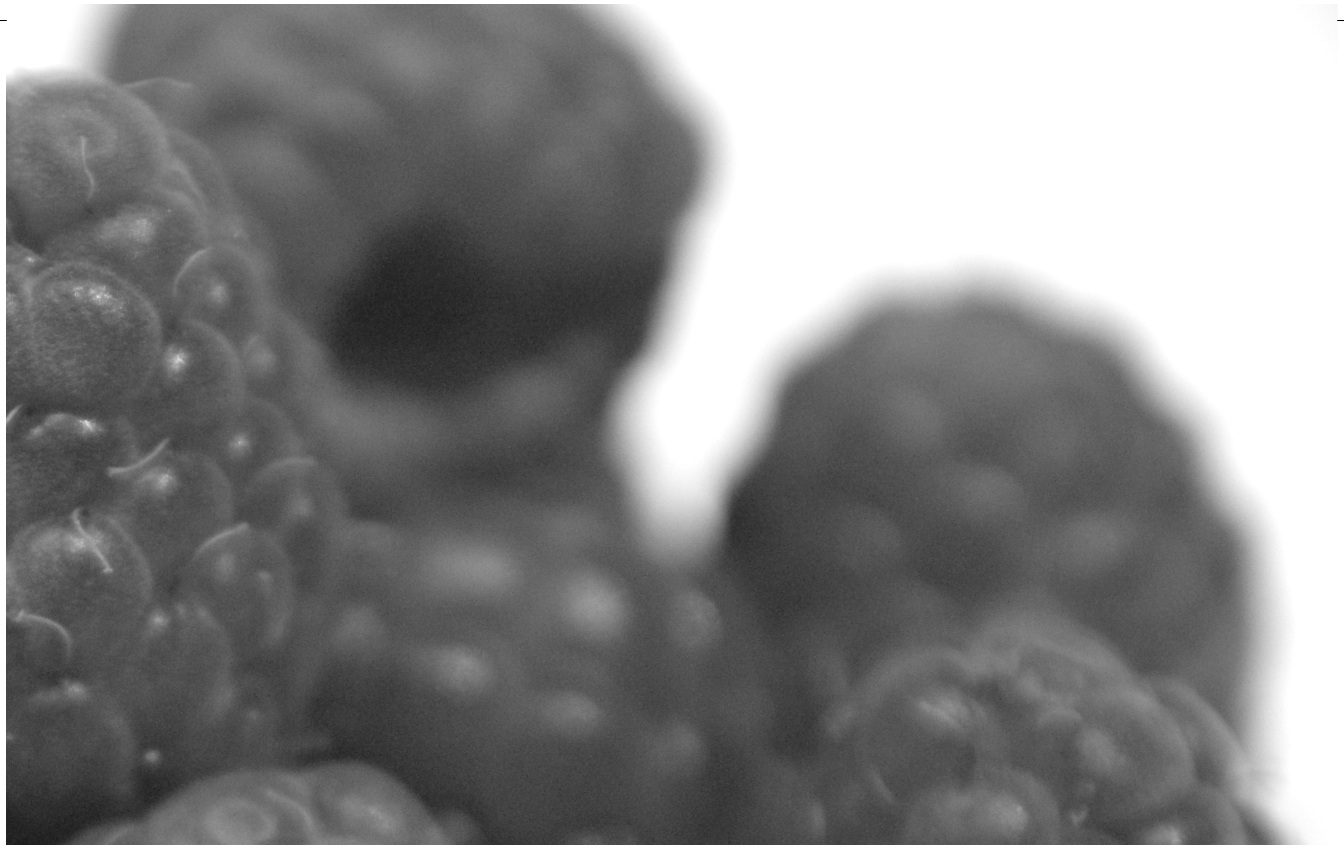
This work was supported by the EU FP7 project 'Integrated monitoring and control of foodborne viruses in European food supply chains (VITAL)' (Grant no. KBBE 213178), led by the coordination team of Nigel Cook, Martin D'Agostino and Franco M. Ruggeri, and was conducted in part for the account of the National Institute of Public Health and the Environment (RIVM) for the Strategic RIVM Research project "Norovirus inactivation". Anna Carratalà was a fellow of the Spanish Ministry of Science and Innovation. We thank Peter Teunis for his contribution on the data analysis of this manuscript and Arno Swart for assisting with the surface measurements.

References

1. Scallan, E., Griffin, P.M., Angulo, F.J., Tauxe, R.V., and Hoekstra, R.M., *Foodborne illness acquired in the United States - unspecified agents.* Emerg Infect Dis, 2011. 17(1):16-22.
2. EFSA, *Scientific Opinion, An update on the present knowledge on the occurrence and control of foodborne viruses.* EFSA Journal, 2011. 9(7):2190-2286.
3. Hall, A.J., Eisenbart, V.G., Etingue, A.L., Gould, L.H., Lopman, B.A., and Parashar, U.D., *Epidemiology of foodborne norovirus outbreaks, United States, 2001-2008.* Emerg Infect Dis, 2012. 18(10):1566-73.
4. Baert, L., Uyttendaele, M., Stals, A., van Coillie, E., Dierick, K., Debevere, J., and Botteldoorn, N., *Reported foodborne outbreaks due to noroviruses in Belgium: the link between food and patient investigations in an international context.* Epidemiol Infect, 2009. 137(3):316-25.
5. Widdowson, M.A., Sulka, A., Bulens, S.N., Beard, R.S., Chaves, S.S., Hammond, R., Salehi, E.D., Swanson, E., Totaro, J., Woron, R., Mead, P.S., Bresee, J.S., Monroe, S.S., and Glass, R.I., *Norovirus and foodborne disease, United States, 1991-2000.* Emerg Infect Dis, 2005. 11(1):95-102.
6. Friedman, D.S., Heisey-Grove, D., Argyros, F., Berl, E., Nsubuga, J., Stiles, T., Fontana, J., Beard, R.S., Monroe, S., McGrath, M.E., Sutherby, H., Dicker, R.C., DeMaria, A., and Matyas, B.T., *An outbreak of norovirus gastroenteritis associated with wedding cakes.* Epidemiol Infect, 2005. 133(6):1057-63.
7. Teunis, P.F., Moe, C.L., Liu, P., Miller, S.E., Lindesmith, L., Baric, R.S., Le Pendu, J., and Calderon, R.L., *Norwalk virus: how infectious is it?* J Med Virol, 2008. 80(8):1468-76.
8. Atmar, R.L., Opekun, A.R., Gilger, M.A., Estes, M.K., Crawford, S.E., Neill, F.H., and Graham, D.Y., *Norwalk virus shedding after experimental human infection.* Emerg Infect Dis, 2008. 14(10):1553-7.
9. Rockx, B., De Wit, M., Vennema, H., Vinje, J., De Bruin, E., Van Duynhoven, Y., and Koopmans, M., *Natural history of human calicivirus infection: a prospective cohort study.* Clin Infect Dis, 2002. 35(3):246-53.
10. Okabayashi, T., Yokota, S., Ohkoshi, Y., Ohuchi, H., Yoshida, Y., Kikuchi, M., Yano, K., and Fujii, N., *Occurrence of norovirus infections unrelated to norovirus outbreaks in an asymptomatic food handler population.* J Clin Microbiol, 2008. 46(6):1985-8.
11. Phillips, G., Tam, C.C., Rodrigues, L.C., and Lopman, B., *Prevalence and characteristics of asymptomatic norovirus infection in the community in England.* Epidemiol Infect, 2010. 138(10):1454-8.
12. Lopman, B.A., Adak, G.K., Reacher, M.H., and Brown, D.W., *Two epidemiologic patterns of norovirus outbreaks: surveillance in England and wales, 1992-2000.* Emerg Infect Dis, 2003. 9(1):71-7.
13. Wobus, C.E., Thackray, L.B., and Virgin, H.W., *Murine norovirus: a model system to study norovirus biology and pathogenesis.* J Virol, 2006. 80(11):5104-12.
14. Pires, S.M., Evers, E.G., van Pelt, W., Ayers, T., Scallan, E., Angulo, F.J., Havelaar, A., Hald, T., and 6, M.-V.-N.W.W.G., *Attributing the Human Disease Burden of Foodborne Infections to Specific Sources.* Foodborne Pathogens and Disease, 2009. 6:417–424.
15. Sharps, C.P., Kotwal, G., and Cannon, J.L., *Human norovirus transfer to stainless steel and small fruits during handling.* J Food Prot, 2012. 75(8):1437-46.
16. Stals, A., Uyttendaele, M., Baert, L., and Van Coillie, E., *Norovirus Transfer between Foods and Food Contact Materials.* J Food Prot, 2013. 76(7):1202-9.
17. Nauta, M.J., *Separation of uncertainty and variability in quantitative microbial risk assessment models.* International Journal of Food Microbiology 2010. 57:9–18.
18. Hundesa, A., Maluquer de Motes, C., Bofill-Mas, S., Albinana-Gimenez, N., and Girones, R., *Identification of human and animal adenoviruses and polyomaviruses for determination of sources of fecal contamination in the environment.* Appl Environ Microbiol, 2006. 72(12):7886-93.
19. Martin, L.R. and Palmenberg, A.C., *Tandem mengovirus 5' pseudoknots are linked to viral RNA synthesis, not poly(C)-mediated virulence.* J Virol, 1996. 70(11):8182-6.
20. Verhaelen, K., Bouwknegt, M., Lodder-Verschoor, F., Rutjes, S.A., and de Roda Husman, A.M., *Persistence of human norovirus GII.4 and GI.4, murine norovirus, and human adenovirus on soft berries as compared with PBS at commonly applied storage conditions.* Int J Food Microbiol, 2012. 160(2):137-44.

21. Wyn-Jones, A.P., Carducci, A., Cook, N., D'Agostino, M., Divizia, M., Fleischer, J., Gantzer, C., Gawler, A., Girones, R., Holler, C., de Roda Husman, A.M., Kay, D., Kozyra, I., Lopez-Pila, J., Muscillo, M., Nascimento, M.S., Papageorgiou, G., Rutjes, S., Sellwood, J., Szewzyk, R., and Wyer, M., *Surveillance of adenoviruses and noroviruses in European recreational waters.* Water Res, 2011. 45(3):1025-38.
22. Diez-Valcarce, M., Kovač, K., Cook, N., Rodríguez-Lázaro, D., Hernández, M., *Construction and Analytical Application of Internal Amplification Controls (IAC) for Detection of Food Supply Chain-Relevant Viruses by Real-Time PCR-Based Assays.* Food Analytical Methods 2011. 4(3):437-445.
23. Martinez-Martinez, M., Diez-Valcarce, M., Hernandez, M., and Rodriguez-Lazaro, D., *Design and Application of Nucleic Acid Standards for Quantitative Detection of Enteric Viruses by Real-Time PCR.* Food Environ Virol, 2011. 3(2):92-98.
24. Julian, T.R., Leckie, J.O., and Boehm, A.B., *Virus transfer between fingerpads and fomites.* J Appl Microbiol, 2010. 109(6):1868-74.
25. Rusin, P., Maxwell, S., and Gerba, C., *Comparative surface-to-hand and fingertip-to-mouth transfer efficiency of gram-positive bacteria, gram-negative bacteria, and phage.* J Appl Microbiol, 2002. 93(4):585-92.
26. Butot, S., Putallaz, T., Amoroso, R., and Sanchez, G., *Inactivation of enteric viruses in minimally processed berries and herbs.* Appl Environ Microbiol, 2009. 75(12):4155-61.
27. Gilks, W.R., Richardson, S., and Spiegelhalter, D.J., *Markov Chain Monte Carlo in Practice*. 1996, London: Chapman&Hall.
28. Teunis, P., Takumi, K., and Shinagawa, K., *Dose response for infection by Escherichia coli O157:H7 from outbreak data.* Risk Anal, 2004. 24(2):401-7.
29. Ferrari, S.L.P. and Cribari-Neto, F., *Beta Regression for Modelling Rates and Proportions.* Journal of Applied Statistics, 2004. 31(7):799–815.
30. Simas, A.B., Barreto-Souza, W., and Rocha, A.V., *Improved estimators for a general class of beta regression models.* Computational Statistics & Data Analysis, 2010. 54(2):348-366.
31. Butot, S., Putallaz, T., and Sanchez, G., *Effects of sanitation, freezing and frozen storage on enteric viruses in berries and herbs.* Int J Food Microbiol, 2008. 126(1-2):30-5.
32. Bidawid, S., Malik, N., Adegbunrin, O., Sattar, S.A., and Farber, J.M., *Norovirus cross-contamination during food handling and interruption of virus transfer by hand antisepsis: experiments with feline calicivirus as a surrogate.* J Food Prot, 2004. 67(1):103-9.
33. D'Souza, D.H., Sair, A., Williams, K., Papafragkou, E., Jean, J., Moore, C., and Jaykus, L., *Persistence of caliciviruses on environmental surfaces and their transfer to food.* Int J Food Microbiol, 2006. 108(1):84-91.
34. Escudero, B.I., Rawsthorne, H., Gensel, C., and Jaykus, L.A., *Persistence and Transferability of Noroviruses on and between Common Surfaces and Foods.* Journal of Food Protection, 2012. 75(5):927–935.
35. Bidawid, S., Farber, J.M., and Sattar, S.A., *Contamination of foods by food handlers: experiments on hepatitis A virus transfer to food and its interruption.* Appl Environ Microbiol, 2000. 66(7):2759-63.
36. de Wit, M.A., Widdowson, M.A., Vennema, H., de Bruin, E., Fernandes, T., and Koopmans, M., *Large outbreak of norovirus: the baker who should have known better.* Journal of Infection, 2007. 55(2):188–193.
37. Allwood, P.B., Jenkins, T., Paulus, C., Johnson, L., and Hedberg, C.W., *Hand washing compliance among retail food establishment workers in Minnesota.* J Food Prot, 2004. 67(12):2825-8.
38. Moe, C.L., *Preventing norovirus transmission: how should we handle food handlers?* Clin Infect Dis, 2009. 48(1):38-40.

4



Persistence of human norovirus in reconstituted pesticides – Pesticide application as a possible source of viruses in fresh produce chains

Katharina Verhaelen[a,b], Martijn Bouwknegt[a], Saskia Rutjes[a], Ana Maria de Roda Husman[a,b]

[a]Laboratory for Zoonoses and Environmental Microbiology, Centre for Infectious Disease Control Netherlands, National Institute for Public Health and the Environment (RIVM), P.O. Box 1, NL-3720 BA Bilthoven, The Netherlands

[b]Faculty of Veterinary Medicine, Institute for Risk Assessment Sciences (IRAS), Utrecht University, P.O. Box 80.178, NL-3508 TD Utrecht, The Netherlands

International Journal of Food Microbiology 160 (2013) 323-328

Abstract

The consumption of fresh produce is frequently associated with outbreaks of human norovirus (hNoV) disease. To prevent the contamination of fresh produce with hNoV, knowledge of the possible introduction sources of the viruses, such as water, is needed to be able to implement appropriate and efficient preventive measures. Contaminated water used to reconstitute pesticides could be a relevant source of infectious hNoV, determined by the initial level of virus contamination and the persistence of these viruses in reconstituted pesticides. We studied the persistence of hNoV GI.4, hNoV GII.4 and murine norovirus (MNV-1), the only culturable norovirus, in eight different pesticides after 0 and 2 h. Virus concentrations were determined by reverse transcriptase PCR, and infectivity of MNV-1 was determined by endpoint dilutions followed by maximum likelihood estimations. MNV-1 was found to remain infectious in seven of the eight tested pesticides at the highest concentration applied in practice. In the presence of the insecticide Vertimec, MNV-1 infectivity decreased rapidly with a 1.9 $\log_{10}$-unit reduction at time point T_0. Also, the concentration of NoV GI.4 RNA decreased considerably with a 1.7 $\log_{10}$-unit reduction; whereas the detected PCR fragment of hNoV GII.4 remained stable. Assuming a similar persistence of infectious MNV-1 and hNoV we can conclude that water containing hNoV used to dilute pesticides may be an important source of infectious hNoV in fresh produce chains. The application of pesticides may therefore not only be a chemical hazard, but also a microbiological hazard for public health. The inclusion of antiviral substances in pesticides may be appropriate to reduce the virological health risk posed by the application of pesticides.

Introduction

Human noroviruses (hNoV) are the most common cause of foodborne viral disease worldwide [1]. Food commodities may become contaminated via human faecal pollution or aerosolized vomits either due to infected food handlers or contaminated water, fomites or surfaces in contact with the product. Typical risk products with respect to virus contamination are those that are not heated before consumption and prone to human faecal pollution, such as fresh produce. Raspberries and lettuce especially have been associated with hNoV outbreaks [2-8]. Primary production plays an important role in the possible introduction of viruses into fresh produce chains. Besides infected food handlers, virus contaminated water used for irrigation, for dilution of agrochemicals and water-soluble fertilizers, or for hydroponic cultures may be an introduction source [9, 10]. Often water used for irrigation is seen as an important virus source [11-13], whereas water used to dilute pesticides is less considered.

Farmers use various water sources in the primary production of fresh fruits and vegetables, including well water but also different types of surface water such as river water or lake water [10]. Different types of surface waters and also well wate, were previously found to harbour hNoV [14-20]. Moreover, the use of wastewater in agriculture will increase in the future as fresh water becomes increasingly scarce in the world [21]. Water used in primary production may therefore have a great potential as an introduction source of pathogenic viruses into fresh produce chains. Guidelines such as the 'Code of hygienic practice for fresh fruits and vegetables' by the Codex Alimentarius (2010) state the importance of the use of 'clean water' in the production of fresh produce, especially if the water is in contact with the edible part of the plant and applied closely before harvest. However, a definition of clean water including limits on permitted virus concentrations in water is not given. Due to the potential presence of viruses, such as hNoV, in water sources, and the scarcity of potable water in several regions in the world, the application of virus-free water in the production of fresh produce may be difficult.

Pesticides are commonly applied in the production of fresh produce and insects, fungi and weeds are currently controlled with about 700 pesticides operating by perhaps 95 different mechanisms [22]. Fungicides and insecticides especially are often applied just before harvest to the edible parts of the produce [23], whereas herbicides are applied in the initial stage of the production. Dependent on the crop, pesticides are diluted in different amounts of water and sprayed onto the fields in volumes ranging from 200 L to 1000 L per hectare. Thereby, pesticides sprayed on the fields in large volumes of water may result in a greater risk of viral contamination of the crop, because (i) the probability is higher that potentially contaminated water is in contact with the crops and (ii) the concentration of pesticides is usually lower because of a greater dilution, resulting possibly in a greater persistence of the virus.

The possibility of pesticides being an important introduction source of hNoV is largely determined by the presence of viruses in the water used to reconstitute the pesticides, by the potential of viruses to persist in reconstituted pesticides, and lastly by the number of viruses adhering to the edible parts of the produce, studied by Stine et al. (2011). At present, no data is available on the persistence of viruses in the presence of pesticides. Therefore, the aim of this research was to investigate the persistence of hNoV GI.4, hNoV GII.4, and MNV-1 a culturable surrogate of hNoV, in eight different reconstituted insecticides and fungicides over time. Viral persistence was tested in the highest permitted pesticide concentrations.

Materials and methods

Viruses and cells

HNoV GI.4 and hNoV GII.4 strains from clinical stool samples were kindly provided by Dr. Erwin Duizer (Laboratory for Infectious Diseases and Perinatal Screening, RIVM, The Netherlands). MNV-1 (kindly provided by Dr. Herbert W. Virgin, Washington University, St. Louis, USA) was made available, under agreement, by the group of Dr. Franco M. Ruggeri (Istituto Superiore de Sanitá, Rome, Italy). Mengovirus (vMC_0) (Martin and Palmenberg, 1996), was kindly provided by Dr. David Lees, (CEFAS, Dorset, UK). MNV-1 was propagated in RAW-264.7 cells (ATCC-TIB-71) (for details see Verhaelen et al., 2012). A virus mixture of hNoV GI and GII (10% wt/vol stool suspension) and MNV-1 was prepared, comprising of 2×10^8 genomic copies of hNoV GI.4, 1×10^8 genomic copies of hNoV GII.4 and 1×10^8 genomic copies of MNV-1 equivalent to 2×10^5 infectious virus particles per 200 µL (volume used to spike pesticides).

Pesticide preparation and experimental design

A total of eight pesticides were tested, including four fungicides and four insecticides (see Table 1). The choice of pesticides investigated in this study was based on frequency of use in the production of high risk fresh produce products (lettuce and soft berries). Besides common use, the permitted period between application and harvest was a decisive selection criterion (i.e., ≤4 days prior to harvest). Herbicides are applied comparatively long before harvest and were therefore not included in the study. Pesticides were freshly prepared just before the experiment at an ambient temperature of 21 °C. The pesticides Vertimec and Karate Zeon were liquid while all other tested pesticides consisted of small granules. Chemicals were diluted as recommended by the manufacturer in a concentration equal to the smallest volume used (200 L/ha) and thus the highest pesticide concentration used in practice. Pesticides were diluted in sterile tap water, which was previously autoclaved. Of the reconstituted pesticides, 1.8 mL was spiked with 200 µL of virus mixture. One sample was taken after 0 h (T_0) and one after

Table 1. Used pesticides and their specifications.

Pesticide	Company	Active compound and concentration	Recommended amounts[a]	pH	PHI[b] in days
Fungicides					
Rovral	BASF	Iprodion (750g/kg)	0.7 kg/ha	6.5	3
Aliette	Bayer	Fosetyl Al (746 g/L)	2 kg/ha	4	4
Teldor	Bayer	Fenhexamid (510g/kg)	2 kg/ha	8.5	1
Signum	BASF	Boscalid (267g/kg) + Pyraclostrobin (67g/kg)	1.5 kg/ha	7	3
Insecticides					
Calypso	Bayer	Thiacloprid (480 g/L)	0.2 l/ha	6.5	3
Pirimor Granulat	Syngenta	Pirimicarb (50%)	0.3 kg/ha	7	3
Vertimec	Syngenta	Abamectin (18g/L)	1.25 l/ha	6.0	3
Karate Zeon	Syngenta	Lambda-Cyhalothrin (100 g/l)	75 ml/ha	6.5	3

[a] Pesticides were reconstituted based on a used water volume of 200 l/ha. (To calculate the pesticide concentration per litre one needs to divide the recommended amount by 200.)
[b] PHI = Pre-harvest interval, which is the minimum number of days between the last pesticide application and crop harvest

2 h (T_2). Extended periods were not studied, because pesticides are commonly freshly prepared before application and promptly sprayed onto the fields. Each experiment was performed in triplicate. If needed the pH of the samples was adjusted to 7 with 4 N NaOH or 1 N HCl. The samples were split, one part for PCR detection and the other for detection by cell culture, and stored at -80 °C. Several controls were implemented to monitor the experiment. A negative treatment control was included with 1.8 mL of sterile tap water inoculated with 200 µL of virus mix to monitor the effect of e.g. temperature on viral decay and loss of viruses to e.g. binding of viruses to the wall of the used glass bottles. The reduction of viruses in pesticides was calculated relative to this control ($\log_{10}$ (virus concentration in water/virus concentration in pesticides)). Negative-virus pesticide controls (pesticide dilution without added virus mix) were prepared to study a possible toxic effect of the pesticides on the RAW 264.7 cells, allowing us to distinguish between actual cytopathic effect and cell death due to the pesticides. Therefore, the negative virus pesticide controls of the different pesticides were added to the cells in serial dilutions, to find the dilution where no cell damage was observed. To control the extraction efficiency of nucleic acids, 10 µL of vMC_0 (C_q~29) was added to the sample [26]. To monitor a possible inhibition of the pesticides on the PCR reaction, a competitive internal amplification control (IAC), which uses the same primers as the target virus [25, 27], was included into each PCR reaction. However, due to the low C_q values of the target, this control could not always be detected by PCR. Therefore, an additional experiment was performed to monitor the effect of the used pesticides on the PCR reaction. For this end, we added 9 µL of the nucleic acid extract of a water sample spiked with the target viruses and 1 µL of the 'nucleic acid extract' of the negative-virus pesticide control to the master mix, mimicking a ten times diluted sample. In this way the contact time of genomic material and pesticide matrix was minimized being the best

option to distinguish between an increase of the C_q value due to (i) actual degradation of genomes owing to the pesticides or (ii) possible inhibition of the sample on the PCR reaction, resulting as well in an increase of the C_q value. By comparing the C_q values of the PCR reactions spiked with 1 µL of the negative-virus pesticide control to the same 9 µL of nucleic acid extract, but spiked with 1 µL of water instead, the effect of the pesticides on the PCR reaction was quantified, expressed as ΔC_q values: C_q value of nucleic acid extract spiked with negative-virus pesticide control – C_q value of nucleic acid extract spiked with H_2O. The dilution resulting in a ΔC_q value of 0 was used to test the actual samples. Lastly, negative controls were included in each PCR run consisting solely of the sterile tap water used in the experiment.

Determination of MNV-1 titer

To determine the titer of infective MNV-1 particles endpoint dilutions were performed (see Verhaelen et al., 2012). Briefly, 96 well plates (Corning, The Netherlands) were seeded with 100 µL RAW-265.7 cells in maintenance medium containing 2% fetal calf serum at a concentration of 4×10^5 cells/mL. Eight serial 0.5 $\log_{10}$-unit sample dilutions in medium without FCS were analyzed in 10-fold per dilution for each sample. Plates were incubated for 6 days at 37 °C with 5% CO_2 and observed for cytopathic effect.

Molecular detection

Nucleic acids were extracted using the NucliSens miniMag magnetic extraction kit (bioMérieux, The Netherlands) following the manufactures' instructions with minor modifications. Briefly, lysis buffer was added to the sample in a volume four times the amount of sample. Subsequent 50 µL of well mixed magnetic silica solution was added, incubated for 10 min and the sample was washed twice with 400 µL provided wash buffer 1, twice with 500 µL of wash buffer 2, once with wash buffer 3 and subsequently eluted in 200 µL elution buffer at 60 °C with shaking at 1400 rpm for 5 min. In each extraction cycle, a negative control containing no target was included. The previously included vMC_0 served as a positive control.

A Lightcycler 480 (Roche Diagnostics, The Netherlands) was used for quantitative real time reverse transcriptase PCR, using TaqMan hydrolysis probes. The used primers and probes for hNoV GI.4, hNoV GII.4 and MNV targeted the ORF1/ORF 2 junction of the viral genome. For detection of viral RNA the UltraSense One-Step Quantitative RT-PCR System (Invitrogen, The Netherlands) was used. To control for PCR inhibition, a competitive internal amplification control (IAC) (Yorkshire Bioscience Ltd., UK), specific for each target primer set [27], was added to each reaction, at a concentration previously tested to not influence the detection of the target signal. Instead of FAM, VIC was used as a reporter dye of the IAC. Virus quantification of MNV-1, hNoV GI.4

and hNoV GII.4 was performed using standard curves. Six tenfold dilution of the standards were included in each PCR run in duplicate. Further details of the performed PCRs and genome quantification are as described by Verhaelen et al. (2012).

Data analysis

Virus concentrations were estimated by the maximum likelihood approach. Infectivity counts of MNV-1 were estimated based on the presence/absence profile forMNV-1 obtained from the performed endpoint dilutions, assuming homogeneous ('Poisson') mixing [25]. For genome counts based on RT-PCR, the intercept and slope parameter of the standard curve were estimated simultaneously with the concentration of genomic copies as joint likelihood; allowing the inclusion of the uncertainty of the standard curve in the estimate. Genomic copies were considered to be lognormally distributed. Analyses were performed in Mathematica 8.0 (Wolfram).

Results

The persistence of hNoV GI.4, hNoV GII.4, and MNV-1 (a culturable surrogate of hNoV) was researched over time in eight different reconstituted pesticides. To evaluate the experimental set-up and therefore the validity of the results, several controls were implemented. The effect of pesticides on RAW-264.7 cells was tested with negative virus pesticide controls, and it was found that for most pesticides a 100 times dilution was sufficient to prevent cell death. Signum and Calypso solutions needed to be diluted up to 10^4 times. The PCR reaction was inhibited in a virus-dependent fashion by the pesticide Teldor and particularly by the pesticide Signum. Whereas the effect of Teldor and Signum resulted in ΔC_q values of about 1 and 4, for both hNoV GI.4 and GII.4; the PCR reaction of MNV-1 was considerably more inhibited with ΔC_q values of about 2.5 and 7.5, respectively. Diluting Teldor samples 100 times and Signum samples 1000 times was sufficient to prevent the inhibiting effects of the pesticides on the PCR reaction.

The effect of the different reconstituted pesticides on infectious MNV-1 particles is visualised in Fig. 1. MNV-1 remained infectious in all four fungicide dilutions and in three of the four tested insecticide dilutions, also after 2 h of incubation. In the dilution of the insecticide Vertimec a considerable decrease in MNV-1 infectivity was observed resulting in a rapid 2 $\log_{10}$-unit reduction of infectious MNV-1, independent of the studied time.

As was observed for infectious MNV-1 particles, the targeted genome fractions of MNV-1, hNoV GI.4 and hNoV GII.4 remained stable in the insecticide dilutions of Calypso, Pirimor and Karate Zeon as compared with the reference point (Fig. 2).

MNV-1 RNA decayed with a 0.7 log_{10}-unit reduction at T_0 and 0.8 log_{10}-unit reduction at T_2 in the Vertimec dilution. Decay of hNoV GI.4 RNA was higher with a 1.7 log_{10}-unit reduction at T_0 and a 1.4 log_{10}-unit reduction at T_2, whereas hNoV GII.4 RNA remained stable (less than 0.1 log reduction). Also, the observed decay in targeted viral genome fragments was found to be rapid, with similar decay at 0 h as compared with the decay observed at 2 h. Genomes were found to persist in the fungicide dilutions of Rovral, Teldor and Signum as compared to the reference point. However, a substantial decrease in detectable genomes was observed in Aliette for hNoV GI.4, hNoV GII.4 and MNV-1 of about 3, 4 and 5 log_{10}-units respectively, even though MNV-1 particles remained infectious based on the cell culture data (Fig. 1). As previously mentioned, the PCR reaction was not inhibited by the presence of Aliette and the C_q values of the IAC in the tested reactions were in the expected range (data not shown). However, the C_q values of Mengovirus vMC_0, used to monitor the extraction procedure, increased significantly. The C_q values of vMC_0 were constant in all other studied samples varying between 31.1 and 32.1, displaying a constant efficiency of the nucleic acid extraction procedure. Only in the Aliette samples the C_q values increased by at least 8. Based on this increase the nucleic extraction procedure in the samples containing Aliette was declared invalid. Considering, that infectious MNV-1 particles remained stable in the Aliette samples and thus MNV-1 genomes, we assumed that also hNoV GI.4 and GII.4 remained stable in the pesticide Aliette.

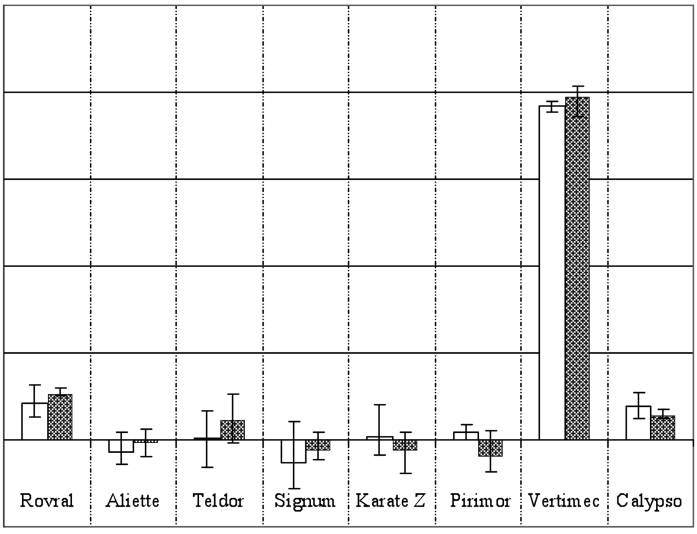


Figure 1. Persistence of infectious MNV-1 particles in reconstituted pesticides after 0 and 2 h expressed as log reduction (virus concentration in pesticide solution relative to virus concentration in water after 0 and 2 hours, respectively). The error bars represent the minimum and maximum values of the measured virus titers.

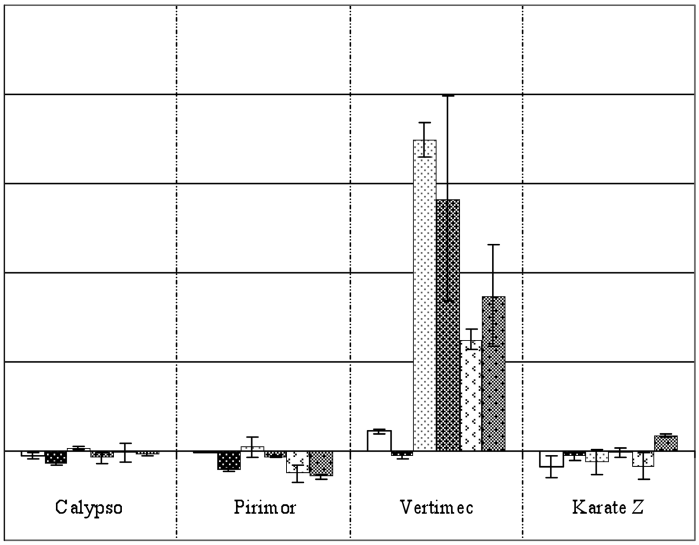


Figure 2. Persistence of hNoV GII.4, hNoV GI.4 and MNV-1 in the four reconstituted insecticides based on RT-PCR data after 0 and 2 h expressed as log reduction (virus concentration in pesticide solution relative to virus concentration in water after 0 and 2 hours, respectively). The error bars represent the minimum and maximum values of the measured virus titers.

Discussion

Human noroviruses and other proven waterborne pathogens such as pathogenic *Escherichia coli* or Cyclospora can be frequently found in water sources used for the dilution of pesticides [28-30]. The application of pesticides to crops may therefore lead to a potential infection risk to the consumer's health. Since only infectious pathogens pose a public health concern, it is of interest whether pathogens remain infectious or even multiply in dilutions of pesticides. Bacteria and parasites were previously shown to persist, or even grow in reconstituted pesticides [31-34] and an outbreak of cyclosporiasis was associated with raspberries, which were assumed to be contaminated with Cyclospora present in the water used to dilute the applied fungicides and insecticides [23]. HNoV cannot replicate in water, but is known to remain infectious in water for prolonged periods of at least 61 days, based on human challenge studies [35]. We tested the persistence of noroviruses in commonly used pesticide dilutions at the highest concentration prescribed to be applied in practice. Our data show that infectivity of murine noroviruses, the only available culturable norovirus, was not affected by the presence of pesticides in water, except for one tested insecticide (Vertimec). Unlike for hNoV GI.4, the targeted RNA fragment of hNoV GII.4 remained stable; suggesting that hNoV GII.4 may be more stable in reconstituted pesticides than hNoV GI.4. The PCRs for both genotypes targeted the same genome fragment (the ORF1/ ORF2 junction) of a similar length.

This suggested that the difference in viral persistence of hNoV GI.4 and GII.4 is unlikely to be explained by a different likelihood to detect the genome damage of the pesticide treatment by PCR. Yet, the data is inconclusive because only a small fragment of nucleotides was analyzed and the correlation between detected GII.4 and GI.4 RNA fragments and infectious hNoV particles remains unknown. However, assuming a similar persistence of infectious MNV-1 and hNoV, we can conclude that water containing hNoV used to dilute pesticides, may be an important source for the introduction of infectious hNoV into fresh produce chains. Whereas for bacteria and parasites, just a few pesticides allowed microbial growth or survival, noroviruses appeared to persist in most pesticide dilutions commonly used in the production of lettuce and raspberries.

The outcome of this study demonstrated moreover the crucial role of appropriate controls in the experimental set-up to validate obtained results. Omission of the nucleic acid extraction control and the controls monitoring the inhibition of the samples on the PCR reaction would have resulted in a false interpretation of the data. The importance of internal quality assurance controls of PCR performance and extractions controls has been described previously [26, 36, 37]. Nevertheless, the inclusion of such controls is not a standard practice at the moment.

The decay observed in infectious MNV-1 particles of about 2 $\log_{10}$- units at time point T_0 in the dilution of the insecticide Vertimec, represents a rapid decrease of a susceptible part of the virus population. A rapid decrease in MNV-1 infectivity after only a short contact time has been also described for other chemical substances such as free chlorine [38]. Except for Vertimec, noroviruses were found to be robust not only against the active compound of the pesticides tested, but also against the added inerts (other ingredients). Inerts are substances that are not actively pesticidal, but are included to promote efficacy and stability of the pesticide [39]. There are approximately 1200 inert ingredients in pesticide formulations [40], whereby the exact composition of pesticides is confidential. Therefore, it is difficult to conclude which compound of the insecticide Vertimec is responsible for the decrease in viral infectivity. The active compound is abamectin. Abamectin targets the voltage-gated chloride channel of insects, one of the four nerve targets, on which insecticides act primarily [22]. Whether abamectin is responsible for the reduction of infectious MNV-1 remains unknown. Other active compounds, such as the tested thiacloprid (Calypso) which affects acetyl-cholinesterase, the target site of most insecticides used [22], were observed to have no effect on viral stability.

The application of pesticides poses the greatest microbiological risk to public health if the time between application and harvest is minimal, and if produce is minimally processed after harvest. Under these circumstances, the viruses present on the produce

are highly probable to remain infectious until consumption. Fresh produce, especially soft berries, are therefore potentially of high risk. Pesticides are commonly applied in their production just before harvest to optimize product quality and to extend the shelf life. Soft berries are often sprayed with fungicides just before harvest to prolong their shelf life, since this group of fruits is rather sensitive to fungal spoilage [41, 42]. In the case of mechanically harvested raspberries, insecticides are also applied shortly before harvest to minimize insect contamination of the harvest [43]. Mechanical harvesters are machines that straddle a row of raspberries and detach the berries by shaking the plant causing not only ripe berries, but also insects to fall off, which results in insect contamination of harvested fruits. In addition, it has been shown previously that once infectious viruses are introduced onto for example raspberries, the viruses are likely to remain infectious under all possible postharvest conditions; both on freshly consumed raspberries [25] and frozen raspberries [44]. We can therefore conclude that the application of pesticides may not only be a chemical, but also a microbiological hazard to public health.

Whereas for irrigation water the contact of the edible parts of the plant with water can be minimized by using drip irrigation, the contact of pesticides with the crop is desired or unavoidable for the successful application of pesticides. Measures previously suggested to prevent the growth of bacteria in pesticides [33], such as an immediate reconstitution of pesticides before application, optimally at low temperatures, are likely to increase the risk of introducing infectious virus particles into fresh produce chains. To prevent the introduction of pathogenic viruses into fresh produce chains by pesticide application, the use of pathogen free water to reconstitute the pesticides may be an effective approach. WHO and Codex Alimentarius documents [10, 21] give guidance on sanitary surveys to chose appropriate water sources; and if necessary state water treatment options. Nevertheless, farmers may choose water sources based on practical or economical considerations, especially due to missing awareness of the associated health risks. A promising approach to minimize the microbiological health risk of pesticide application, which is not depending on the compliance and awareness of the farmers, is the inclusion of antiviral substances into pesticides. For example, different chlorine compounds are approved as inert substances in pesticide formulations [40], which may reduce the number of infectious norovirus particles in the reconstituted pesticide.

Acknowledgments

This work was supported by the EU FP7 project 'Integrated monitoring and control of foodborne viruses in European food supply chains (VITAL)' (Grant no. KBBE 213178), led by the coordination team of Nigel Cook, Martin D'Agostino and Franco

M. Ruggeri, and was conducted in part for the account of the National Institute of Public Health and the Environment (RIVM) for the Strategic RIVM Research project "Norovirus inactivation". We thank Michael Ophey (Agri V Raiffeisen eG, Germany) and Marcel Krumbach (Syngenta, Germany) for information on pesticide applications and supply of the used pesticides.

References

1. Koo, H.L., Ajami, N., Atmar, R.L., and DuPont, H.L., *Noroviruses: The leading cause of gastroenteritis worldwide.* Discov Med, 2010. 10(50):61-70.
2. Cotterelle, B., Drougard, C., Rolland, J., Becamel, M., Boudon, M., Pinede, S., Traore, O., Balay, K., Pothier, P., and Espie, E., *Outbreak of norovirus infection associated with the consumption of frozen raspberries, France, March 2005.* Euro Surveill, 2005. 10(4):E050428 1.
3. Falkenhorst, G., Krusell, L., Lisby, M., Madsen, S.B., Bottiger, B., and Molbak, K., *Imported frozen raspberries cause a series of norovirus outbreaks in Denmark, 2005.* Euro Surveill, 2005. 10(9):E050922 2.
4. Hjertqvist, M., Johansson, A., Svensson, N., Abom, P.E., Magnusson, C., Olsson, M., Hedlund, K.O., and Andersson, Y., *Four outbreaks of norovirus gastroenteritis after consuming raspberries, Sweden, June-August 2006.* Euro Surveill, 2006. 11(9):E060907 1.
5. Maunula, L., Roivainen, M., Keranen, M., Makela, S., Soderberg, K., Summa, M., von Bonsdorff, C.H., Lappalainen, M., Korhonen, T., Kuusi, M., and Niskanen, T., *Detection of human norovirus from frozen raspberries in a cluster of gastroenteritis outbreaks.* Euro Surveill, 2009. 14(49).
6. Showell, D., Sundkvist, T., Reacher, M., and Gray, J., *Norovirus outbreak associated with canteen salad in Suffolk, United Kingdom.* Euro Surveill, 2007. 12(11):E071129 6.
7. Schmid, D., Stuger, H.P., Lederer, I., Pichler, A.M., Kainz-Arnfelser, G., Schreier, E., and Allerberger, F., *A food-borne norovirus outbreak due to manually prepared salad, Austria 2006.* Infection, 2007. 35(4):232-9.
8. Sarvikivi, E., Roivainen, M., Maunula, L., Niskanen, T., Korhonen, T., Lappalainen, M., and Kuusi, M., *Multiple norovirus outbreaks linked to imported frozen raspberries.* Epidemiol Infect, 2012. 140(2):260-7.
9. Brassard, J., Gagne, M.J., Genereux, M., and Cote, C., *Detection of human food-borne and zoonotic viruses on irrigated, field-grown strawberries.* Appl Environ Microbiol, 2012. 78(10):3763-6.
10. CAC, *Code of hygienic practice for fresh fruits and vegetables. Adopted 2003. Revision 2010 (new Annex III for Fresh Leafy Vegetables).* 2010, Codex Alimentarius Commission.
11. Gerba, C.P. and Choi, C.Y., *Role of Irrigation Water in Crop Contamination by Viruses*. Viruses in Foods, ed. S.M. Goyal. 2006, US: Springer.
12. Newell, D.G., Koopmans, M., Verhoef, L., Duizer, E., Aidara-Kane, A., Sprong, H., Opsteegh, M., Langelaar, M., Threfall, J., Scheutz, F., van der Giessen, J., and Kruse, H., *Food-borne diseases - the challenges of 20 years ago still persist while new ones continue to emerge.* Int J Food Microbiol, 2010. 139:3-15.
13. Stine, S.W., Song, I., Choi, C.Y., and Gerba, C.P., *Application of microbial risk assessment to the development of standards for enteric pathogens in water used to irrigate fresh produce.* J Food Prot, 2005. 68(5):913-8.
14. Borchardt, M.A., Bertz, P.D., Spencer, S.K., and Battigelli, D.A., *Incidence of enteric viruses in groundwater from household wells in Wisconsin.* Appl Environ Microbiol, 2003. 69(2):1172-80.
15. Fout, G.S., Martinson, B.C., Moyer, M.W., and Dahling, D.R., *A multiplex reverse transcription-PCR method for detection of human enteric viruses in groundwater.* Appl Environ Microbiol, 2003. 69(6):3158-64.
16. Gabrieli, R., Maccari, F., Ruta, A., Pana, A., Divizia, M., , *Norovirus Detection in Groundwater.* Food and Environmental Virology, 2009. 1:92-96.
17. Haramoto, E., Katayama, H., Oguma, K., and Ohgaki, S., *Application of cation-coated filter method to detection of noroviruses, enteroviruses, adenoviruses, and torque teno viruses in the Tamagawa River in Japan.* Appl Environ Microbiol, 2005. 71(5):2403-11.
18. Horman, A., Rimhanen-Finne, R., Maunula, L., von Bonsdorff, C.H., Torvela, N., Heikinheimo, A., and Hanninen, M.L., *Campylobacter spp., Giardia spp., Cryptosporidium spp., noroviruses, and indicator organisms in surface water in southwestern Finland, 2000-2001.* Appl Environ Microbiol, 2004. 70(1):87-95.
19. Koh, S.J., Cho, H.G., Kim, B.H., and Choi, B.Y., *An outbreak of gastroenteritis caused by norovirus-contaminated groundwater at a waterpark in Korea.* J Korean Med Sci, 2011. 26(1):28-32.
20. Lodder, W.J. and de Roda Husman, A.M., *Presence of noroviruses and other enteric viruses in sewage and surface waters in The Netherlands.* Appl Environ Microbiol, 2005. 71(3):1453-61.

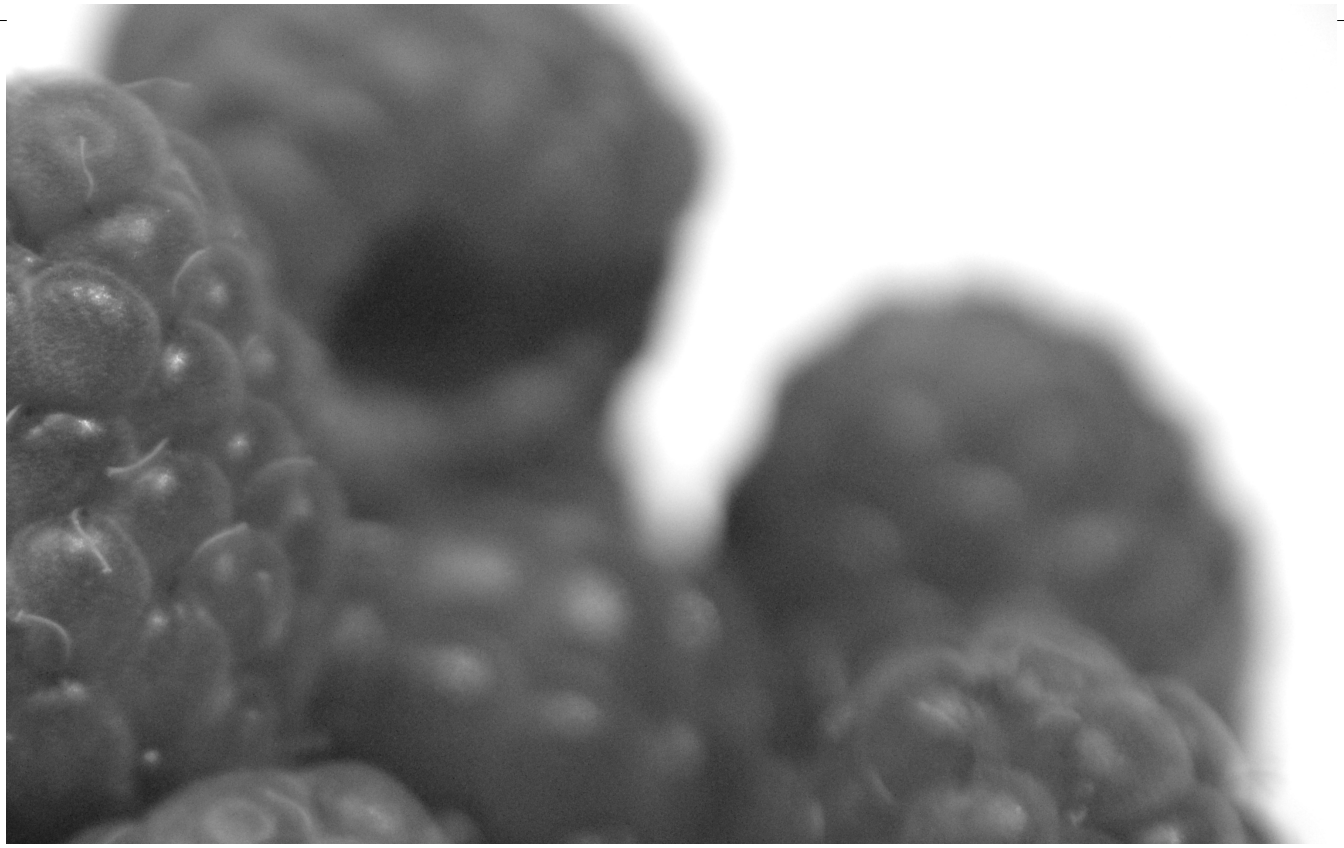
21. WHO, *Guidelines for the safe use of wastewater, excreta and greywater.* Wastewater use in agriculture, 2006. 2: http://whqlibdoc.who.int/publications/2006/9241546832_eng.pdf
22. Casida, J.E., *Pest toxicology: the primary mechanisms of pesticide action.* Chem Res Toxicol, 2009. 22(4):609-19.
23. Herwaldt, B.L. and Ackers, M.L., *An outbreak in 1996 of cyclosporiasis associated with imported raspberries. The Cyclospora Working Group.* N Engl J Med, 1997. 336(22):1548-56.
24. Stine, S.W., Song, I., Choi, C.Y., and Gerba, C.P., *Application of Pesticide Sprays to Fresh Produce: A Risk Assessment for Hepatitis A and Salmonella.* Food and Environmental Virology, 2011. 3:86-91.
25. Verhaelen, K., Bouwknegt, M., Lodder-Verschoor, F., Rutjes, S.A., and de Roda Husman, A.M., *Persistence of human norovirus GII.4 and GI.4, murine norovirus, and human adenovirus on soft berries as compared with PBS at commonly applied storage conditions.* Int J Food Microbiol, 2012. 160(2):137-44.
26. Costafreda, M.I., Bosch, A., and Pinto, R.M., *Development, evaluation, and standardization of a real-time TaqMan reverse transcription-PCR assay for quantification of hepatitis A virus in clinical and shellfish samples.* Appl Environ Microbiol, 2006. 72(6):3846-55.
27. Diez-Valcarce, M., Kovač, K., Cook, N., Rodríguez-Lázaro, D., Hernández, M., *Construction and Analytical Application of Internal Amplification Controls (IAC) for Detection of Food Supply Chain-Relevant Viruses by Real-Time PCR-Based Assays.* Food Analytical Methods 2011. 4(3):437-445.
28. Ferguson, C., de Roda Husman, A.M., Altavilla, N., Deere, D., and Ashbolt, N., *Fate and Transport of Surface Water Pathogens in Watersheds.* Critical Reviews in Environmental Science and Technology, 2003. 33:299-361.
29. Olsen, S.J., Miller, G., Breuer, T., Kennedy, M., Higgins, C., Walford, J., McKee, G., Fox, K., Bibb, W., and Mead, P., *A waterborne outbreak of Escherichia coli O157:H7 infections and hemolytic uremic syndrome: implications for rural water systems.* Emerg Infect Dis, 2002. 8(4):370-5.
30. Sterling, C.R. and Ortega, Y.R., *Cyclospora: an enigma worth unraveling.* Emerg Infect Dis, 1999. 5(1):48-53.
31. Guan, T.Y., Blank, G., Ismond, A., and Van Acker, R., *Fate of foodborne bacterial pathogens in pesticide products.* Journal of the Science of Food and Agriculture, 2001. 81:503-512.
32. Guan, T.T., Blank, G., and Holley, R.A., *Survival of pathogenic bacteria in pesticide solutions and on treated tomato plants.* J Food Prot, 2005. 68(2):296-304.
33. Ng, P.J., Fleet, G.H., and Heard, G.M., *Pesticides as a source of microbial contamination of salad vegetables.* Int J Food Microbiol, 2005. 101(2):237-50.
34. Sathyanarayanan, L. and Ortega, Y., *Effects of pesticides on sporulation of Cyclospora cayetanensis and viability of Cryptosporidium parvum.* J Food Prot, 2004. 67(5):1044-9.
35. Seitz, S.R., Leon, J.S., Schwab, K.J., Lyon, G.M., Dowd, M., McDaniels, M., Abdulhafid, G., Fernandez, M.L., Lindesmith, L.C., Baric, R.S., and Moe, C.L., *Norovirus infectivity in humans and persistence in water.* Appl Environ Microbiol, 2011. 77(19):6884-8.
36. Rutjes, S.A., Lodder, W.J., Bouwknegt, M., and de Roda Husman, A.M., *Increased hepatitis E virus prevalence on Dutch pig farms from 33 to 55% by using appropriate internal quality controls for RT-PCR.* J Virol Methods, 2007. 143(1):112-6.
37. D'Agostino, M., Cook, N., Rodriguez-Lazaro, D., and Rutjes, S., *Nucleic Acid Amplification-Based Methods for Detection of Enteric Viruses: Definition of Controls and Interpretation of Results.* Food and Environmental Virology, 2011. 2:55-60.
38. Cromeans, T.L., Kahler, A.M., and Hill, V.R., *Inactivation of adenoviruses, enteroviruses, and murine norovirus in water by free chlorine and monochloramine.* Appl Environ Microbiol, 2010. 76(4):1028-33.
39. Hochberg, E.G., *The market for agricultural pesticide inert ingredients*, in *Pesticide Formulation and Adjuvant Technology*, C.L. Foy and D.W. Pritchard, Editors. 1996, CRC Press: Florida. p. 203-208.
40. EPA. *Inert Ingredients in Pesticide Products*. 2012, 15-05-2012.
41. Bayer. *Teldor, Botrytis control for improving quality and shelf life*. 2011, 15-05-2012.

42. Goulart, B.L., Hammer, P.E., Evensen, K.B., Janisiewicz, W., and Takeda, F., *Pyrrolnitrin, Captan + Benomyl, and High CO2 Enhance Raspberry Shelf Life at 0 or 18C.* Journal of the American Society for Horticultural Science, 1992. 117:265-270.

43. Gianessi, L. *The Benefits of Insecticide Use: Raspberries*. 2009, 15-05-2012.

44. Butot, S., Putallaz, T., and Sanchez, G., *Effects of sanitation, freezing and frozen storage on enteric viruses in berries and herbs.* Int J Food Microbiol, 2008. 126(1-2):30-5.

5



Quantitative farm-to-fork risk assessment model for norovirus and hepatitis A virus in European leafy green vegetable and berry fruit supply chains

Martijn Bouwknegt[a*], Katharina Verhaelen[a,b], Artur Rzeżutka[c], Iwona Kozyra[c], Leena Maunula[d], Carl-Henrik von Bonsdorff[d], Apostolos Vantarakis[e], Petros Kokkinos[e], Tamas Petrovic[f,] Sava Lazic[f], Ivo Pavlik[g], Petra Vasickova[g], Kris A. Willems[h], Arie H. Havelaar[a,b], Saskia A. Rutjes[a] and Ana Maria de Roda Husman[a,b]

[a] Centre for Infectious Disease Control, National Institute for Public Health and the Environment, Bilthoven, The Netherlands, [b] Institute for Risk Assessment Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands, [c] Department of Food and Environmental Virology, National Veterinary Research Institute, Puławy, Poland, [d] Department of Food Hygiene and Environmental Health, Faculty of Veterinary Medicine, University of Helsinki, Helsinki, Finland, [e] Department of Public Health, Medical School, University of Patras, Patras, Greece, [f] Virology Department, Scientific Veterinary Institute “Novi Sad”, Novi Sad, Serbia, [g] Veterinary Research Institute, Brno, Czech Republic, [h] Department of Microbial and Molecular Systems, KU Leuven University, Leuven, Belgium

Article in press in the International Journal of Food Microbiology

Abstract

Fresh produce that is contaminated with viruses may lead to infection and viral gastroenteritis or hepatitis when consumed raw. It is thus important to reduce virus numbers on these foods. Prevention of virus contamination in fresh produce production and processing may be more effective than treatment, as sufficient virus removal or inactivation by post-harvest treatment requires high doses that may adversely affect food quality. To date knowledge of the contribution of various potential contamination routes is lacking. Models were developed for human norovirus (NoV), hepatitis A virus (HAV) and human adenovirus (hAdV) in raspberry and salad vegetable supply chains to quantify contributions of potential contamination sources to the contamination of produce at retail. These models were further used to estimate public health risks for NoV and HAV. Model parameterization was based on monitoring data from European supply chains and literature data. No human pathogenic viruses were found in the soft fruit supply chains; hAdV was detected and used as indicator of faecal pollution to assess the contribution of potential contamination points. Estimated risks per serving of lettuce based on the models were 3×10^{-4} (6×10^{-6}–5×10^{-3}) for NoV infection and 3×10^{-8} (7×10^{-10} – 3×10^{6}) for hepatitis A jaundice. The contribution to virus contamination of hand-contact was larger as compared with the contribution of irrigation, the conveyor belt or the water used for produce rinsing. In conclusion, viral contamination in the lettuce and soft fruit supply chains occurred and estimated health risks were generally low. Nevertheless, the 97.5% upper limit for the estimated NoV contamination of lettuce suggested that infection risks up to 50% per serving might occur. Our study suggests that attention to full compliance for hand hygiene will improve fresh produce safety related to virus risks most as compared to the other examined sources, given the monitoring results. This effect will be further aided by compliance with other hygiene and water quality regulations in production and processing facilities.

Introduction

In the Netherlands, 23% of all food (incl. water)-related gastroenteritis cases are estimated to be caused by norovirus (NoV), rotavirus and hepatitis A virus (HAV), compared to 9% by bacteria [1]. When combining these estimates with the severity of illness following infection, rotavirus and NoV were the third and fourth most important food-related pathogens, following *Toxoplasma gondii* and *Campylobacter* spp. [1]. To provide safe food it is thus important to direct efforts at reducing the numbers of viruses on consumed food items, preferably by prevention as recommended by EFSA [2]. Reported foodborne virus outbreaks among humans are frequently associated with fresh produce such as raspberries, salad vegetables and sun-dried tomatoes [e.g., 3, 4, 5]. The consumption of fresh produce does not involve preparation steps that inactivate viruses, and therefore the infection risks need to be reduced prior to retail. Inactivation processes applicable before retail, however, may not suffice to eliminate norovirus contamination and may lead to unacceptable food quality [6, 7].

As an alternative to inactivating treatments, effective prevention of contamination could reduce virus numbers on produce and thereby decrease consumer risks of viral foodborne infections and therefore illness. Therefore, knowledge on the contribution of potential contamination points to the overall virus-contamination of food, the effects of food handling on the virus contamination and virus-specific characteristics such as persistence and transfer proportions due to surface contact is required. Such data were gathered in the European FP7 project 'VITAL', which focused on the integrated approach of data collection and data analysis for use in risk assessment and management. Potential contamination points along three raspberry, one strawberry and three lettuce supply chains were identified using HACCP-based questionnaires and site visits by HACCP experts (Table 1). Required sample sizes were then calculated and delivered in chain-specific sampling plans to the data gathering laboratories. Samples were collected longitudinally per chain [8, 9] and analyzed for norovirus (NoV; genogroups 1 and 2) and hepatitis A virus (HAV) with optimized, standardized detection procedures [10, 11]. In addition to these human pathogenic viruses, the presence of human adenoviruses (hAdV) was examined to demonstrate that a route of contamination existed from infected humans to the sampling point, which other enteric viruses could follow. These monitoring data are subsequently used in a quantitative microbiological risk assessment model to estimate the human health risks associated with the consumption of fresh produce and the contribution of potential contamination points to the overall virus contamination. The model consists of newly developed mathematical descriptions of the contamination points along supply chains to examine the most important contamination points among those considered. The model was parameterized using the raw data collected in VITAL, completed with literature data to fill remaining data gaps. That model and the model outcomes are presented in the current paper.

Materials and methods

Conceptual model

Food supply chains differed within and between countries. For instance, harvested produce was sold at farmers' markets without further processing, others were rinsed and further handled prior to transport to supermarkets. Each process step was represented by a specific module, with each module describing the net contribution to the overall virus concentration on the produce. The full conceptual model that was considered is shown in Figure 1. The appropriate modules were selected per supply chain and linked in a fixed chronological order: irrigation, harvesters' hands, conveyor belt, food handlers' hands, rinsing, consumption and dose response. Contamination occurring in kitchens of consumers was not considered in the current model. Other potential sources that might contribute to virus contamination, such as direct contamination with faeces in the production fields and addition of viruses through pesticides that are prepared with surface water [12], were not included at present due to lack of data. Furthermore, potential intrinsic contamination of viruses through uptake via roots or leafs was not considered in the current model.

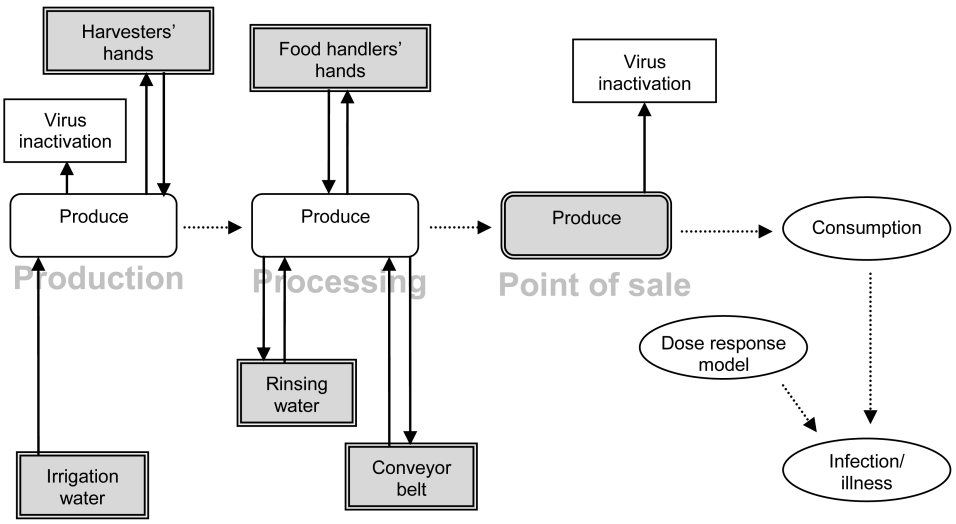


Figure 1. Full conceptual model of the soft fruit and leafy green vegetable production chains. Each box represents a module. The actual models differ per production chain based on the practice applied in that chain. Double-lined, shaded boxes indicate where samples were collected in the monitoring. Ovals indicate processes that occur in the consumer phase.

Supply chains

Three salad vegetable chains were studied. The practices in supply chain A could be represented by the irrigation module, the harvesters' hands module and the rinsing water module (Table 1). The type of lettuce produced was romaine lettuce. The second and third supply chains (B & C) could be represented by the irrigation module and harvester's hands module only. The type of lettuce produced was butterhead lettuce.
Two raspberry chains were studied. These chains were short with no processing of fruits involved, and spray irrigation was not used in the examined supply chains. One of the chains, chain D, involved mechanical harvesting and food handlers inspecting and touching the berries on the conveyor belt. Chain D was therefore represented by two modules: conveyor belt and food handlers' hands (Table 1). Chain E involved manual harvesting of berries, followed by transport on a conveyor belt. Chain E was therefore represented by two modules: harvesters' hands and conveyor belt.

The strawberry production chain (chain F) employed drip irrigation and manual harvest followed by immediate transport of berries to retail after harvest. This chain was therefore modeled using only the harvesters' hands module.

Table 1. Overview of potential contamination points modelled per production chain, including results (positive/total) from the production chain monitoring [8, 9].

Chain	Product	Irrigation	Harvesters	Food handlers	Rinsing	Conveyor belt	Consumption & Dose response
A	Romaine lettuce	■ hAdV: 17/22 NoV: 1/5	■ hAdV: 31/87 NoV: 1/12		■ hAdV: 2/6 NoV: n.a.[a]		■
B	Butterhead lettuce	■ hAdV: 0/17	■ hAdV: 3/66				■
C	Butterhead lettuce	■ hAdV: 0/22 HAV: 0/20	■ hAdV: 1/86 HAV: 2/87				■
D	Raspberries[b]			■ hAdV: 1/51		■ hAdV: 0/15	
E	Raspberries[b]		■ hAdV: 4/64			■ hAdV: 0/24	
F	Strawberries[b]		■ hAdV: 1/60				

[a] na: not available
[b] No consumption and dose-response, because no human pathogenic viruses were found in the monitoring and only hAdV was model

Modules

Irrigation

The production sites that were monitored in VITAL applied drip irrigation, spray irrigation or no irrigation. Drip irrigation supplies water at the branches or roots of crops and therefore was not considered for external contamination of produce in the current study. The irrigation water module thus considered contamination with virus through spray irrigation, which was used only in the lettuce head production chains.

Irrigation schemes are developed to provide each plant with a certain volume of water. It was therefore considered reasonable to assume a uniform distribution of the sprayed water across the crop field during irrigation. Under this assumption, the volume of water that falls on the lettuce head was estimated from the average intensity of irrigation across the field (I_{irw}; in L per m^2). Assuming the water falls onto the heads from one direction, and the lettuce head is a round sphere with diameter d (in m), then the surface area of the produce that is watered (ω_{prod}; m^2) can be estimated as half a sphere by $0.5 \times 4\pi(0.5d)^2$. The estimated number of viruses deposited per unit of product item due to spray irrigation (n_{irw}) was calculated as:

$$n_{irw} = C_{irw} I_{irw} \omega_{prod} \quad (1)$$

where C_{irw} is the estimated virus concentration per L water. Data about the probability of human pathogenic viruses attaching to the produce are lacking. The worst-case situation involving the attachment of all viruses falling onto the produce was therefore modeled, which may lead to an overestimation of the risk.

Petterson et al. [13] showed experimentally that for bacteriophages the last irrigation event before harvest is most determining for the virus contamination of lettuce heads by irrigation. Therefore the irrigation module considered one single irrigation event (although an extension to multiple irrigation events could easily be made).
If spray irrigation was not applied, then n_{irw} was considered to be zero.

Harvester's hands

The proportion of virus that transfers from harvesters' hands to produce depended on the surface concentration of viruses on the whole hand (C_{harv}; n per hand), the surface area of the hand that touches the product (ω_{harv}; in cm^2) and the proportion of viruses transferred from hand to product (f_{hand}). Furthermore, a proportion of viruses already present on the product may be transferred from the product to hands (f_{rasp} and f_{lett}, generally referred to as f_{prod}), reducing the number of viruses in this module. For products that are harvested manually, the number of viruses per product after harvest (n_{harv}) was calculated as:

$$n_{harv} = n_{irw} - f_{prod}\frac{\omega_{harv}}{\omega_{prod}}n_{irw} + f_{hand}\frac{\omega_{harv}}{\omega_{hand}}C_{harv} \quad (2)$$

with ω_{hand} being the total surface area of a harvesters' hand.

The surface area of both hands, including palm, back and fingers, is reported to be on average 0.107 m^2 for males and 0.089 m^2 for females [14]. These estimates were further combined to a single mean estimate of 0.1 m^2, or 1000 cm^2, for both hands, because the ratio between male and female harvesters was unknown. Lettuce heads were assumed to be picked using one hand only (the other hand is used for cutting) and therefore the palm surface area of 245 cm^2 was used for ω_{harv}. Soft fruits are generally picked with three fingers. Verhaelen et al. [15] estimated the surface area of the thumb, index finger and middle finger for touching a raspberry at 0.7 cm^2 per finger and found no statistical difference between the surface area of the three finger types. The value for ω_{harv} of 2.1 cm^2 was therefore used for soft fruits in the current study.

The fraction of virus transfer from hands to product (raspberry, strawberry and lettuce) and product (raspberry and lettuce) to hand after contact was estimated experimentally for hAdV and NoV genogroup 1 and 2 by Verhaelen et al. [15]. The values appropriate for the virus and transfer route modeled in the current study were used. These values include the mean transfer proportion and the associated uncertainties.

If produce is picked mechanically (*e.g.*, by shacking bushes and collecting berries), as was done in one of the raspberry supply chains, then n_{harv} was assumed to equal n_{irw} (i.e., no handling of produce occurred).

Conveyor belt

The conveyor belt was only used in the soft fruit production chains and was modeled to contribute to the number of viruses per product after this stage (n_{belt}) by transfer of viruses from the conveyor belt to the produce and by transfer of previously introduced viruses from the produce to the conveyor belt according to equation 3:

$$n_{belt} = n_{harv} - f_{prod}\pi_{belt}n_{harv} + f_{hand}\pi_{belt}\omega_{prod}C_{belt} \quad (3)$$

where π_{belt} is the proportion of the surface area of the fruit item that touches the conveyor belt, ω_{prod} is the total surface area of the fruit item (cm^2) and C_{belt} is the number of viruses per cm^2 of belt. Specific transfer proportions from conveyor belts to fruits could not be retrieved from literature and therefore were assumed to be equal to the transfer proportion from produce to hands (f_{hand}) as described in section Harvester's hands. The value

for π_{belt} was unknown and modeled with a uniform distribution between 0.25 and 1 to simulate its uncertainty, from one side of a berry touching the belt up to all of the berry. The value of C_{belt} was based on swab samples collected during the monitoring. The swab samples were collected from a recorded area of the conveyor belt, allowing for estimation of the virus contamination per cm^2 under the assumption that viruses are distributed homogeneously. If a conveyor belt was not used, then n_{belt} equaled n_{harv}.

Food handlers' hands

Food handlers touching lettuce heads occurred in a single food supply chain, for cutting. For soft fruits, touching by food handlers occurred on occasion during transport on a conveyor belt to turn the berries for visual inspection. For these supply chains, the number of viruses per product after food handling (n_{touch}) was modeled with equation 4:

$$n_{touch} = n_{belt} - f_{prod}\pi_{food}n_{belt} + f_{hand}\frac{\omega_{food}}{\omega_{hand}}C_{food} \quad (4)$$

with C_{food} being the virus number per hand of a food handler's hand and π_{food} being the proportion of the surface area of the food handlers' hand touching the product. For lettuce heads, the touching surface of a hand (ω_{food}) was modeled similarly as for harvesters' hands. For soft fruits, π_{food} was unknown and modeled using a uniform distribution between 0 and 1, because part of the berries likely remain untouched whereas other berries made a full turn when touched as based on visual observations in one of the production chain. If berries were untouched, then n_{touch} equaled n_{belt}.

Rinsing water

Rinsing was applied in a single salad vegetable supply chain. This process can affect the virus contamination by addition and removal of virus. The addition can result from viruses already present in the rinsing water before start of washing, or due to contamination of formerly clean water by already-contaminated produce. Removal of virus can occur due to washing-off. The number of viruses after rinsing (n_{rinse}) was estimated using equation 5:

$$n_{rinse} = n_{touch}10^{-f_{rinse}} + C_{rinse}V_{rinse} \quad (5)$$

where f_{rinse} is the decimal removal rate of viruses due to rinsing, C_{rinse} is the virus concentration in the rinsing water and V_{rinse} is the volume of rinsing water that clings to the produce. C_{rinse} was estimated from the data obtained during monitoring. V_{rinse} was obtained from Shuval et al. [16], who experimentally assessed the volume of water retained by long leaf lettuce after full immersion under water to be 10.8 ml per 100 g of lettuce.

This figure was considered the maximum carrying capacity of water by lettuce. The examined lettuce type that was rinsed in the current study weighted on average 1.8 kg at retail, and thus would carry at most about 200 ml of water after rinsing when assuming the 10.8 ml per 100 g is representative for each 100 g of the lettuce head. The virus concentration in the rinsing water was not modelled dynamically in this model.
Mohktari and Jaykus [17] synthesized several experimental studies on virus removal due to washing and concluded that the effect of rinsing with clean water (f_{rinse}) was best described by a uniform distribution of 1 to 2 $\log_{10}$ units removal. In absence of more recent and virus-specific data for the current study, the same approach was used.

Virus inactivation
Virus inactivation for NoV and hAdV was modeled as exponential reduction using the general virus inactivation model presented by Verhaelen et al. [18]. This model estimates the temperature-dependent average daily reduction (ADR) in virus numbers. For viruses detected by PCR on fruits kept refrigerated at 5 °C, the mean predicted $\log_{10}$ ADR was 0.011 $\log_{10}$-units per day. For viruses on fruits at 20 °C, the mean predicted $\log_{10}$ ADR was 0.151 $\log_{10}$-units. For lettuce, the same values as for raspberries were used regarding ADR and temperature.

The inactivation rate of hepatitis A virus for exponential reduction was taken from Bertrand et al. [19], who provided estimates for HAV inactivation at temperatures <50 °C. Using their results, the estimated time to first $\log_{10}$-unit reduction (TFL) of infectious HAV at 4 °C and 20 °C was 76 (95% prediction interval: 6–928) and 25 (2–302) days, respectively, with associated average ADRs of 1/76=0.013 and 1/25=0.040 $\log_{10}$ units per day. Note that these estimates are for culturable HAV. However, estimates based on PCR detection have not been reported.

The times of inactivation considered per module were: 14 days for irrigation (assuming the last irrigation event occurs two weeks before harvest) applied to n_{irw} and subsequently 7 days for one other module (assuming that fresh produce is consumed seven days after harvest) applied to either n_{harv}, n_{rin}, n_{touch} or n_{belt} (whichever is modeled last in the specific food production chains; only one variable is corrected to prevent double accounting for inactivation).

Consumption
The exposure and public health risks were estimated for consumption of lettuce only, because no human pathogenic viruses were found on berries. The amount of lettuce consumed was set to 200 g per event, which is the advised amount of vegetables to be consumed per day in the Netherlands.

Risk characterization

Estimation of virus concentrations per source

Virus counts in positive samples were expressed as PCR-detectable units (PDU), with one PDU representing an unknown number of virus particles (1 or more) depending on the properties of the (RT-)PCR. By definition, the lower limit of detection for any (RT-)PCR is 1 PDU in a reaction vessel. The estimated virus concentration for each sample was based on the actual portion of the sample that was examined in the (RT-)PCR. This portion was back-calculated using the concentration and dilution factors in nucleic acid isolation and detection procedures. The PDU were assumed to be distributed randomly within samples, and to be gamma-distributed between samples. The most likely parameter value for the Gamma distribution was obtained by maximum likelihood estimation as described by Bouwknegt, Teunis [20]. Briefly, the likelihood of an actual virus concentration per sample, c_k, was assessed using the presence/absence profiles for neat and serial 10-fold diluted RNA solutions for sample k according to:

$$g_k(c_k \mid V, p) = \prod_{i=1}^{J} (1 - \mathrm{Exp}[-c_k \cdot V_{pcr,i}])^{p_i} \cdot (\mathrm{Exp}[-c_k \cdot V_{pcr,i}])^{1-p_i} \qquad (6)$$

where $V_{pcr,i}$ represents the actual portion of the sample represented in solution i (e.g., 1 = neat, 2 = 10-fold dilution, etc.), and p_i denotes the presence (p_i=1) or absence (p_i=0) of ≥1 PDU in solution i. The likelihood for the parameters r and λ of a gamma distribution based on n samples per sampling point was subsequently assessed by

$$\ell(r, \lambda) = \prod_{k=1}^{n} \int_{c=0}^{\infty} \frac{c^{r-1} e^{-\frac{c}{\lambda}}}{\lambda^k \Gamma(r)} \cdot g_k(c) \qquad (7)$$

When the likelihood function (7) did not converge, then the virus concentration was assumed to be homogeneously distributed between samples (i.e., all observations were treated as if obtained from a single sample k) and eqn. (6) was used. When no positive samples are found for a potential contamination point, then the most likely estimate of the PDU concentration is zero. The 95% upper limit of the virus concentration was subsequently estimated by solving eqn. (6) for a -2 Log transformation of the likelihood value of 3.84 (these transformed likelihoods are approximately chi-square distributed with 1 degree of freedom). This 95% upper limit of the virus concentration was included in respective modules for estimating the approximate 95% upper limit of the total estimated virus contamination per product.

Dose response models

The used hypergeometric dose-response model for norovirus (combined 8fIIa and 8fIIb) was developed by Teunis et al. [21], who based the model on volunteer experiments with Norwalk virus, a particular NoV strain. This model allows for heterogeneity in host susceptibility to NoV infection.

The exponential dose-response model for hepatitis A virus was initially described by Haas et al. (1998), based on data from Ward et al. [22] who inoculated institutionalized individuals with HAV and monitored the occurrence of jaundice (hence the prediction of jaundice cases for this dose-response model). The dose administered to the individuals was expressed as g of faeces. The ingested doses in the VITAL risk assessments were PDUs per consumption event. To harmonize the estimated dose in the model with the dose inoculated by Ward better, the likelihood function of the dose response model using the original doses reported by Ward et al. [22] was expanded with a likelihood function for the number of HAV genome copies per g of faeces as reported by Kamel et al. [23]. This model assumes that all individuals are equally susceptible to develop jaundice for a certain exposure dose.

Risk estimation

The estimated virus concentrations on the end product were estimated by Monte Carlo simulation in Mathematica version 8 taking 10,000 random samples from the uncertainty distributions. Table 2 listed all parameters and their values or distributions used in the risk assessment.

The estimates from the model were compared to the estimates based on the point-of-sale monitoring of produce. Virus numbers per food product were estimated as described in section Estimation of virus concentrations per source, and the exposure was assessed using the consumption data as described in section Consumption.

Sensitivity analysis

The sensitivity analysis was done for two production chains to cover each module (lettuce supply chain A and raspberry chain A). The sensitivity of the model to each parameter was assessed multivariably using Monte Carlo simulation (10,000 iterations). For each parameter a value was randomly drawn from the distributions as listen in Table 3 per iteration and used to estimate the contamination level for the produce. Spearman rank correlations coefficients (SCCs) were subsequently assessed in SAS v9.3 (SAS Institute, Cary, USA) to rank the correlation between the estimated contamination levels and input parameter values [24]. The SCCs were used, because the risk assessment model is nonlinear and monotonic. Those parameters with the highest SCC were considered most influential.

Table 2: List of parameters, their values and/or uncertainty distributions used in the risk assessment models.

Parm.	Explanation	Value or distribution	Ref
C_{irw}	Virus PDU concentration in irrigation water (*n* per L water)	NoV, romaine lettuce (chain A): Gamma[0.084, 0.039] hAdV romaine lettuce (chain A): Gamma[0.577, 0.031] hAdV, butterhead lettuce (chain B): most likely 0; 95% upper limit: 0.12 HAV, butterhead lettuce (chain C): most likely 0; 95% upper limit: 0.12 hAdV, butterhead lettuce (chain C): most likely 0; 95% upper limit: 0.11	Estimated in this study
V_{irw}	Volume of water (V_{irw}) sprayed per unit surface	50,000 L per ha or 0.5 ml per cm^2 (provided by suppliers)	Determined in this study
ω_{prod}	Surface area of produce	Raspberry: Normal[1064, 167] mm^2 Strawberry: Normal[1064, 167] mm^2 Butterhead lettuce: 1400 cm^2 Romaine lettuce: 226 cm^2	Estimated in this study
f_{prod}	Transferred proportion per touch from produce to hand	NoV, lettuce: Beta[18.55, 49.05] HAV, lettuce: data for NoV used: Beta[18.55, 49.05] hAdV, lettuce: Beta[13.36, 430] hAdV, raspberry: Beta[15.64, 41.94] NoV, strawberry: data for raspberries used, Beta[7.42, 39.51]	[15]
f_{hand}	Transferred proportion per touch from hand to produce	NoV, lettuce: LogNormal[-2.22, 0.17] hAdV, lettuce: Beta[15.14, 46.72] hAdV, raspberry: LogNormal[-8.34, 0.58] NoV, strawberry: LogNormal[-2.32, 0.15]	[15]
ω_{harv}	Surface area of hands that touch produce	Lettuce: 245 cm^2 Raspberry and strawberry: 2.1 cm^2	[14] [15]
ω_{hand}	Total surface area of one side of one hand	245 cm^2	[14]
π_{food}	Proportion of the food handlers' hand touching produce	Uniform[0, 1]	Assumed in this study
C_{harv}	Virus PDU number on harvesters' hands (*n*)	NoV, romaine lettuce (chain A): Gamma[1.11, 4.46] hAdV, romaine lettuce (chain A): Gamma[1.22, 24.53] hAdV, butterhead lettuce (chain B): Gamma[0.06, 117] HAV, butterhead lettuce (chain C): Gamma[0.98, 1.55] hAdV, butterhead lettuce (chain C): Gamma[0.009, 141.2]	Estimated in this study
C_{harv}	Virus PDU number on harvesters' hands (*n*)	hAdV, raspberry (chain E): Gamma[0.14, 54.6] hAdV, strawberry (chain F): Gamma[0.002, 40135]	Estimated in this study
C_{food}	Virus PDU concentration on food handlers' hands (*n*)	hAdV, raspberry site A: Gamma[0.67, 1.62]	Estimated in this study
C_{belt}	Virus concentration on conveyor belts (*n* per m^2)	hAdV: most likely 0; 95% upper limit: 70	
π_{belt}	Proportion of the berry surface touching the conveyor belt	Uniform[0.25, 1]	Assumed in this study
f_{rinse}	Decimal removal rate of viruses due to rinsing	Uniform[1, 2]	[17]
δ	Temperature-dependent virus decay (log_{10} units per day)	NoV, MCMC post., *T* was set at 20°C for irrigation and 5°C for all other modules	[18]
		HAV, for *T*=20: Normal[1.88,0.555], for *T*=5: Normal[1.40, 0.555]	[19]
$(\alpha, \beta)_{NoV}$	Set of parameters for the NoV dose response model	MCMC post. obtained from corresponding author of reference	[21]
r_{HAV}	Infectivity of HAV in exponential dose response model	MCMC post., mean: 4×10^{-6}; 95% interval: 1×10^{-6} – 1×10^{-5}	Estimated in this study

Table 3: Alternative parameter values for the sensitivity analysis (see Table 2 for an explanation of the parameters).

	Probabilistic analysis	Scenario-based analysis				
Parameter	Distribution	lowest	low	baseline	high	highest
ω_{lett}	$r \sim$ Uniform[6, 24]*	57	127	226	509	905
I_{irw}	$0.5\times10^{\text{Uniform}[-2,2]}$	0.005	0.05	0.5	5	50
C_{irw}	$0.02\times10^{\text{Uniform}[-2,2]}$	2×10^{-4}	2×10^{-3}	2×10^{-2}	2×10^{-1}	2
ω_{harv}	Uniform[0.25×2.1, 4×2.1]	0.5	1.1	2.1	4.2	8.4
f_{lett}	Uniform[0, 1]	0	0.003	0.03	0.3	1
f_{hand}	Uniform[0, 1]	0	0.01	0.1	.	1
ω_{hand}	Uniform[123, 490]	123	184	245	368	490
C_{harv}	$30\times10^{\text{Uniform}[-2,2]}$	0.3	3	30	300	3,000
f_{rinse}	Uniform[0, 1]	0	0.5	1.5	2.5	3.5
π_{belt}	Uniform[0, 1]	0	.	0.625	.	1
ω_{rasp}	Uniform[6.8, 14.5]**	6.8	9.5	10.6	11.8	14.5
C_{belt}	$10^{\text{Uniform}[0,4]}$	1	10	100	1,000	10,000
f_{rasp}	Uniform[0, 1]	0	0.03	0.3	.	1
π_{food}	Uniform[0, 1]	0	.	0.5	.	1
C_{food}	$10^{\text{Uniform}[0,4]}$	1	10	100	1,000	10,000

* r is the radius of a lettuce head, ω_{lett} is calculated as $\frac{1}{2}4\pi r^2$; ** Parameters are based on the 1% and 99% limits of the distribution for raspberry surface as described in Table 1.

The SCC does not provide insight in the magnitude of change of the outcome due to alternative parameter value. The relative change in estimated contamination of produce was therefore assessed using a scenario-based approach with alternative parameter values as listed in Table 3. Each parameter was adjusted individual per scenario. Alternative parameter values were based on modifications of the baseline value (virus concentrations based on hAdV), except for the hAdV concentration on the conveyor belt and food handlers' hands. The most likely values for the latter two parameters were 0, and therefore the sensitivity was based on the hypothetical concentration of 1 up to 10^4 PDU. Note that the assumption that a whole hand touches a lettuce head removes the proportion $\omega_{harv}/\omega_{hand}$ from Eqn. 2 for lettuce heads, and thus also from the sensitivity analysis.

Results

Salad vegetables

Human adenovirus was included in the VITAL study to demonstrate an existing route of human faecal contamination, and used to estimate the contribution of irrigation water and harvesters' hands to the overall produce contamination. Human adenovirus

was found in none of 17 (Chain B) and 22 (Chain C) irrigation water samples, respectively, whereas hAdV was detected on 3 of 66 and 1 of 86 harvesters' hands for Chain B and C, respectively (Table 1). In supply chain A, hAdV was detected in irrigation water and on harvesters' hands. Using the model, the estimated average contamination was 0.01 (6×10^{-4}-0.08) for chain A (romaine lettuce) and 0.08 (95% interval: ~0 – 8) and 5×10^{-5} (~0 – 7) for chains B and C, respectively (butterhead lettuce) (Table 4), with harvesters' hands contributing most to the contamination (Figure 2A).

Human pathogenic viruses were detected in samples taken in supply chains A and C. In chain A, NoV was found in 1 of 5 irrigation water samples and in 1 of 12 harvesters' hands swabs (Table 1). Rinsing water was not examined for human pathogenic viruses. The most likely NoV concentrations in the water and on hands were 3 PDU (95% interval: 0 – 34) per L water and 5 PDU (95% interval: 0.2 – 17) per hand, respectively. The median estimated virus number per lettuce head at point of sale was 9×10^{-3} (3×10^{-4} – 0.08), yielding a median estimated infection risk of 3×10^{-4} (6×10^{-6} – 5×10^{-3}) for the consumption of 100 g of romaine lettuce. Harvesters' hands contributed more to the estimated NoV contamination than irrigation water (Figure 2B). The estimated infection risk based on point-of-sale data was 0, with a 97.5% upper limit of 0.5.

In chain C, HAV was found in none of 20 irrigation water samples and in 2 of 87 harvesters' hands swabs (Table 1). The most likely HAV concentrations in the water and on hands were 0 PDU per L water (95% upper limit: 0.12) and 1 (95% interval: 0.07 – 18), respectively. The median estimated HAV PDU concentration at point of sale per lettuce head based on the model was 8×10^{-2} (5×10^{-3} – 7), yielding an estimated risk for HAV jaundice of 3×10^{-8} (7×10^{-10} – 3×10^{-6}) for the consumption of 100 g (Table 4). No

Table 4. Estimated virus concentration (mean PDU and 95% interval) per produce item, risk of infection after consumption or risk of jaundice after consumption for fresh produce. Estimates are either based on the production chain model or on the point of sale measurements as described in section Risk estimation.

		Estimate based on	
Produce	Virus	Chain model	Point of sale monitoring
Contamination per item			
Romaine lettuce (chain A)	hAdV	0.01 (6×10^{-4} – 0.08)	600 (42 – 3×10^{3})
Butterhead lettuce (chain B)	hAdV	5×10^{-5} (~0[a] – 7)	0.02 (3×10^{-3} – 0.06)
Butterhead lettuce (chain C)	hAdV	0.08 (~0 – 8)	50 (~0 – 586)
Raspberry (chain D)	hAdV	1×10^{-5} (5×10^{-8} – 0.1)	0.03 (0.003 – 0.08)
Raspberry (chain E)	hAdV	1×10^{-5} (~0 –1×10^{-3})	0.01 (8×10^{-4} – 0.07)
Strawberry (chain F)	hAdV	9×10^{-6} (0 – 1×10^{-7})	1.2 (~0 – 1.3)
Infection risk per serving			
Romaine lettuce (chain A)	NoV	3×10^{-4} (6×10^{-6} – 5×10^{-3})	0 (0 – 0.5)
Jaundice risk per serving			
Butterhead lettuce (chain C)	HAV	3×10^{-8} (7×10^{-10} – 3×10^{-6})	0 (0 – 2×10^{-6})

[a] Estimate <10^{-10}

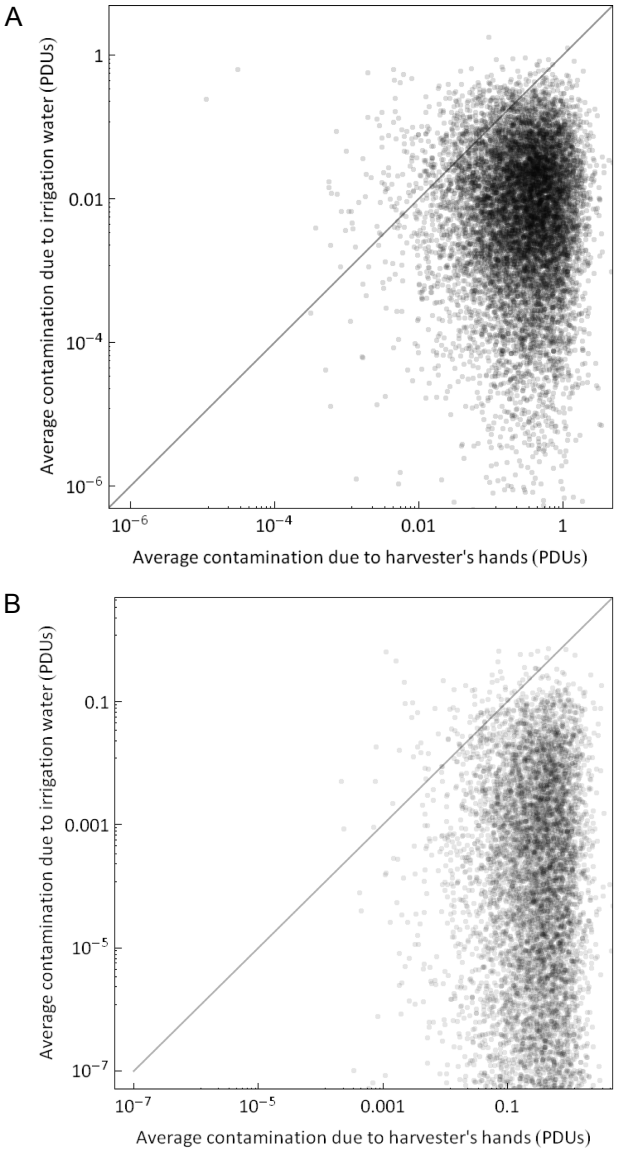


Figure 2. Contribution of harvesters' hands and irrigation water to the estimated contamination of lettuce heads with hAdV (A) and NoV (B) for supply chain A. Each marker represents a single iteration from the Monte Carlo simulation. The grey line represents an equal contribution for both potential contamination points. Markers positioned above this line indicate a greater contribution for irrigation water (than hands) and markers below the line indicate a greater contribution for hands.

HAV was found on lettuce heads at point of sale, leading to an associated 97.5% upper limit of 1×10^{-6} for the jaundice risk based on monitoring data.

Soft fruits

No human pathogenic viruses were detected in samples from either of the two raspberry supply chains, while hAdV was detected [9]. The estimated average number of hAdV per raspberry at point of sale using the model was 1×10^{-5} (95% interval: 2×10^{7} – 0.09) for chain D and 6×10^{-6} (95% interval: 8×10^{7} – 2×10^{-5}) for chain E (Table 4). At both chains, the majority of the virus contamination was estimated to originate from hands, because no hAdV was found on the conveyor belts. The monitoring at points of sale, with the locations linked to these two production sites, identified 2 of 28 and 1 of 37 raspberry samples to be contaminated with hAdV, leading to estimated virus concentrations of 0.04 (0.01 – 0.12) PDU and 0.01 (0.001 – 0.07) PDU per berry for site D and E, respectively (Table 4).

In the strawberry monitoring (chain F), NoV was not detected on harvester hands' [9], while hAdV was detected on 1 of 60 hands. The most likely hAdV PDU concentrations on hands was 70 PDU (28 - 131) per hand. The estimated NoV PDU concentration at point of sale for strawberries was 2×10^{-4} PDU (3×10^{-5} – 5×10^{-4}) per strawberry. In the point of sale monitoring hAdV was detected on 1 of 51 strawberries obtained at point of sale, giving a most likely average contamination of 3 (1 - 5) PDU per strawberry.

Sensitivity analysis

The most influential parameters for the lettuce head production chain was f_{rinse}, for the raspberry production chain C_{belt}. Overall, various parameters for estimated virus concentrations at potential contamination points (C_{irw}, C_{harv}, C_{food}) were among the top-ranked

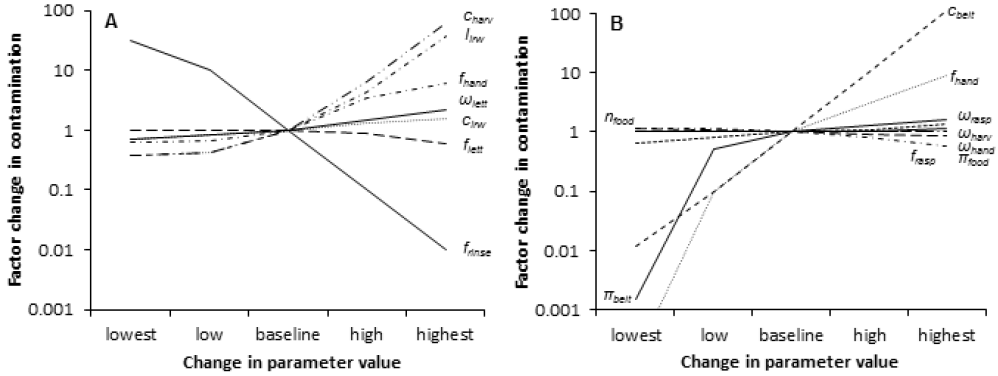


Figure 3. Results of the sensitivity analysis using the irrigation, the harvester hand and the rinsing modules for lettuce (A) and conveyor belt and food handler hand modules for raspberries (B). Details of the scenarios represented on the x-axis are described in Table 2.

Table 5. Spearman's Rank Correlation Coefficients (SCCs) for the correlation between model parameters and estimated contamination level of lettuce and raspberries, as part of the sensitivity analyses (see Table 2 for an explanation of the parameters).

Lettuce head model		Raspberry model	
Parameter	SCC	Parameter	SCC
f_{rinse}	-0.88	C_{belt}	0.86
C_{harv}	0.22	f_{hand}	0.31
I_{irw}	0.11	π_{belt}	0.24
C_{irw}	0.10	N_{food}	0.09
f_{hand}	0.06	f_{rasp}	-0.09
ω_{lett}	0.03	π_{food}	-0.07
f_{lett}	-0.01	ω_{rasp}	0.06
		ω_{hand}	-0.01
		ω_{harv}	0.00

influential parameters (Table 5). The parameter π_{belt} had an overall important impact on the results (Table 5), but the effect on the estimated virus concentration was one-sided (Figure 3). Alternative parameter values led to an increase or decrease up to two orders of magnitude on the final product within the range examined (Figure 3). The parameters describing proportions, such as transfer and surface area proportions, generally had smaller effects on the estimated contamination levels than the concentration estimates.

Discussion

The magnitude of a public health risk posed by viruses in the food chain cannot be assessed solely from detected presence or absence of viruses in the products at retail. A single sampling is usually not representative of the actual public health risk due to e.g. temporal and geographic variation, virus levels below the detection limit that results in false-negative results, and a non-homogeneous distribution of viruses on the product. Ideally, data on the pathogen concentration in the contamination source, the fate and behavior of the pathogen, the level of eventual exposure of humans and the probability of an adverse health event associated with that exposure are integrated, and a QMRA provides a valuable tool for this purpose [25, 26]. The model presented in the current study is developed with that aim and is relatively easily applicable to other situations when quantitative data on virus contamination in sources are available.

Ranking the contribution of potential contamination points showed that contact with hands was the most dominant contamination source given the current monitoring data and the modeling used. Hand hygiene may thus be a prime starting point for prevention

of contamination, as is the case for bacteria. Full (i.e., 100%) compliance at all times to current HACCP guidelines therefore likely contributes to virus-safe production of soft fruits and vegetables. The efficacy of proposed or implemented hygiene practices for viruses, however, needs to be assessed. For instance, the bactericidal activity of a hand rub is not per se similar to that for viruses [27]. Nonetheless, even washing with water is effective in reducing virus numbers on hands, as concluded from a quantitative meta-analysis using data from multiple experiments [17], and therefore is a recommended preventive measure.

As always with QMRA, restrictions in the current parameterization and generalizations are present. Firstly, the applied parameters of the dose-response models cause large uncertainties in results due to the heterogeneity among hosts (including susceptibility) and pathogens and the small sample size associated with parameterization of the dose-response models [21, 25]. Furthermore, an important aspect relates to the translation of estimated PDU concentrations into the ingested dose of infectious organisms. The detection of viral DNA or RNA by (RT-)PCR indicates that contamination has occurred in the production chain, but the presence of infectious viruses at the moment of sampling is not confirmed [28, 29]. Detected RNA could originate from defective virus particles. Furthermore, the exposure dose was estimated in PDU per event. Applying this dose to the dose response model implies that the ratio between infective and defective particles of the monitoring samples is identical to that of the dose-response samples. This ratio is however highly variable [30] and that assumption likely does not hold. However, no further information is available on this ratio and therefore it is not possible to indicate whether risks are over- or underestimated. Furthermore, no objective alternatives are currently available to include this ratio properly in risk assessment, but several methods are emerging for the determination of viability by PCR, including cell-culture PCR, long-template PCR and enzymatic pre-treatment with RNAses and propidium monoazide [31-35]. When fully validated for foodborne viruses in food environments, such approaches might be used to more accurately estimate the (likely variable) fraction of infectious viruses among all viruses detected by PCR. Similar efforts also need to be made for data supporting dose response models.

The estimated risks based on the production chain model and the point-of-sale samples agreed for the human pathogenic viruses. Yet the estimated contamination levels per produce item for hAdV differed several orders of magnitude with respect to most likely values. The uncertainty for the latter estimates, however, was large due to the relatively low sample sizes per sampling point and few positive samples and 95% intervals overlapped in four of five cases. Discrepancies between the model estimates and point-of-sale estimates can have several causes: 1) not all contamination points were included as

modules in the monitoring and the model; 2) the modules poorly reflected the essence of the included contamination processes; and 3) the contamination of produce is episodic in nature and sampling was too limited in time and space to provide data on the likelihood and the extent of incidental contamination. The virus concentrations for potential contamination points were shown in the sensitivity analysis to be most influential on the risk outcome, possibly altering the risk estimates several orders of magnitude. The higher estimates based on the point of sale monitoring might be the result of an episodic contamination event in the production of that particular batch, whereas similar events might not have been encountered during the production chain monitoring. The discrepancies between model estimates and point-of-sale estimates, the model sensitivity, but also the observed variety of processes employed in the supply chains, impact the generalizability of our results and shows the need for case specific parameterization of the risk model.

Given the relatively low risk estimates in the current study, but the occasional large extent of outbreaks, possibly the incidental contamination events contribute to a larger extent to the adverse public health effect than the general production practices. Episodic events may occur due to e.g. a single noncompliance event for hand hygiene or due to combined sewer overflows after heavy rainfall events. In other instances, prolonged outbreaks, such as for HAV in semi-dried tomatoes, suggest a structural contamination source along that food supply chain [36, 37]. Gaining insights into the effect of structural as well as episodic contamination points is important for accurate estimation of the effectiveness of implemented intervention measures.

Human pathogenic viruses were found at low numbers in the monitoring. These low numbers provide limited information on the virus concentrations for contamination points and thus affects the uncertainty of the virus concentration estimates. Hence the effort in the current study to model explicitly the uncertainty. Human adenovirus was found in a larger number of samples, providing more robust estimates of the virus concentrations, the exposure levels and the larger contribution of hand hygiene compared to irrigation water to the virus contamination. Nevertheless, the uncertainty for hAdV was also large. Future monitoring efforts for human pathogenic viruses along food production chains would benefit from even larger sample sizes than those examined in VITAL, combined with highly sensitive detection methods.

In conclusion, the current study showed that viral contamination in each of the different food production chains occurred. In addition to large epidemiological studies that have been conducted and have shown a significant number of people falling ill due to the consumption of virus-contaminated foods, the use of model-based risk assessments

adds value to the development of targeted intervention measures. It was shown here that the estimated mean risks were in general low, although some 95% upper limits did not exclude the potential for considerable risks (i.e., 50% infection risk for NoV on romaine lettuce) per serving. Furthermore, hand transfer was found to be a more likely contamination source for lettuce than irrigation water, based on the monitoring data and subsequent modeling. Our study suggests that attention to full compliance for hand hygiene will improve fresh produce safety related to virus risks most as compared to the other examined sources, given the monitoring results. This effect will be further aided by compliance with other hygiene and water quality regulations in production and processing facilities.

Acknowledgements

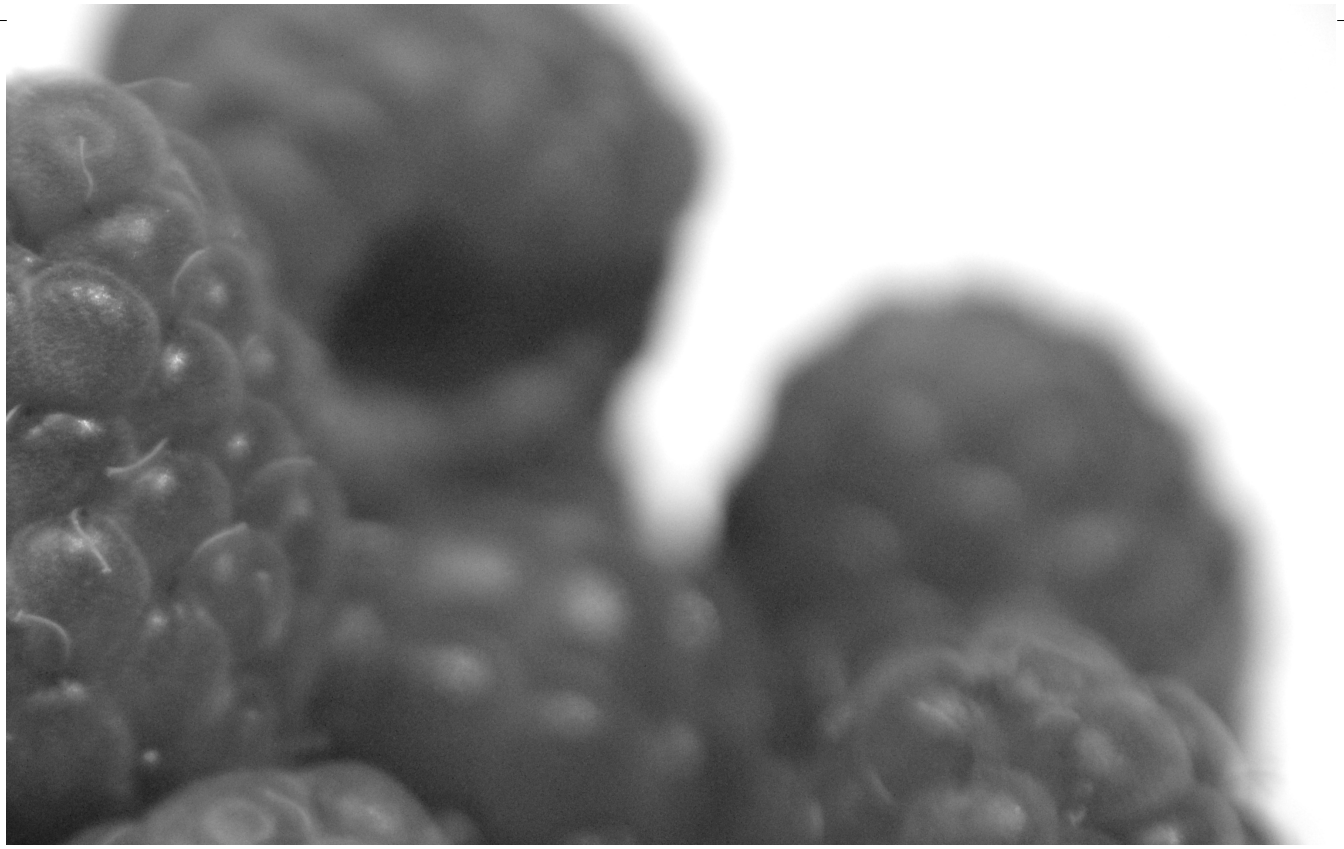
This work was supported by the European Commission Framework Program 7 project "Integrated monitoring and control of foodborne viruses in European food supply chains (VITAL)" (Grant No. KBBE 213178), led by the coordination team of Nigel Cook (FERA, UK), Martin D'Agostino (FERA, UK) and Franco M. Ruggeri (ISS, Italy). The work done by the Veterinary Research Institute (Czech Republic) was also financially supported by AdmireVet (CZ 1.05/2.1.00/01.0006; ED0006/01/01).

References

1. Havelaar, A.H., Haagsma, J.A., Mangen, M.J., Kemmeren, J.M., Verhoef, L.P., Vijgen, S.M., Wilson, M., Friesema, I.H., Kortbeek, L.M., van Duynhoven, Y.T., and van Pelt, W., Disease burden of foodborne pathogens in the Netherlands, 2009. Int J Food Microbiol, 2012. 156(3):231-8.
2. EFSA, Scientific Opinion on an update on the present knowledge on the occurrence and control of foodborne viruses. EFSA Journal, 2011. 9(7):2190.
3. Ethelberg, S., Lisby, M., Bottiger, B., Schultz, A.C., Villif, A., Jensen, T., Olsen, K.E., Scheutz, F., Kjelso, C., and Muller, L., Outbreaks of gastroenteritis linked to lettuce, Denmark, January 2010. Euro Surveill, 2010. 15(6).
4. Gallot, C., Grout, L., Roque-Afonso, A.M., Couturier, E., Carrillo-Santisteve, P., Pouey, J., Letort, M.J., Hoppe, S., Capdepon, P., Saint-Martin, S., De Valk, H., and Vaillant, V., Hepatitis A associated with semidried tomatoes, France, 2010. Emerg Infect Dis, 2011. 17(3):566-7.
5. Sarvikivi, E., Roivainen, M., Maunula, L., Niskanen, T., Korhonen, T., Lappalainen, M., and Kuusi, M., Multiple norovirus outbreaks linked to imported frozen raspberries. Epidemiol Infect, 2012. 140(2):260-7.
6. Niemira, B.A., Irradiation of fresh and minimally processed fruits, vegetables, and juices, in Microbial safety of minimally processed foods, J.S. Novak, G.M. Sapers, and V.K. Juneja, Editors. 2003, CRC Press. p. 279-300.
7. Baert, L., Debevere, J., and Uyttendaele, M., The efficacy of preservation methods to inactivate foodborne viruses. Int J Food Microbiol, 2009. 131(2-3):83-94.
8. Kokkinos, P., Kozyra, I., Lazic, S., Bouwknegt, M., Rutjes, S., Willems, K., Moloney, R., de Roda Husman, A.M., Kaupke, A., Legaki, E., D'Agostino, M., Cook, N., Rzezutka, A., Petrovic, T., and Vantarakis, A., Harmonised investigation of the occurrence of human enteric viruses in the leafy green vegetable supply chain in three European countries. Food Environ Virol, 2012. 4(4):179-91.
9. Maunula, L., Kaupke, A., Vasickova, P., Söderberg, K., Kozyra, I., Lazic, S., van der Poel, W.H.M., Bouwknegt, M., Rutjes, S.A., Willems, K., Moloney, R., D'Agostino, M., de Roda Husman, A.M., von Bonsdorff, C.H., Rzeżutka, A., Pavlik, I., Petrovic, T., and Cook, N., Tracing enteric viruses in the European berry fruit supply chain. Int J Food Microbiol, 2013. 167(2):177-185.
10. Martinez-Martinez, M., Diez-Valcarce, M., Hernandez, M., and Rodriguez-Lazaro, D., Design and Application of Nucleic Acid Standards for Quantitative Detection of Enteric Viruses by Real-Time PCR. Food Environ Virol, 2011. 3(2):92-98.
11. D'Agostino, M., Cook, N., Rodríguez-Lázaro, D., and Rutjes, S.A., Nucleic acid amplification-based methods for detection of enteric viruses: definition of controls and interpretation of results. Food and Environmental Virology, 2011. 3:55-60.
12. Verhaelen, K., Bouwknegt, M., Rutjes, S.A., and de Roda Husman, A.M., Persistence of human norovirus in reconstituted pesticides--pesticide application as a possible source of viruses in fresh produce chains. Int J Food Microbiol, 2013. 160(3):323-8.
13. Petterson, S.R., Teunis, P.F., and Ashbolt, N.J., Modeling virus inactivation on salad crops using microbial count data. Risk Anal, 2001. 21(6):1097-108.
14. USEPA, Exposure factors handbook. 1997, Washington: United States Environmental Protection Agency.
15. Verhaelen, K., Bouwknegt, M., Carratalà, A., Lodder-Verschoor, F., Diez-Valcarce, M., Rodríguez-Lázaro, D., De Roda Human, A.M., and Rutjes, S.A., Virus transfer proportions between gloved fingertips, soft berries, and lettuce, and associated health risks. Int J Food Microbiol, 2013. 166(3):419-25.
16. Shuval, H., Lampert, Y., and Fattal, B., Development of a risk assessment approach for evaluating wastewater reuse standards for agriculture. Water Sci Technol, 1997. 35(11-12):15-20.
17. Mokhtari, A. and Jaykus, L.A., Quantitative exposure model for the transmission of norovirus in retail food preparation. Int J Food Microbiol, 2009. 133(1-2):38-47.
18. Verhaelen, K., Bouwknegt, M., Lodder-Verschoor, F., Rutjes, S.A., and de Roda Husman, A.M., Persistence of human norovirus GII.4 and GI.4, murine norovirus, and human adenovirus on soft berries as compared with PBS at commonly applied storage conditions. Int J Food Microbiol, 2012. 160(2):137-44.

19. Bertrand, I., Schijven, J.F., Sanchez, G., Wyn-Jones, P., Ottoson, J., Morin, T., Muscillo, M., Verani, M., Nasser, A., de Roda Husman, A.M., Myrmel, M., Sellwood, J., Cook, N., and Gantzer, C., The impact of temperature on the inactivation of enteric viruses in food and water: a review. J Appl Microbiol, 2012. 112(6):1059-74.
20. Bouwknegt, M., Teunis, P.F., Frankena, K., de Jong, M.C., and de Roda Husman, A.M., Estimation of the likelihood of fecal-oral HEV transmission among pigs. Risk Anal, 2011. 31(6):940-50.
21. Teunis, P.F., Moe, C.L., Liu, P., Miller, S.E., Lindesmith, L., Baric, R.S., Le Pendu, J., and Calderon, R.L., Norwalk virus: how infectious is it? J Med Virol, 2008. 80(8):1468-76.
22. Ward, R., Krugman, S., Giles, J.P., Jacobs, A.M., and Bodansky, O., Infectious hepatitis; studies of its natural history and prevention. N Engl J Med, 1958. 258(9):407-16.
23. Kamel, A.H., Ali, M.A., El-Nady, H.G., Deraz, A., Aho, S., Pothier, P., and Belliot, G., Presence of enteric hepatitis viruses in the sewage and population of Greater Cairo. Clin Microbiol Infect, 2011. 17(8):1182-5.
24. Mokhtari, A. and Frey, H.C., Sensitivity analysis of a two-dimensional probabilistic risk assessment model using analysis of variance. Risk Anal, 2005. 25(6):1511-29.
25. Haas, C.N., Rose, J.R., and Gerba, C.P., Quantitative Microbial Risk Assessment. 1999, New York, USA: John Wiley & Sons.
26. Vose, D., Risk Analysis: A Quantitative Guide. 3rd ed. 2008, West Sussex, England: John Wiley & Sons.
27. Sattar, S.A., Springthorpe, V.S., Tetro, J., Vashon, R., and Keswick, B., Hygienic hand antiseptics: should they not have activity and label claims against viruses? Am J Infect Control, 2002. 30(6):355-72.
28. Havelaar, A.H. and Rutjes, S.A., Risk assessment of viruses in food: opportunities and challenges, in Food-borne viruses: progress and challenges, M.P.G. Koopmans, D.O. Cliver, and A. Bosch, Editors. 2008, ASM Press: Washington, DC.
29. Stals, A., Van Coillie, E., and Uyttendaele, M., Viral genes everywhere: public health implications of PCR-based testing of foods. Curr Opin Virol, 2013. 3(1):69-73.
30. De Roda Husman, A.M., Lodder, W.J., Rutjes, S.A., Schijven, J.F., and Teunis, P.F., Long-term inactivation study of three enteroviruses in artificial surface and groundwaters, using PCR and cell culture. Appl Environ Microbiol, 2009. 75(4):1050-7.
31. Schielke, A., Filter, M., Appel, B., and Johne, R., Thermal stability of hepatitis E virus assessed by a molecular biological approach. Virol J, 2011. 8:487.
32. Greening, G.E., Hewitt, J., and Lewis, G.D., Evaluation of integrated cell culture-PCR (C-PCR) for virological analysis of environmental samples. J Appl Microbiol, 2002. 93(5):745-50.
33. Parshionikar, S., Laseke, I., and Fout, G.S., Use of propidium monoazide in reverse transcriptase PCR to distinguish between infectious and noninfectious enteric viruses in water samples. Appl Environ Microbiol, 2010. 76(13):4318-26.
34. Allain, J.P., Hsu, J., Pranmeth, M., Hanson, D., Stassinopoulos, A., Fischetti, L., Corash, L., and Lin, L., Quantification of viral inactivation by photochemical treatment with amotosalen and UV A light, using a novel polymerase chain reaction inhibition method with preamplification. J Infect Dis, 2006. 194(12):1737-44.
35. Sanchez, G., Elizaquível, P., and Aznar, R., Discrimination of Infectious Hepatitis A Viruses by Propidium Monoazide Real-Time RT-PCR. Food Environ Virol, 2012. 4(1):21-25.
36. Carvalho, C., Thomas, H., Balogun, K., Tedder, R., Pebody, R., Ramsay, M., and Ngui, S., A possible outbreak of hepatitis A associated with semi-dried tomatoes, England, July-November 2011. Euro Surveill, 2012. 17(6).
37. Fournet, N., Baas, D., van Pelt, W., Swaan, C., Ober, H., Isken, L., Cremer, J., Friesema, I., Vennema, H., Boxman, I., Koopmans, M., and Verhoef, L., Another possible food-borne outbreak of hepatitis A in the Netherlands indicated by two closely related molecular sequences, July to October 2011. Euro Surveill, 2012. 17(6).

6



Wipes Coated with a Singlet Oxygen Producing Photosensitizer are Effective Against Human Influenza Virus but not Against Norovirus

Katharina Verhaelen[a,b], Martijn Bouwknegt[a], Saskia Rutjes[a], Ana Maria de Roda Husman,[a,b] Erwin Duizer[a]

[a] Centre for Infectious Disease Control, National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands;
[b] Faculty of Veterinary Medicine, Institute for Risk Assessment Sciences (IRAS), Utrecht University, Utrecht, The Netherlands

Applied and Environmental Microbiology, 80(14).

Abstract

Transmission of enteric and respiratory viruses, including human norovirus (hNoV) and human influenza virus, may involve surfaces. In food-preparation and health-care settings, surfaces are cleaned with wipes, however, wiping may not efficiently reduce contamination or even spread viruses, increasing a potential public health risk. The virucidal properties of wipes with a singlet oxygen generating immobilized photosensitizer (IPS) coating were compared to similar but uncoated wipes (non-IPS) and to commonly used viscose wipes. Wipes were spiked with hNoV GI.4 and GII.4, murine norovirus (MNV-1), human adenovirus (hAdV-5) and influenza virus H1N1 to study viral persistence. We also determined residual and transferred virus proportions on steel carries after successively wiping a contaminated, and uncontaminated steel carrier. Only on IPS-wipes, influenza viruses were promptly inactivated with a 5 $\log_{10}$ reduction. D-values of infectious MNV-1 and hAdV-5 were 8.7 and 7.0 h on IPS-wipes, 11.6 and 9.3 h on non-IPS wipes and 10.2 and 8.2 h on viscose wipes. Independent of the type of wipe, dry cleaning removed, or drastically reduced, initial spot contamination of hNoV on surfaces. All wipes transferred hNoV to an uncontaminated carrier, however, the risk of continued transmission by reuse of wipes after 6 and 24 h was limited for all viruses. We concluded that cleaning wet spots with dry wipes efficiently reduced spot contamination on surfaces, but that cross-contamination of noroviruses by wiping may result in an increased public health risk at high initial virus loads. For influenza, IPS-wipes present an efficient one-step procedure for cleaning and disinfecting contaminated surfaces.

Introduction

Viruses are the most common cause of infectious disease acquired in the indoor environment and cause considerable impact on human health [1]. Transmission of human norovirus (hNoV) and human influenza virus is assumed to occur mostly direct from person to person followed by indirect transmission through contaminated fomites, hands and surfaces [2-4]. The public health risk resulting from exposure to contaminated surfaces depends on a number of factors, including the level of shedding of infectious particles, their stability on surfaces and resistance to decontamination procedures [2, 5].

HNoV are non-enveloped RNA viruses and recognized as the leading cause of epidemics of gastroenteritis worldwide and an important cause of sporadic gastroenteritis in all age groups [6]. The virus is transmitted via feces and vomit of infected individuals and transmission routes are difficult to separate [1, 7]. HNoV shows a great potential for surface transmission. The virus is (i) abundantly shed via vomit and feces [8, 9], (ii) persistent in the environment and resistant to disinfectants [5, 10, 11], (iii) transferable between inanimate and animate surfaces [12-16], (iv) highly infectious [17], and (v) constantly evolving into new antigenic types [18, 19]. Contamination of hard (steel, glass or plastic) surfaces with hNoV in different facilities such as food and health care settings has been described [2, 3, 20-23], as well as hNoV transmission via surfaces sustaining a succession of outbreaks in closed settings such as airplanes, cruise ships and hotels [24-27]. In foodborne outbreaks in which surfaces played a role in the transmission, initial contamination occurred mostly when kitchen staff vomited in a sink [26, 28]. Influenza viruses are an important cause of respiratory disease frequently resulting in epidemics and occasionally pandemics [29]. It was concluded that the predominant route of influenza virus spreading was by airborne transmission, but transfer of infectious influenza virus via frequently touched non-porous surfaces such as door handles and light switches could make contact transmission a key transmission mode [2, 4]. This conclusion was based on the findings that influenza virus could remain infectious for several days on these surfaces and that contamination levels could be quite high due to sneezing and coughing [4, 30].

Cleaning and disinfection procedures by wiping fomites with cloths, with or without disinfectants, are applied commonly in food preparation areas and health care settings to minimize the transmission of human pathogens from surfaces to humans and foods. At present, a wide variety of cleaning products, disinfectants and cloths are on the market. Chlorine is considered the most efficient disinfectant against norovirus contamination [31, 32], however, the mandated chlorine concentration of 200 ppm chlorine is unlikely to prevent transfer of norovirus on surfaces by wiping [12]. For influenza chlorine, but also other cleaning agents such as vinegar and quaternary ammonium are effective

disinfectants [5, 29, 30]. Not only the used disinfectant, but also the wiping cloth affects decontamination efficacies and cross-contamination by wiping [33-35]. Gibson et al. [34] showed the effect of the type of cloth on the removal of hNoV surrogates from surfaces and their transfer to surfaces and especially the transfer to surfaces by wiping with contaminated wipes was greatly affected by the type of cloth; with cotton terry bar towels cross-contaminating surfaces to the greatest extent and microfiber cloth to the least. The use of wipes coated with antimicrobials may allow combined cleaning and disinfection of surfaces and prevent cross contamination via the cleaning cloth if the pathogen is inactivated efficiently in the cloth. Cloths that incorporate antimicrobials such as light activated polymers or copper-based biocides have been shown to be efficient in inactivating pathogens [36-40].

We studied nonwoven wipes with an immobilized photosensitizer that generates singlet oxygen on activation by light for their virucidal capacities. Additionally, their potential to decontaminate stainless steel surfaces and to minimize virus transfer to clean surfaces was assessed. The commonly found hNoV GI.4 and hNoV GII.4 strains were included as examples of their respective genogroups. MNV-1, the only cultivable norovirus, was used as a proxy to study hNoV infectivity. Human influenza A virus (H1N1) was studied as an examples of a surface transmittable enveloped respiratory virus. Human adenovirus type 5 (hAdV-5), was included in the virucidal activity testing of the wipes since it is a non-enveloped DNA virus that can be detected in respiratory secretions and in feces and the European Committee for Standardisation suggests testing hAdV-5 to assess the efficacy of disinfectants [41]. As far as we know, this is the first study on the use of immobilized photosensitizer fabrics as cleaning wipes for surfaces.

Materials and methods

Viruses and cells

HNoV GI.4 (Hu/NoV/GI.4/10001/2009/Netherlands) and hNoV GII.4(Hu/NoV/GII.4/10496/2010/Netherlands) were obtained from 10% fecal solutions. In addition, cultivatable MNV-1 (Mu/NoV/GV/MNV1/2002/USA), hAdV-5 (reference strain, Hu/adenovirus/type 5/6270/1988/Ethiopia) and influenza A (H1N1) virus (clinical isolate, Hu/influenza A/266/2008/Netherlands (H1N1) virus) were included in the experiments. Virus stocks were obtained by three freeze-thaw cycles of infected cells and subsequent centrifugation at 1500 g for 15 min. Cell lines used to propagate viruses are described by [42].

Wipes

Both coated and uncoated Serqet wipes were a gift from LAAMScience Inc, (Morris-

ville, NC, USA). The wipes were nonwoven and consisted of a blend of mostly cotton, rayon, and bamboo fibres with less than 8% polypropylene fibres. The fibres of coated wipes were coated with the immobilized photosensitizer rose bengal [43, 44] and are referred to as immobilized photosensitizer (IPS) wipes. Rose bengal-styrene monomers were mixed with acrylic acid for polymerization, and the polymers were subsequently covalently bound to the fibres by multiple amide links at a final dye level of 0.04% to 0.08 % (w/w). The immobilized rose bengal produced singlet oxygen ($^1O_2$) from molecular oxygen (O_2) during exposure to visible light (i.e., photosensitized generation of singlet oxygen). Besides coated IPS and the uncoated non-IPS-wipes, commonly used viscose wipes (non-woven cotton) that can be purchased in any household supply store were studied. Viscose wipes were included to compare IPS-wipes to commonly used cleaning wipes. Non-IPS-wipes were included to study the effect of the IPS on virus inactivation.

Preparation and spiking of wipes

The wipes were cut into 1-cm^2 pieces under sterile conditions in a safety cabinet with the light off and placed in petri dishes wrapped in aluminium foil for storage until use. Wipes were spiked with 50 µl of a mixture of hNoV GII.4 (~ 10^5 PCR detectable units (PCRU)), hNoV GI.4 (~ 10^6 PCRU) and MNV-1 (~ 3 x 10^4 infectious particles and 10^6 PCRU) or with 50 µL of influenza A (H1N1) virus (~ 10^5 infectious particles and 10^6 PCRU) or with 50 µL hAdV-5 virus stock (~ 10^6 infectious particles and 10^7 PCRU). The spiked virus mix was completely absorbed by the wipes, and wetted the previously dry wipes.

Viral persistence on wipes

Experiments were performed in a safety cabinet. The temperature in the cabinet was monitored with a probe every half hour and varied between 25.5°C and 27.5°C. The light intensity inside the cabinet was 1300 Lux. Viruses were spiked on the three types of wipes and exposed to light for 0 min, 5 min, 1 h, 6 h and 24 h, and afterwards, were eluted from the fabrics. Studying viral persistence for up to 24 h was of interest regarding wipe reuse before discarding or washing. To study the effect of singlet oxygen release on virus persistence, IPS-wipes were stored in the dark (wrapped in aluminium foil in a petri dish) for 1 h, 6 h and 24 h to prevent the production of singlet oxygen. Each experimental condition was performed in triplicate. A negative control was included (no virus spiked on the wipes), to control for possible contamination of used chemicals and equipment and to monitor a potentially toxic effect of the eluent on the cells used to determine viral infectivity.

Reduction and transfer of viruses due to wiping

To study virus spread through wiping, a steel carrier, A, (2 x 2 cm) was inoculated with 20 µL of the previously used virus suspension containing MNV-1, hNoV GI.4, hNoV GII.4, and influenza A (H1N1) virus. HAdV-5 was not studied in this experiment. Carrier A was then wiped with a dry 1 cm by 1 cm viscose, non-IPS and IPS-wipe, in a circular fashion for 10 s with gloved fingertips. Afterwards, a second, uncontaminated steel carrier, B, was wiped in the same manner with the now damp wipes that were used to clean carrier A. Viruses were eluted from both carriers A and B and from the wipe. As a reference point, viruses were spiked on a steel carrier and subsequently eluted from the carrier without any wiping. Experiments were performed in duplicate.

Virus elution

Viruses were eluted from the wipes in 1.5 mL of Tris-base-glycine-beef-extract (TGBE) buffer, pH 9.5. Tubes were covered with aluminium foil to prevent further viral inactivation by the treated wipes. Tubes were rotated for 1 h at 4°C. The elution buffer was transferred to a new tube, the pH was adjusted to 7, and the sample was split into two portions; one was used to determine viral infectivity and the other for molecular detection. Samples were stored at – 80°C before usage. To elute viruses from the steel carriers, the carriers were transferred to 6-well plates and covered with 3 mL of TGBE pH 9.5; they were then shaken at 100 rpm at 4 °C for 1 h. The liquid was recovered from the wells, the pH adjusted to 7 and the samples were stored at – 80°C in aliquots.

Virus enumeration

Viruses were enumerated by endpoint titration in 96-well plates [42, 45]. Cells were seeded in a concentration of 2×10^5 cells/mL, and 10-fold serial dilutions in fetal calf serum (FCS)-free medium were prepared (eight dilutions, 10 replicates with each), followed by inoculation in a 96-well plate. HAdV-5 virus dilutions were added to 100 µL of freshly trypsinized Hep-2 cells in medium containing 10% FCS. MNV-1 was replicated on RAW-264.7 cells that were previously incubated for at least 3 h in culture medium containing 2% FCS. We titrated influenza A (H1N1) virus on 3-day-old MDCK-1 cell monolayers after washing twice with phosphate buffer solution prior to infection. The influenza A (H1N1) virus suspensions were prepared in Dulbecco's Modified Eagle Medium (DMEM) with 2.5 µg/ml TPCK (l-(tosylamido- 2-phenyl) ethyl chloromethyl ketone) treated trypsin. A cytopathic effect (CPE) of the different cells was observed after 6 to 7 days of incubation at 37 °C in 5% carbon dioxide, whereupon Hep-2 cells and MDCK-1 cells were stained with crystal violet to confirm CPE.
A negative control (inoculation of wells with only dilution medium and no virus) was included on each plate. Furthermore, we tested for toxic effects of the negative sample matrix (eluent of each of the three different wipes that were spiked with water) on the cells to be able to distinguish between CPE and cell death by sample toxicity. We also

analysed whether the negative sample matrix affected the detection of virus infectivity, for example, by modifying the virus cell-receptor, which would not allow replication of infectious virus particles, but would not necessarily affect the cell monolayer. To accomplish this, we inoculated the cells with 100 µL of the negative sample matrix and, after an incubation of 2 h, added 100 µL of stock virus dilution to the cells; we then compared the infectious-virus titre of those cells to that of cells incubated solely with dilution medium.

Molecular detection

Nucleic acids were extracted with the NucliSens miniMag magnetic extraction kit (bioMérieux, Zaltbommel, The Netherlands), following the manufacturer's instructions with minor modifications [46]. To monitor the extraction efficacy, samples were spiked with mengovirus (vMC_0) before extraction. In each extraction cycle, a negative control without a target was included. HAdV-5 and MNV-1 were detected as described by Verhaelen et al. [45] and hNoV GI, hNoV GII and influenza were detected as described by Tuladhar et al. [47] and Svraka et al. [48]. Inhibition controls were included in all polymerase chain reactions (PCRs) to monitor PCR inhibition [42, 45, 48]. The fragment length analyzed was small and varied between 95 to 149 nucleotides. PCRU of the virus stocks were determined, by the slope of standard curves, with the highest dilution resulting in a positive PCR signal being equal to a value of 1 PCRU [47].

Data analysis

Infectivity counts of MNV-1, hAdV-5 and influenza H1N1 were estimated by maximum likelihood from the presence-absence profile of the endpoint dilutions, assuming homogeneous (Poisson) mixing [45]. The $\log_{10}$-unit reductions of infectious virus particles were calculated on the basis of the virus count at time t relative to the count at t=0 (viruses eluted from wipes immediately after spiking). The reductions of viral genomes were calculated on basis of the difference in quantification cycles (C_q values) and not on absolute genome numbers. Therefore, C_q values were determined at time t and t=0, and a difference in C_q value of log(10)/log(2) (equivalent to a difference of 3.3 C_q values) was assumed equal to a 1 $\log_{10}$-unit reduction of viral genomes. The statistical significance of differences in reduction between treated and untreated IPS-wipes and among viruses was determined with the likelihood ratio test using a general linear model with infectious virus counts or C_q values as response variable and treatment and virus as explanatory variables (α=0.05).

The residual viral contamination after wiping carriers A was expressed as the proportion of viruses recovered from the wiped carriers and the viruses recovered from the reference carrier (spiked unwiped carrier). The transferred viral contamination was expressed as

the proportion of viruses recovered from the B carriers and the viruses recovered from the reference carrier (spiked unwiped carrier), assuming similar recoveries. To express the proportions of residual viral genomes on carriers A and transferred viral genomes on B, the $\log_{10}$-unit differences between wiped and reference carriers were determined in the same way and subsequently translated into percentages.

Results

We analyzed the persistence of hNoV GI.4 and GII.4, MNV-1, hAdV-5 and H1N1 on viscose, non-IPS and IPS wipes, and determined furthermore the residual and transferred virus proportions on and to steel carriers after successive wiping.

Controls

The efficiency of viral genome-extraction from wipes was monitored using $vMC_{0.}$ The extraction efficiency was found to be constant for all wipes, with a maximum difference of 1 C_q value between the samples. The yields of infectious viruses varied between 10-100% for MNV-1, influenza H1N1 and hAdV-5. No toxic effect of the sample matrix of the wipe eluates on the cells was detected and the virus titre was not affected by the sample matrix (data not shown). The internal and external amplification controls showed that the sample matrix did not inhibit the amplification of the PCRs.

Viral persistence on viscose wipes, non-IPS-wipes and IPS-wipes

Both, the type of wipe ($p \leq 0.002$) and the studied virus ($p < 0.001$), influenced the persistence of the infectious viruses significantly. Enveloped influenza viruses were least persistent, followed by hAdV-5 and MNV-1 (Figure 1). The IPS-coating resulted in a rapid reduction of influenza infectivity. After immediate elution of influenza from the IPS-wipes ($t=0$), no infectious viruses could be recovered, which relates to a ≥4 $\log_{10}$-unit reduction of infectious influenza virus for a recovery of ≥10% (estimated recovery of influenza viruses from non-IPS-wipes) and a 5 $\log_{10}$-unit reduction assuming complete virus inactivation. Consequent to the immediate influenza decay on IPS-wipes, after 1 h, no infectious influenza viruses could be recovered from IPS-wipes stored at dark conditions (Figure 1). On viscose and non-IPS-wipes, no rapid inactivation of influenza-virus infectivity was observed, but a reduction of over 2 $\log_{10}$-units was found after 1 h (Figure 1).

Inactivation of infectious non-enveloped viruses was not rapid on any wipe, with mean D-values of 7 to 9 h, and 9 to 10 h for infectious MNV-1 and hAdV-5 particles, respectively (Table 1). Infectious MNV-1 and hAdV-5 were marginally but significantly less persistent on IPS-wipes than on non-IPS wipes ($p < 0.001$), and the difference in viral

persistence on viscose and non-IPS-wipes was also small but significant (p=0.002) (Figure 1, Table 1). After storage for 6 h, wipes of the dark control were still wet, whereas the wipes stored at light were completely dried.

Concomitantly, MNV-1 and hAdV-5 viruses were more persistent on IPS-wipes at dark conditions as compared to light conditions, with a difference of about 2 $\log_{10}$ units (Figure 1). After 24 h storage, wipes stored at light and dark conditions were dried.

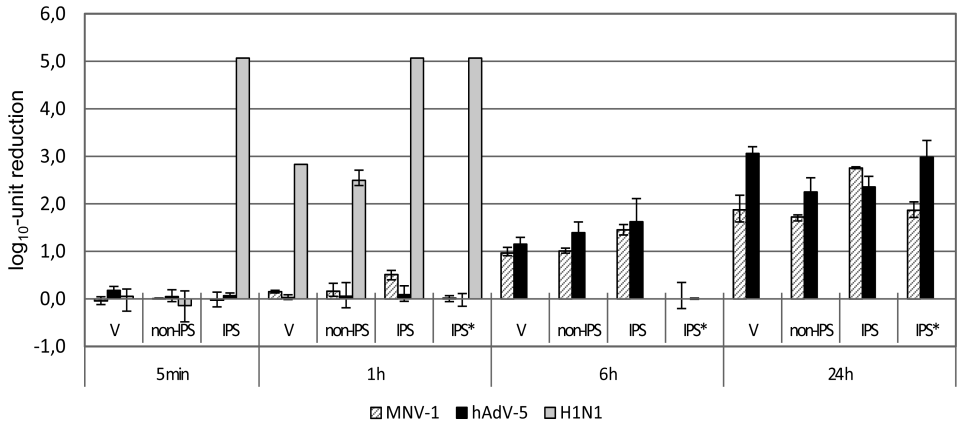


Figure 1. Average reductions of infectious MNV-1, hAdV-5 and H1N1 particles on viscose wipes (V), non-IPS wipes (non-IPS), IPS wipes (IPS), and IPS wipes stored in the dark (IPS*). The error bars represent the minimum and maximum log10-reduction values of the tested condition. Because rapid and complete decay of infectious H1N1 viruses was observed on IPS- wipes, persistence of this virus was not determined after 6 and 24 h. The letter (a) indicates that the maximum inactivation of infectious influenza is represented.

Table 1. Mean D-values (time needed for the first 1log10-unit reduction) of infectious influenza, MNV-1 and hAdV-5 particles on the three tested wipes and their 95% confidence intervals. The D-values were calculated on basis of the linear regression model.

Virus	D-value [h]		
	Viscose	non-IPS	IPS
Influenza H1N1	0.6 (0.55 – 0.67)	0.71 (0.6 – 0.87)	≤ 0.02[a]
MNV-1	10.2 (8.7 – 12.2)	11.6 (9.0 – 15.1)	8.7 (6.8 – 11.2)
hAdV-5	8.2 (7.6 – 8.8)	9.3 (7.9 – 10.9)	7.0 (6.0 – 8.1)

[a] Because no infectious influenza viruses could be detected at time point 0, an exact D-value could not be calculated.

For MNV-1 decay of nucleic acids and infectious particles was comparable (Figure 2). For influenza and hAdV-5 decay of viral nucleic acids was less pronounced, but followed similar trends compared to infectious particles (Figure 2). As was the case for infectivity, genome persistence of the tested viruses was significantly influenced by the type of wipe (p=0.002) and virus (p<0.0001). After 1 hour, the reduction of influenza genomes on IPS wipes relative to IPS wipes stored at dark was 1 $\log_{10}$-unit higher. After 6 and 24 h the hAdV-5 DNA genomes were consistently more persistent than the RNA NoV genomes. The effect of any wipe on viral genome persistence was not statistically different among MNV-1, hNoV GI.4 and hNoV GII.4 (p = 0.32).

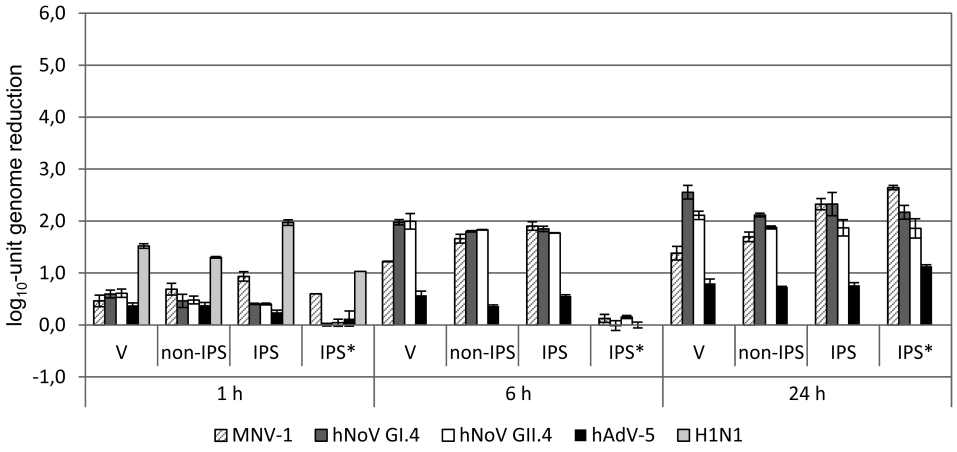


Figure 2. Average genomic copy reductions of MNV-1, hNoV GI.4, hNoV GII.4, hAdV-5 and influenza virus H1N1 on viscose wipes (V), non-IPS-wipes (non-IPS), IPS-wipes (IPS), and IPS wipes stored in the dark (IPS*). The error bars represent the minimum and maximum log10-unit reduction values of the tested condition. Influenza genomes were only analyzed for up to an hour because of the observed rapid decay of infectious influenza virus on IPS-wipes. No decay of viral genomes was observed after 5 min.

Residual and transferred contamination on steel carriers after wiping

No residual contamination of infectious MNV-1 and influenza particles could be recovered from the spiked steel carriers after wiping. Residual contamination was only determined for norovirus genomes, with similar residual virus proportions of 0.2 to 0.6 % for the three wipes and both genotypes (Figure 3). No transferred infectious influenza virus particles could be recovered from the subsequently wiped steel carriers B. Transferred contamination proportions of infectious MNV-1 particles and hNoV genomes to steel carriers B were similar and ranged, as the residual virus proportions, between 0.2% to 0.6%; whereas transferred MNV-1 genome proportions where higher (Figure 3). The three different wipes had little impact on the transfer of hNoV GI.4 and GII.4 genomes to steel carriers. The presence or absence of viruses was also determined on the used

wipes. Infectious MNV-1, and hNoV GI and GII genomes, were detected on all wipes, whereas infectious influenza was detected only on non-IPS-wipes and viscose wipes, but not on IPS-wipes.

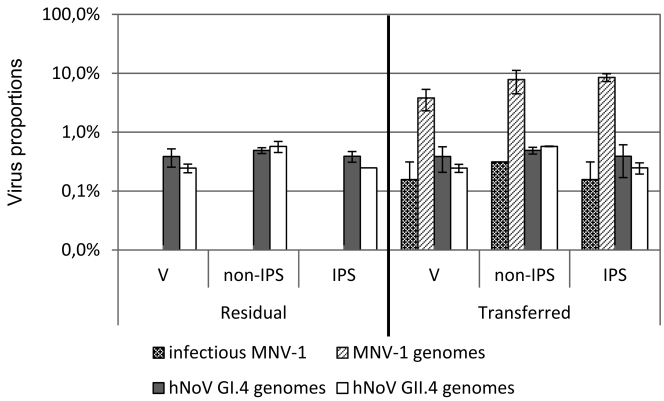


Figure 3. Residual and transferred virus proportions of hNoV genomes and infectious MNV-1 particles and genomes after wiping sequentially with viscose wipes (V), non-IPS-wipes (non-IPS) and IPS-wipes (IPS). The error bars represent the minimum and maximum residual and transferred virus proportions.

Discussion

Efficient cleaning and disinfection procedures are needed to reduce the infectious load of pathogenic viruses on surfaces and interrupt virus transmission. The actual efficiency of the cleaning and disinfection procedures in practice depends on several factors, including the cleaning method, disinfectant and cloth used. Wiping with or without disinfectant has been shown to reduce the residual contamination of hard surfaces significantly, but not always to a level low enough to exclude continued transmission [5, 12]. Additionally, wiping may result in a more extensive spread of viruses on surfaces by transferring pathogens from a highly contaminated spot to a larger surface or a another surface. This is especially problematic for highly persistent and highly infectious pathogens such as hNoV. Both the efficiency of the removal of infectious viruses by wiping of contaminated surfaces and the rapid viral inactivation on the wipes, and/ or irreversible binding of viruses to the wipes, are relevant for the overall efficiency of cleaning procedures to prevent spread of viruses via surfaces.

Wiping is a short duration activity and to have a significant effect in decontamination or transfer prevention, the virucidal activity should be very fast, i.e. within seconds. Neither the viscose wipes, nor the IPS-wipes caused rapid inactivation of infectious

MNV-1 or rapid reduction of hNoV GI.4 and GII.4 genomes. IPS-wipes are, therefore, not likely to prevent the spread of noroviruses better than viscose wipes. Nevertheless, residual norovirus contamination on spiked steel carriers after wiping with any of the tested wipes was little and no infectious MNV-1 virus and only a small fraction of human norovirus genomes was recovered. In food and health settings it is recommended to clean surfaces subjected to liquid norovirus soiling by, for example, vomit or fecal contamination, with dry absorbing material such as paper towels or wipes [49, 50]. Initial virus removal from surfaces by cleaning with dry wipes after spot contamination with 10^4 noroviruses was found to be efficient.

We did show that infectious MNV-1 virus particles and hNoV genomes were transferred from one steel carrier to another by reusing wipes, indicating that viruses are not irreversibly bound to the wipes. The transferred norovirus genome proportions are thereby likely to represent infectious viruses, given the short time interval of wiping and the persistence data of infectious MNV-1 on wipes. Noroviruses are thus possibly spread to surfaces by continued use or reuse of wipes. Cross contamination may be prevented by discarding or by laundering (depending on the initial virus load) [51, 52] of wipes direct after contamination. The use of chlorine in preventing cross-contamination of hNoV on surfaces by wiping is not well investigated and a study by Barker and coworkers [12] showed that even the use of high chlorine concentrations were not always able to prevent transfer of hNoV RNA to secondary surfaces by wiping, especially where there was fecal soiling. However, research showed that detectability of RNA (fragments) is not necessarily a good measure for infectivity [47, 53].

Whether the by cross-contamination transferred viral loads on surfaces lead to an enhanced health risk through continued transmission of the virus depends on (i) the initial virus load on the surface, (ii) on the virus proportion transferred to secondary surfaces and (iii) on the viral transfer proportion from the secondary surface to a human host. We found that about 0.2% of the initial norovirus contamination was transferred to a clean spot through cross-contamination by wiping. Assuming a target level of residual contamination of 50 noroviruses per spot, a level resulting in minimal risk of continuous virus transmission or infection [5], we can deduce that the contamination of the initial spot should be above $2.5x10^4$ infectious viruses per spot to pose a risk. Considering that spots of about 1 mg fecal contamination are just barely visible and thus realistic fecal contaminations, shedding of over $2.5x10^7$ hNoV particles per gram feces is required. Projectile vomiting in the acute disease phase may add as well to the spread of the virus [54],with a release of 10^4 genomic norovirus copies/mL and a total excreted volume of up to 1.7 L [55]. This indicates that cross-contaminated surfaces may pose a risk when the initial contamination occurred during a period of about 10 days around

the peak of viral shedding [9, 56] or after an episode of vomiting. We may thus conclude that cross-contamination through wiping of surfaces could pose a public health risk and should be considered in implementing transmission intervention measures in food production and health care facilities.

In contrast to noroviruses, infectious influenza viruses were found to be inactivated promptly by 4 to 5 $\log_{10}$ units by IPS-wipes, but not by regular viscose wipes and non-IPS-wipes. Unlike noroviruses and adenoviruses, influenza viruses have a lipoprotein envelope, which makes them less persistent to disinfectants compared to non-enveloped viruses, as has been shown for several disinfectants [5, 30, 57]. In a study on the virucidal activity of rose bengal on influenza viruses, it was proposed that singlet oxygen had a direct effect on the fusion protein of the virus after enrichment of the photosensitizer rose bengal in the viral envelope [58]. In our study, the rose bengal was immobilized by covalent binding to the fibers of the wipes, thus not allowing for enrichment of the photosensitizer in the viral envelope. The prompt inactivation of the influenza viruses on IPS-wipes suggests the relevance of singlet oxygen in the pathogen inactivation as was shown before for other enveloped viruses [58, 59] and for bacteria such as *Escherichia coli* [60]. Yet we could not show that the efficient inactivation of influenza virus was due to released singlet oxygen, or due to other mechanisms (e.g. antiviral effect of the coating), as complete viral decay on IPS-wipes was immediate (t=0) and therefore also on wipes stored in the dark no infectious influenza viruses could be detected. However, the design of our study was not aimed to elucidate the exact mechanism of the very efficient influenza inactivation, but to determine the overall effect on (infectious) virus reduction on wipes and surfaces including the transfer of viruses by different cloths. On the basis of our study, wiping with IPS-wipes is likely to be an efficient transmission intervention method for surface related influenza transmission, because infectious viruses are efficiently removed and inactivated, thus preventing transmission and cross contamination. Similar effects of IPS-wipes may be expected for other enveloped viruses [58].

Wipes for cleaning kitchen or bathroom surfaces, in food settings such as restaurants, but also in private households, may not be instantly discarded as is prescribed in health care settings, and thus they may contribute to viral spread over an extended period of time. Therefore, we analyzed the persistence of viruses on wipes after 6 h and 24 h and observed substantial reductions between 2 and 3.5 $\log_{10}$-units of infectious MNV-1 and hAdV-5 viruses on all wipes. IPS-wipes showed a 1 $\log_{10}$-unit higher reduction of infectious MNV-1 particles after 24 h than viscose wipes, indicating an increased potential of these wipes to contain norovirus spread by reused wipes as compared to viscose wipes. Instead of discarding the wipe, drying IPS-wipes for a day may thus prevent potential viral cross-contamination of both enveloped and non-enveloped viruses, which is espe-

cially of interest in countries with restricted resources. An important factor of viral decay after prolonged times on wipes may be desiccation.
In summary, we conclude that cleaning wet spots with dry wipes efficiently reduced spot contamination of the viruses present on surfaces. Yet, IPS-wipes, as well as the other wipes we used, were not able to prevent surface cross-contamination of the non-enveloped viruses by wiping, which may result in an increased public health risk at high initial virus loads. However, the IPS-wipes tested present a one-step procedure for cleaning and disinfecting influenza contaminated surfaces. In addition, we show that the risk for continued virus transmission by reuse of viscose or IPS-wipes is limited, if wipes are allowed to dry before reuse.

Acknowledgements

We wish to acknowledge Anne-Marie van den Brandt for performing the hNoV and influenza A (H1N1) PCRs. In addition, we thank Joe S. Schneider from LaamScience, Inc. for providing the wipes and product details and Sally Ebeling for language editing.

References

1. Barker, J., Stevens, D., and Bloomield, S., *Spread and prevention of some common viral infections in community facilities and domestic homes.* Journal of Applied Microbiology, 2001. 91:7-21.
2. Boone, S.A. and Gerba, C.P., *Significance of fomites in the spread of respiratory and enteric viral disease.* Appl Environ Microbiol, 2007. 73(6):1687-96.
3. Lopman, B., Gastanaduy, P., Park, G.W., Hall, A.J., Parashar, U.D., and Vinje, J., *Environmental transmission of norovirus gastroenteritis.* Curr Opin Virol, 2012. 2(1):96-102.
4. Weber, T.P. and Stilianakis, N.I., *Inactivation of influenza A viruses in the environment and modes of transmission: a critical review.* J Infect, 2008. 57(5):361-73.
5. Tuladhar, E., Hazeleger, W.C., Koopmans, M., Zwietering, M.H., Beumer, R.R., and Duizer, E., *Residual viral and bacterial contamination of surfaces after cleaning and disinfection.* Appl Environ Microbiol, 2012. 78(21):7769-75.
6. Glass, R.I., Parashar, U.D., and Estes, M.K., *Norovirus gastroenteritis.* N Engl J Med, 2009. 361(18):1776-85.
7. Verhoef, L., Vennema, H., van Pelt, W., Lees, D., Boshuizen, H., Henshilwood, K., and Koopmans, M., *Use of norovirus genotype profiles to differentiate origins of foodborne outbreaks.* Emerg Infect Dis, 2010. 16(4):617-24.
8. Caul, E.O., *Small round structured viruses: airborne transmission and hospital control.* Lancet, 1994. 343(8908):1240-2.
9. Atmar, R.L., Opekun, A.R., Gilger, M.A., Estes, M.K., Crawford, S.E., Neill, F.H., and Graham, D.Y., *Norwalk virus shedding after experimental human infection.* Emerg Infect Dis, 2008. 14(10):1553-7.
10. Abad, F.X., Pinto, R.M., Diez, J.M., and Bosch, A., *Disinfection of human enteric viruses in water by copper and silver in combination with low levels of chlorine.* Appl Environ Microbiol, 1994. 60(7):2377-83.
11. Lamhoujeb, S., Fliss, I., Ngazoa, S.E., and Jean, J., *Evaluation of the persistence of infectious human noroviruses on food surfaces by using real-time nucleic acid sequence-based amplification.* Appl Environ Microbiol, 2008. 74(11):3349-55.
12. Barker, J., Vipond, I.B., and Bloomfield, S.F., *Effects of cleaning and disinfection in reducing the spread of Norovirus contamination via environmental surfaces.* J Hosp Infect, 2004. 58(1):42-9.
13. Escudero, B.I., Rawsthorne, H., Gensel, C., and Jaykus, L.A., *Persistence and Transferability of Noroviruses on and between Common Surfaces and Foods.* Journal of Food Protection, 2012. 75(5):927–935.
14. Stals, A., Uyttendaele, M., Baert, L., and Van Coillie, E., *Norovirus Transfer between Foods and Food Contact Materials.* J Food Prot, 2013. 76(7):1202-9.
15. Verhaelen, K., Bouwknegt, M., Carratala, A., Lodder-Verschoor, F., Diez-Valcarce, M., Rodriguez-Lazaro, D., de Roda Husman, A.M., and Rutjes, S.A., *Virus transfer proportions between gloved fingertips, soft berries, and lettuce, and associated health risks.* Int J Food Microbiol, 2013. 166(3):419-25.
16. Tuladhar, E., Hazeleger, W.C., Koopmans, M., Zwietering, M.H., Duizer, E., and Beumer, R.R., *Transfer of noroviruses between fingers and fomites and food products.* International Journal of Food Microbiology, 2013. 167:346–352.
17. Teunis, P.F., Moe, C.L., Liu, P., Miller, S.E., Lindesmith, L., Baric, R.S., Le Pendu, J., and Calderon, R.L., *Norwalk virus: how infectious is it?* J Med Virol, 2008. 80(8):1468-76.
18. Bull, R.A. and White, P.A., *Mechanisms of GII.4 norovirus evolution.* Trends Microbiol, 2011. 19(5):233-40.
19. Siebenga, J.J., Vennema, H., Zheng, D.P., Vinje, J., Lee, B.E., Pang, X.L., Ho, E.C., Lim, W., Choudekar, A., Broor, S., Halperin, T., Rasool, N.B., Hewitt, J., Greening, G.E., Jin, M., Duan, Z.J., Lucero, Y., O'Ryan, M., Hoehne, M., Schreier, E., Ratcliff, R.M., White, P.A., Iritani, N., Reuter, G., and Koopmans, M., *Norovirus illness is a global problem: emergence and spread of norovirus GII.4 variants, 2001-2007.* J Infect Dis, 2009. 200(5):802-12.
20. Boxman, I.L., Dijkman, R., te Loeke, N.A., Hagele, G., Tilburg, J.J., Vennema, H., and Koopmans, M., *Environmental swabs as a tool in norovirus outbreak investigation, including outbreaks on cruise ships.* J Food Prot, 2009. 72(1):111-9.
21. Boxman, I.L., Verhoef, L., Dijkman, R., Hagele, G., Te Loeke, N.A., and Koopmans, M., *Year-round prevalence of norovirus in the environment of catering companies without a recently reported outbreak of gastroenteritis.* Appl Environ Microbiol, 2011. 77(9):2968-74.

22. Kokkinos, P., Kozyra, I., Lazic, S., Bouwknegt, M., Rutjes, S., Willems, K., Moloney, R., de Roda Husman, A.M., Kaupke, A., Legaki, E., D'Agostino, M., Cook, N., Rzezutka, A., Petrovic, T., and Vantarakis, A., *Harmonised investigation of the occurrence of human enteric viruses in the leafy green vegetable supply chain in three European countries.* Food Environ Virol, 2012. 4(4):179-91.

23. Ronnqvist, M., Ratto, M., Tuominen, P., Salo, S., and Maunula, L., *Swabs as a tool for monitoring the presence of norovirus on environmental surfaces in the food industry.* J Food Prot, 2013. 76(8):1421-8.

24. Cheesbrough, J.S., Green, J., Gallimore, C.I., Wright, P.A., and Brown, D.W., *Widespread environmental contamination with Norwalk-like viruses (NLV) detected in a prolonged hotel outbreak of gastroenteritis.* Epidemiol Infect, 2000. 125(1):93-8.

25. Marks, P.J., Vipond, I.B., Carlisle, D., Deakin, D., Fey, R.E., and Caul, E.O., *Evidence for airborne transmission of Norwalk-like virus (NLV) in a hotel restaurant.* Epidemiol Infect, 2000. 124(3):481-7.

26. Patterson, W., Haswell, P., Fryers, P.T., and Green, J., *Outbreak of small round structured virus gastroenteritis arose after kitchen assistant vomited.* Commun Dis Rep CDR Rev, 1997. 7(7):101-3.

27. Thornley, C.N., Emslie, N.A., Sprott, T.W., Greening, G.E., and Rapana, J.P., *Recurring norovirus transmission on an airplane.* Clinical Infectious Disease, 2011. 53(6):515-520.

28. de Wit, M.A., Widdowson, M.A., Vennema, H., de Bruin, E., Fernandes, T., and Koopmans, M., *Large outbreak of norovirus: the baker who should have known better.* Journal of Infection, 2007. 55(2):188–193.

29. Greatorex, J.S., Page, R.F., Curran, M.D., Digard, P., Enstone, J.E., Wreghitt, T., Powell, P.P., Sexton, D.W., Vivancos, R., and Nguyen-Van-Tam, J.S., *Effectiveness of common household cleaning agents in reducing the viability of human influenza A/H1N1.* PLoS One, 2010. 5(2):e8987.

30. Tuladhar, E., de Koning, M.C., Fundeanu, I., Beumer, R., and Duizer, E., *Different virucidal activities of hyperbranched quaternary ammonium coatings on poliovirus and influenza virus.* Appl Environ Microbiol, 2012. 78(7):2456-8.

31. Tung, G., Macinga, D., Arbogast, J., and Jaykus, L.A., *Efficacy of commonly used disinfectants for inactivation of human noroviruses and their surrogates.* J Food Prot, 2013. 76(7):1210-7.

32. Division of Viral Diseases, N.C.f.I., Respiratory Diseases, C.f.D.C., and Prevention, *Updated norovirus outbreak management and disease prevention guidelines.* MMWR Recomm Rep, 2011. 60(RR-3):1-18.

33. Diab-Elschahawi, M., Assadian, O., Blacky, A., Stadler, M., Pernicka, E., Berger, J., Resch, H., and Koller, W., *Evaluation of the decontamination efficacy of new and reprocessed microfiber cleaning cloth compared with other commonly used cleaning cloths in the hospital.* Am J Infect Control, 2010. 38(4):289-92.

34. Gibson, K.E., Crandall, P.G., and Ricke, S.C., *Removal and transfer of viruses on food contact surfaces by cleaning cloths.* Appl Environ Microbiol, 2012. 78(9):3037-44.

35. Koo, O.K., Martin, E.M., Story, R., Lindsday, D., and Ricke, S.C., *Comparison of cleaning fabrics for bacterial removal from food-contact surfaces.* Food Control, 2013. 30:292-297.

36. Arenbergerova, M., Arenberger, P., Bednar, M., Kubat, P., and Mosinger, J., *Light-activated nanofibre textiles exert antibacterial effects in the setting of chronic wound healing.* Exp Dermatol, 2012. 21(8):619-24.

37. Hamilton, D., Foster, A., Ballantyne, L., Kingsmore, P., Bedwell, D., Hall, T.J., Hickok, S.S., Jeanes, A., Coen, P.G., and Gant, V.A., *Performance of ultramicrofibre cleaning technology with or without addition of a novel copper-based biocide.* Journal of Hospital Infection, 2010. 74(1):62-71.

38. Imai, K., Ogawa, H., Bui, V.N., Inoue, H., Fukuda, J., Ohba, M., Yamamoto, Y., and Nakamura, K., *Inactivation of high and low pathogenic avian influenza virus H5 subtypes by copper ions incorporated in zeolite-textile materials.* Antiviral Res, 2012. 93(2):225-33.

39. Lhotakova, Y., Plistil, L., Moravkova, A., Kubat, P., Lang, K., Forstova, J., and Mosinger, J., *Virucidal nanofiber textiles based on photosensitized production of singlet oxygen.* PLoS One, 2012. 7(11):e49226.

40. Perni, S., Piccirillo, C., Pratten, J., Prokopovich, P., Chrzanowski, W., Parkin, I.P., and Wilson, M., *The antimicrobial properties of light-activated polymers containing methylene blue and gold nanoparticles.* Biomaterials, 2009. 30(1):89-93.

41. Steinmann, J., *Surrogate viruses for testing virucidal efficacy of chemical disinfectants.* J Hosp Infect, 2004. 56 Suppl 2:S49-54.

42. Tuladhar, E., Terpstra, P., Koopmans, M., and Duizer, E., *Virucidal efficacy of hydrogen peroxide vapour disinfection.* J Hosp Infect, 2012. 80(2):110-5.

43. DeRosa, M.C. and Crutchley, R.J., *Photosensitized singlet oxygen and its applications.* Coordination Chemistry Reviews 2002. 233-234:351-371.

44. Schafer, M., Schmitz, C., Facius, R., Horneck, G., Milow, B., Funken, K.H., and Ortner, J., *Systematic study of parameters influencing the action of Rose Bengal with visible light on bacterial cells: comparison between the biological effect and singlet-oxygen production.* Photochem Photobiol, 2000. 71(5):514-23.

45. Verhaelen, K., Bouwknegt, M., Lodder-Verschoor, F., Rutjes, S.A., and de Roda Husman, A.M., *Persistence of human norovirus GII.4 and GI.4, murine norovirus, and human adenovirus on soft berries as compared with PBS at commonly applied storage conditions.* Int J Food Microbiol, 2012. 160(2):137-44.

46. Verhaelen, K., Bouwknegt, M., Rutjes, S.A., and de Roda Husman, A.M., *Persistence of human norovirus in reconstituted pesticides--pesticide application as a possible source of viruses in fresh produce chains.* Int J Food Microbiol, 2013. 160(3):323-8.

47. Tuladhar, E., Bouwknegt, M., Zwietering, M.H., Koopmans, M., and Duizer, E., *Thermal stability of structurally different viruses with proven or potential relevance to food safety.* J Appl Microbiol, 2012. 112(5):1050-7.

48. Svraka, S., van der Veer, B., Duizer, E., Dekkers, J., Koopmans, M., and Vennema, H., *Novel approach for detection of enteric viruses to enable syndrome surveillance of acute viral gastroenteritis.* J Clin Microbiol, 2009. 47(6):1674-9.

49. EFSA, *Scientific Opinion, An update on the present knowledge on the occurrence and control of foodborne viruses.* EFSA Journal, 2011. 9(7):2190-2286.

50. Anonymous, *Reiniging, desinfectie en sterilisatie in de openbare gezondheidszorg - Standaardmethoden* in *Reiniging, desinfectie en sterilisatie in de openbare gezondheidszorg*. 2003, National Intstiute for Public Health and the Environment (RIVM): The Netherlands.

51. Gerba, C.P. and Kennedy, D., *Enteric virus survival during household laundering and impact of disinfection with sodium hypochlorite.* Appl Environ Microbiol, 2007. 73(14):4425-8.

52. Heinzel, M., Kyas, A., Weide, M., Breves, R., and Bockmuhl, D.P., *Evaluation of the virucidal performance of domestic laundry procedures.* Int J Hyg Environ Health, 2010. 213(5):334-7.

53. de Roda Husman, A.M., Lodder, W.J., Rutjes, S.A., Schijven, J.F., and Teunis, P.F., *Long-term inactivation study of three enteroviruses in artificial surface and groundwaters, using PCR and cell culture.* Appl Environ Microbiol, 2009. 75(4):1050-7.

54. Zelner, J.L., Lopman, B.A., Hall, A.J., Ballesteros, S., and Grenfell, B.T., *Linking time-varying symptomatology and intensity of infectiousness to patterns of norovirus transmission.* PLoS One, 2013. 8(7):e68413.

55. Atmar, R.L., Opekun, A.R., Gilger, M.A., Estes, M.K., Crawford, S.E., Neill, F.H., Ramani, S., Hill, H., Ferreira, J., and Graham, D.Y., *Determination of the 50% Human Infectious Dose for Norwalk Virus.* J Infect Dis, 2013.

56. Sukhrie, F.H., Teunis, P., Vennema, H., Bogerman, J., van Marm, S., Beersma, M.F., and Koopmans, M., *P2 domain profiles and shedding dynamics in prospectively monitored norovirus outbreaks.* J Clin Virol, 2013. 56(4):286-92.

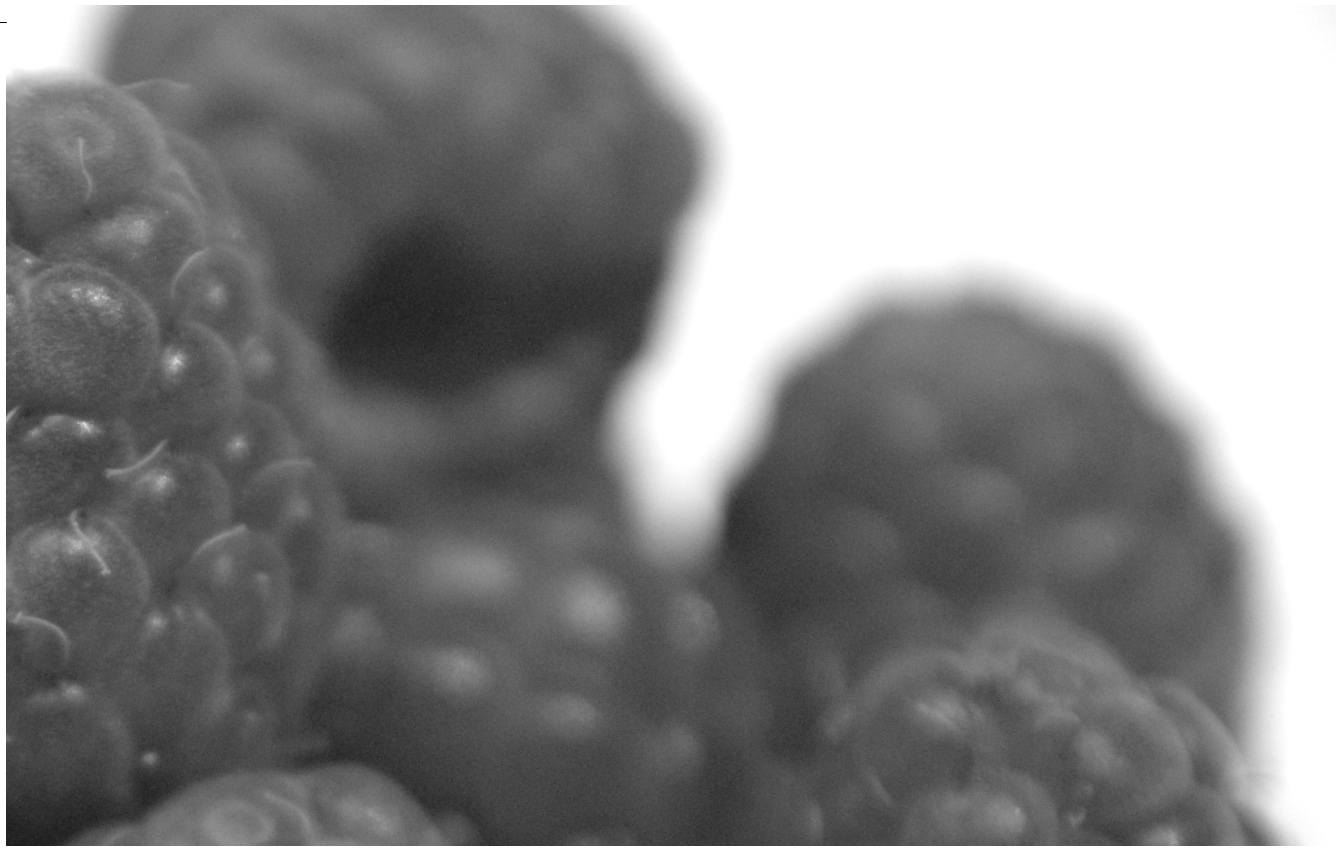
57. Shirai, J., Kanno, T., Tsuchiya, Y., Mitsubayashi, S., and Seki, R., *Effects of chlorine, iodine, and quaternary ammonium compound disinfectants on several exotic disease viruses.* J Vet Med Sci, 2000. 62(1):85-92.

58. Lenard, J. and Vanderoef, R., *Photoinactivation of influenza virus fusion and infectivity by rose bengal.* Photochem Photobiol, 1993. 58(4):527-31.

59. Lenard, J., Rabson, A., and Vanderoef, R., *Photodynamic inactivation of infectivity of human immunodeficiency virus and other enveloped viruses using hypericin and rose bengal: inhibition of fusion and syncytia formation.* Proc Natl Acad Sci U S A, 1993. 90(1):158-62.

60. Bezman, S.A., Burtis, P.A., Izod, T.P., and Thayer, M.A., *Photodynamic inactivation of E. coli by rose bengal immobilized on polystyrene beads.* Photochem Photobiol, 1978. 28(3):325-9.

7



General discussion

General Discussion

Foodborne disease outbreaks associated with the consumption of fruits and vegetables are on the rise, with noroviruses causing the majority of these outbreaks [1-4]. However, currently there are "no effective, realistic and validated risk management options to eliminate viral contamination on fresh produce prior to consumption, without changing the desired characteristics of the food" [5]. The research described in this thesis therefore focused on the persistence of noroviruses on fresh produce (**chapter 2**) and the relevant norovirus introduction sources in food chains (**chapters 3**, **4**, **5** and **7**). Applicable and efficient mitigation strategies to prevent or reduce virus contamination of fresh produce and thereby to diminish the health risk of norovirus disease associated with its consumption were evaluated using a risk assessment approach (**chapters 3**, **6** and **7**).

Norovirus persistence on fresh produce

Knowledge on the persistence of noroviruses under commonly applied storage conditions in fresh produce chains is crucial to estimate health risks associated with the consumption of produce and to set appropriate mitigation strategies. The frequent association of raspberries and lettuce with norovirus outbreaks suggests virus persistence on these fresh produce types. Studies have demonstrated norovirus persistence under commonly applied storage conditions for lettuce [6-8] and frozen soft berries [9]. It was likewise demonstrated in **chapter 2** that noroviruses GI.4, GII.4 and MNV-1 persist during the shelf life of fresh raspberries and strawberries under post-harvest storage conditions at a temperature of up to 10 °C for strawberries and 21 °C for raspberries. The few studies on persistence of norovirus surrogates and other viruses on fresh produce likewise suggest viral persistence [10-12]. Thus, once fresh produce is contaminated, a considerable number of viruses likely remain infectious until produce consumption. The persistence of noroviruses under field conditions is, however, not well studied, even though the decay rate may significantly affect health risk [13], and determine the critical time window for norovirus introduction in the primary production process. Nevertheless, one can conclude that post-harvest contamination and contamination during primary production, at least shortly before harvest, is likely to pose a public health risk.

Food matrix effects on norovirus persistence

Considering fruit consumption patterns, it is striking that especially the consumption of raspberries is frequently associated with norovirus outbreaks but not that of other fruits such as apples, which are one of the most popular fruits [14, 15] and can be eaten raw and unpeeled. Monitoring studies on the presence of norovirus on apples are lacking, but contamination during their production is possible, as they may be spray-irrigated and are manually handled during picking and retail. Different virus loads introduced onto the product, or different persistence of infectious noroviruses during production

of apples as compared to e.g. raspberries, may explain why apples are so far unlinked to norovirus disease. Persistence of noroviruses on apples is unexplored, but, like most fresh produce types, apples are regularly stored at low temperatures that favor persistence [16]. Yet not only production and storage conditions but also the food matrix may determine persistence of noroviruses. It was observed in **chapter 2** that norovirus decay rates differ even among seemingly similar fruits like raspberries and strawberries. As discussed in that chapter, matrix factors such as physiological parameters (e.g., the respiration rate), surface structure and composition, and the presence of degrading enzymes and endogenous antiviral compounds may lead to differences in norovirus persistence on produce types despite comparable conditions. Raspberries are characterized by much higher respiration rates as compared to apples [17-19], and the water vapor released by respiration [20] may prevent the virus from the desiccation that leads to virus reduction, especially at higher temperatures. Moreover, the wax layer of apples provides an excellent barrier against water loss [21] and may therefore hasten the desiccation of norovirus on apples. The effect of food-specific characteristics on norovirus persistence needs to be better understood. Such understanding could enable grouping of food items into different risk categories for the transmission of certain viruses based on matrix characteristics. Aside from such characteristics, the apparent dominance of raspberries in outbreaks may in part reflect the fact that stakeholders, from inspectors to consumers, are aware of its risks and therefore more watchful for berry-related outbreaks than for outbreaks potentially caused by less notorious and well studied types of produce. Furthermore, different binding affinities of norovirus to different fresh produce types [22] may influence the relevance of certain fresh produce types in transmitting noroviruses.

Contribution of introduction sources to the public health risk

In addition to viral persistence, understanding the contribution of norovirus contamination sources to the public health risk is important to develop successful mitigation strategies to reduce viral contamination in fresh produce chains.

Food handlers

Based on investigations of norovirus outbreak with a known source, food handlers are assumed to be the main introduction source [23-25]. Investigations typically look at food handlers at the food preparation steps just before consumption. Norovirus can, however, be introduced early in the food chain by food handlers at harvest or at sorting of produce before distribution. Such and early introduction, results in more diffuse outbreaks, which are difficult to link to the possible role of food handlers when using an epidemiological approach. In **chapter 3,** transfer proportions of noroviruses to fresh produce were experimentally determined, and a scenario in which infected food handlers picked raspberries at harvest was used to simulate the spread of noroviruses and

the associated health risks. This simulation was based on an assumed virus concentration on the hands of those food handlers not following good hygiene practice, as actual data were lacking. Our simulation can be improved by applying the data of a recent study by Liu et al. [26], who determined an average contamination of 7 x 10^3 genomic copies on a hand of infected individuals after defecation. It was assumed that 100% and 10% of the noroviruses present on the hand were in contact with the picked raspberry, representing a worst-case scenario and a scenario in which virus particles were evenly distributed on the hand, with about 10 % of the hand surface in touch with the raspberry, as estimated in **chapter 5**. Applying these two scenarios and rerunning the model discussed in **chapter 3,** it was estimated that about 2.8 kg and 1.2 kg of raspberries may become contaminated by a single food handler, given the model assumptions explained in the chapter. The contaminated raspberries were simulated to be eaten individually, resulting in about 290 and 100 infections, respectively. These simulations illustrate the efficient spread of norovirus by food handlers and indicate a potentially large number of infected people resulting from a single infected food handler. The simulation of **chapter 3** showed in addition that low transfer proportions as found for soft berries result in an increased public health risk as compared to higher transfer rates, possibly promoting norovirus transmission by raspberries. Also, the quantitative risk assessment of **chapter 5** confirmed the contact of hands with produce as one of the dominant sources of contamination as compared, for example, with irrigation water and surfaces, given the low norovirus concentration observed in the VITAL monitoring study and the modeling used. The determined relative contributions of introduction sources, are, however, specific to the monitoring data employed, and cannot be generalized on basis of the limited monitoring data available. The data of both chapters confirmed the potentially high relevance of food handlers to the introduction and spread of noroviruses in fresh produce chains and emphasizes clearly the need to implement preventive measures.

Water

As described in the introduction a great variety of water sources potentially harboring noroviruses are used in the production of fresh produce, and water can contaminate produce by a range of applications such as irrigation [13] and dilution of pesticides before spraying (**chapter 4**). The public health risk associated with the introduction of noroviruses by contaminated water is determined by the i) prevalence and concentration of noroviruses in the used water, ii) volume of water and consequently the number of viruses adhering to the edible parts of the produce, and iii) persistence of viruses on the produce until its consumption. In case of pesticide application, the viruses additionally need to persist in the pesticide dilutions. The persistence of viruses in pesticides had not been studied, and the results discussed in **chapter 4** suggest that noroviruses may frequently persist in even the highest applied concentrations of pesticides. Thus

the application of pesticides may itself pose a risk for virus introduction, if contaminated water is used to dilute pesticides. The type of water used to dilute pesticides is not regulated in Europe [27]. Moreover, one can minimize the risk that irrigation water will introduce viruses onto fresh produce (e.g., by drip irrigation), but pesticides must typically contact the edible part of a plant to achieve adequate pest control. For certain produce types, such as soft berries and lettuce, fungicides and insecticides are not uncommonly approved for application within just a few days of harvest, and some can be used on the day of harvest [28-30]. Because viruses remain infectious during the shelf-life of fresh and frozen raspberries and other produce, the application of pesticides, especially close to harvest, may pose a microbiological risk to public health if the water used to dilute the pesticides is not controlled.

Mitigation strategies

Controlling viruses in fresh produce requires a preventive food chain approach with a focus on avoiding viral contamination rather than reducing the presence of persistent viruses [27]. This is especially true for perishable foods, such as fresh produce, because their nature makes it difficult to apply mitigation measures while maintaining the organoleptic properties. Preventive measures should target food handlers, the water used during the production process, and surfaces such as food preparation surfaces and utensils, conveyor belts, and storage containers.

Food handlers' hygiene

Appropriate and continuous hand hygiene is probably the most relevant measures to prevent virus contamination of fresh produce by food handlers [31, 32]. Currently, washing hands under running water with soap for 20 s and subsequent hand drying with paper towels is recommended [5, 27, 33]. Washing hands for 10 s with soap reportedly reduced about 1 $\log_{10}$-unit of norovirus genomes from hands [34], representing a conservative reduction efficacy of hand washing, because the recommended procedure was not entirely realized. Based on the risk assessment model of **chapter 3** and the two previously described scenarios, in which the hands of raspberry pickers carried about 7 x 10^3 and 7 x 10^2 virus particles, a 1 $\log_{10}$-unit reduction of norovirus genomes on hands would translate to a mean reduction of about 200 from 290 infections and 86 from 100 infections, respectively. For both scenarios, a considerable health risk remained but hand washing resulted in a substantial reduction of the public health risk. Overall, results suggest that hand-washing efficiently reduces the spread of norovirus by food handlers, but may not completely prevent norovirus disease if viral loads are high. Not only the actual virus reduction on hands, but also the compliance to good hand hygiene practices, determines the successful prevention of norovirus introduction by infected food handlers. It has been suggested that hand washing compliance amongst fresh produce

farm workers can be amplified by educational and training programs and easy access to hand washing facilities [35].

Guidelines on water quality

Guidelines on hygiene practice of fresh produce emphasize the use of clean water in productions chains, but do not provide specific criteria on the required water quality [5, 27, 36]. It is stated that clean water shall not contain virus levels in quantities capable of compromising the food safety of fresh produce [5], but, the virus level causing a public health risk remains unspecified. Risk based limits (performance objectives[1]) on virus concentration at various points of the food chain can be derived from QMRA models [37, 38]. The QMRA model of **chapter 5** was used to determine in a scenario analysis the effect of a range of norovirus concentrations in irrigation water on the annual infection risk for the consumption of lettuce (see formula 6, Schijven et al. 2012) (Figure 1). For the calculation of the annual risk an average consumption size of 54 g lettuce and an annual consumption on 80/365 days [40] was used. To account for variability and uncertainty of the parameters determining the health risk, second order Monte Carlo simulations were performed. To this end, a thousand samples from variability (concen-

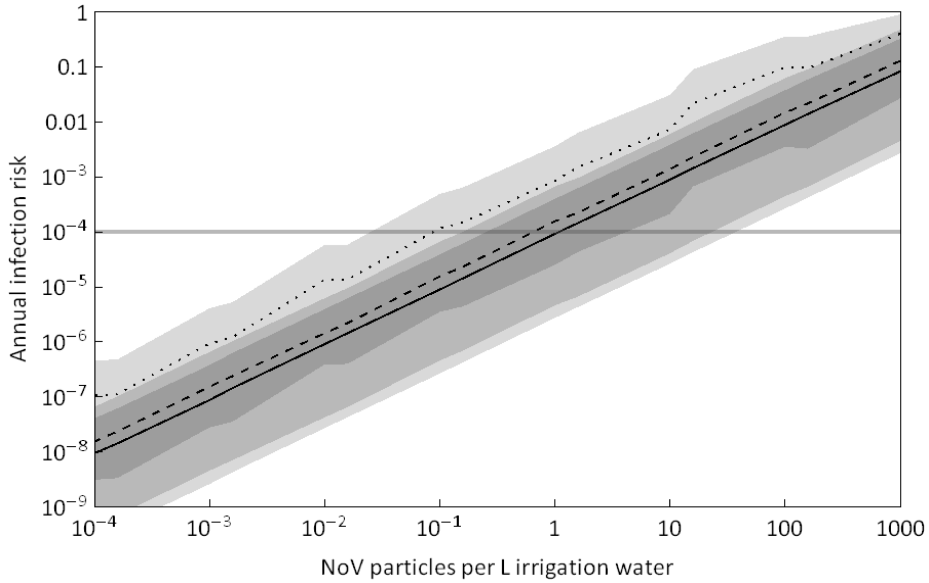


Figure 1. Scenario analysis on the effect of norovirus contamination levels in irrigation water on the annual infection risk of lettuce consumption. Variability in norovirus concentrations is represented by different standard deviations (0.25 = solid line, 0.5=dashed line, 1=dotted line). The grey area around the lines shows the 95 % confidence interval on the uncertainty around the estimates. The grey line represents the commonly used annual health risk of 10^{-4}.

[1] irrigation water falls strictly speaking not within the definition of a performance objective, as it is neither a food nor a food ingredient [38]

tration of noroviruses per L water) or uncertainty distributions (removed norovirus-proportion via harvesters' hands, norovirus inactivation, and dose-response parameters) of the parameters were generated, resulting eventually in the uncertainty distribution for the annual infection risk (mean and 95% upper limit).

A mean concentration around 0.4 norovirus particles per liter was estimated to realize with 95% certainty the conventional health target for drinking water of less than 1 infection per 10,000 people per year [38] (see Table 1, Figure 1). Note that the limit to the mean concentrations is insensitive to its variance, but that the limit to the median (and hence the mean log concentration) decreases as the variance increases. The determined performance objective[1] on the irrigation water quality relates specifically to the described lettuce chain of the risk assessment of **chapter 5** and needs to be re-evaluated for other scenarios, for example, lettuce chains were no rinsing is applied, or chains in which other withholding periods (time between irrigation and harvest) are used.

The estimated performance objective gives an indication of the suitability of water sources in the primary production of fresh produce. However, the ratio of infectious to non-infectious norovirus genomes in water sources cannot be determined and is likely highly variable. The ratio of virus genomes to cultivable viruses may range between

Table 1. Estimated maximum concentrations of norovirus particles per *L* irrigation water that results in an annual infection risk of less than 1 in 10,000 consumers according to the risk assessment from chapter 5.

Mean annual infection risk $<10^{-4}$				95% upper limit of annual infection risk $<10^{-4}$			
Mean (log_{10})	SD (log_{10})	Median	Arithmetic mean	Mean (log_{10})	SD (log_{10})	Median	Arithmetic mean
0.05	0.25	1.1	1.3	-0.47	0.25	0.34	0.4
-0.17	0.5	0.7	1.3	-0.69	0.5	0.20	0.4
-0.99	1	0.1	1.4	-1.50	1	0.03	0.4

20 and 10^4 in surface waters [41, 42], and is generally influenced by the water type, temperature, and the microbial water flora [43, 44]. Noroviruses were shown to remain infectious in groundwater for at least 61 days (length of study period) based on a volunteer study [45]. That study also showed that norovirus particles remained detectable by RT-PCR for over three years, with a final reduction of only 1 log_{10}-unit, and that even pure norovirus RNA was stable for at least 2 weeks. The persistence of norovirus particles and RNA in surface water is likely lower as compared to groundwater [44]. In the absence of a detection system for infectious noroviruses, the implementation of critical virus concentration in irrigation water is challenging. Without knowledge of how to interpret the presence of norovirus genomes, it cannot be concluded that genomic copy numbers above the proposed critical limit pose a health risk. However, it can be inferred

that genome levels below the critical limit do not pose a health risk, based on the applied modeling.

Another issue inherent in the proposed critical limit relates to detection of concentrations in irrigation water that fall below the detection limit of the ISO TS 15216-2 protocol on detection of viruses in water [46]. This issue makes it practically impossible to determine and control the proposed critical virus levels. Nevertheless, it is possible to compare the critical virus levels in irrigation water to those detected in field samples and decide on possible controls. The few data on norovirus genome levels in irrigation water were obtained from river water sources containing up to 10^2 to 10^3 norovirus genomes per liter of irrigation water [46, 47]. As a comparison, the risk assessment of **chapter 5,** based on the VITAL monitoring, used an average norovirus level of 1 virus particle per liter of irrigation water, indicating a wide variation of norovirus concentrations among monitoring studies and locations. Norovirus levels determined in irrigation water may consequently exceed the above-determined critical virus levels by several orders of magnitude, suggesting that the health risk from the use of surface water in irrigation may exceed the prescribed health target.

For the determination of suitable water sources, e.g., the Codex guidelines on hygienic practice for fresh leafy vegetables [36] provide guidance for on-site sanitary surveys to assess the microbial contamination potential of water sources. Additionally, treatments to reduce pathogen loads are advisable when water sources pose a high risk of contamination e.g., surface water, reclaimed wastewater, and shallow, unprotected groundwater [36], especially when produce is eaten raw or minimally processed. To achieve the health target of less than 1 infections in 10,000 consumers, norovirus concentrations of up to 10^2 to 10^3 virus genomes per liter call for measures reducing viral loads by about 2.5 to 3.5 $\log_{10}$-units. This performance criterion is again specific to the lettuce chain of **chapter 5.** The WHO guidelines on the use of wastewater in agriculture [48] provide detailed recommendation on the suitability of water treatment processes applicable in agriculture, such as waste stabilization ponds or disinfection treatments [49-51], and additionally on application techniques for irrigation to minimize water contact with produce, such as drip irrigation. It is estimated that combining various pre-harvest measures such as water treatments, drip irrigation, or withholding periods may result in up to a 6 $\log_{10}$-unit reduction of pathogens on produce [48]. However, data on the actual reduction of norovirus particles on produce by e.g. drip irrigation or withholding periods are lacking [52]. Regarding pesticides, the microbiological health risk related to application can be lowered if pesticides are produced with added antiviral compounds, such as chlorine compounds [53] or natural substances [54], an approach that is independent of user compliance (see **chapter 4**).

Preventing surface cross-contamination

Surfaces in food-processing or preparation areas can be contaminated with noroviruses from, for example, the hands of food handlers' practicing poor hand hygiene, and may thus contribute to the cross-contamination of fresh produce. A contaminated food handler transfers about 15% of the norovirus on his hands to a steel surface [55] and contaminated steel transfers about 5% of norovirus to lettuce [56]. An initial virus concentration on the hands of up to 7×10^3 virus particles [26] would result in a critical contamination level of about 50 virus particles on lettuce, posing a significant risk of infection to the consumer [57]. This rough estimate illustrates the potential health risk from surface cross-contamination. Surfaces in food settings are usually cleaned by wiping them with and without disinfectants. **Chapter 6** showed that cleaning wet spots with dry wipes efficiently reduced norovirus spot contamination of surfaces. On average, about 0.2% of the spiked noroviruses remained on a steel carrier after wiping, and the same proportion was found on a second steel carrier that was subsequently wiped with the same wipe. Based on the given example, cleaning of surfaces may likely prevent cross-contamination from surfaces to food, and although reuse of contaminated wipes can cause further spread of norovirus, its concentration is low and unlikely to increase the public health risk for fresh produce consumption. However, surfaces in food processing areas can also be contaminated with norovirus by vomit, as norovirus disease causes projectile vomiting. Even after routine cleaning of surfaces, continued transmission of norovirus via surfaces contaminated with vomit is reported to contribute to large outbreaks [25, 58]. Assuming a vomit quantity of 30 mL in a vomit event [59], about 10^6 genomic norovirus copies are released [60]. Here, a first cleaning step using dry wipes as described in **chapter 6** and subsequent wiping with soap and a chlorine concentration of 1000 ppm, reducing norovirus genomes by about 3 $\log_{10}$-units [61], may be appropriate to reduce viral spread.

Measures aiming at reducing the number of infectious noroviruses on fresh produce

Due to the properties of noroviruses, their abundance in the environment, and non-compliance to e.g., good hygiene practice, a consistent and complete prevention of norovirus contamination in food chains is illusionary. In addition to measures preventing norovirus contamination, appropriate measures reducing infectious norovirus loads on foods are thus required for adequate food safety. The current possibilities in the fresh produce industry are limited by the perishability of the product. Moreover, legal requirements and consumer concerns narrow the choices. Even though scientifically considered safe, irradiation of produce and washing of produce with recommended chlorine concentrations lack consumer acceptance and legal allowance in several countries [62, 63]. The costs of equipment and treatment measures are an additional concern, especially for smaller producers.

Efficacy of mitigation measures in reducing noroviruses on fresh produce

The effect on the public health risk of measures reducing the number of infectious norovirus particles on fresh produce is largely unknown. We used the risk assessment model of **chapter 3** to evaluate norovirus reduction levels on fresh produce after norovirus introduction by food handlers. The previous scenarios of 7×10^3 and 7×10^2 virus particles on the hands of raspberry pickers were reused to simulate a 1, 2 and 3 $\log_{10}$-unit reduction of norovirus particles on raspberries. For the first scenario, the mean number of 290 infections without treatment decreased to 125, 21 and 2 infections, respectively; for the second scenario, the initial number of 100 infections decreased to 20, 2 and 0 infections, respectively. This example suggests that the first 1 $\log_{10}$-unit reduction prevents the most infections but that a 3 $\log_{10}$-unit reduction still adds to the reduction of the public health risk, especially at higher initial viral loads on produce. Thus, even though treatments achieving a 1 $\log_{10}$-unit reduction of norovirus on fresh produce (e.g., washing or irradiation) may not lower the infectious risk to zero, they may still lower the public health risk substantially, especially if they present one of multiple hurdles in a chain of risk management options The extent of the infectious risk reduction is dependent on the distribution of contamination levels on produce (see **chapter 3**). In addition, the example showed that a 1 $\log_{10}$-unit reduction of norovirus on hands results in a greater reduction of the public health risk than a 1 $\log_{10}$-unit reduction of norovirus on fresh produce.

Potential intervention measures

Numerous studies have lately been published on the efficacies of non-thermal intervention measures that could be applied to fresh produce to reduce the number of infectious noroviruses (see introduction). Reviewing these papers shows that treatment applicability and efficacies strongly depend on virus-matrix combinations and suggest that interventions must be tailored for the needs of specific produce pathogen combinations. For instance human volunteer studies show that high pressure treatment efficiently reduces infectious human noroviruses [64] but negatively affects the quality of fresh berries and lettuce [65]. Nevertheless, this technology may be appropriate to reduce the number of infectious norovirus particles in berry purees [65, 66] or berries to be frozen [65]. Ionizing radiation has the advantage that it penetrates fresh produce, allowing for inactivation of internalized viruses as compared to UV-radiation. At low doses (2 kGy) radiation does not affect produce quality [67], and application in the form of gamma radiation is suitable for fresh lettuce and strawberries achieving about a 1 $\log_{10}$-unit reduction [68-70]. For E-beam radiation, the literature suggests that higher doses as compared to gamma-radiation may be required to achieve norovirus reduction [71]. Other treatments such as cold plasma [72], gaseous sanitizers, and hurdle approaches [73] also have potential in reducing noroviruses on fresh produce while maintaining

produce quality, but their efficacies need to be further investigated. Even though heating changes the sensory and nutritional values of soft berries, it should be considered as an intervention in food settings where a great number of people are served, such as in catering and canteen operations. Some national food safety authorities, such as the Finnish Food Safety Authority (Evira), recommend heating soft berries for 2 min at 90 °C in food service establishments. Also in Germany, heating of frozen berries before consumption is nowadays recommended as a consequence of a large outbreak associated with strawberries served in canteen settings affecting more than 10,000 people. Prevention of such outbreaks in the future may require general European guidelines on heating soft berries in canteen settings, especially when sensitive groups are served, as in health care facilities.

Future Perspectives

The risk assessment described in **chapter 5** can be used to inform risk management plans on the efficiency of potential measures mitigating the risk of norovirus disease from fresh produce and to reach conclusions on control options and their critical limits. To investigate the performance of measures reducing viral contamination, various data gaps must be filled. For example, to evaluate the efficacy of drip irrigation in reducing viral contamination during primary production, the actual amount of water in contact with the produce must be determined, especially if produce grows close to the ground, like lettuce. To set appropriate withholding periods requires determination of produce-specific decay rates of norovirus under field conditions.

Additional monitoring studies on norovirus levels and prevalence on fresh produce and related introduction sources must be targeted to i) validate the effect of mitigation measures on the public health risk (e.g., measures to improve compliance of hand hygiene practices), ii) improve estimates on the contribution of norovirus introduction sources to the public health risk, and iii) decrease model uncertainties. Studies should also look at less considered introduction sources, such as the use of septic tanks that combine animal and human manure [74] or use of water for the dilution of pesticides. For comparability of monitoring studies, standardized and readily verified ISO protocols for the detection of norovirus on fresh produce and water should be applied [75]. Extensive and comparable monitoring data may facilitate the interpretation of the presence of norovirus genomes on fresh produce. For shellfish, batches associated and not-associated to outbreaks were found to differ significantly as to their norovirus genome levels [76, 77]. A similar approach for fresh produce, comparing genome levels on food linked to outbreaks with levels observed in routine monitoring, could help inform the establishment of threshold criteria of norovirus levels on fresh produce.

The farm-to-fork risk assessment described in **chapter 5** indicates that incidental contamination events have a larger adverse public health effect than do general production practices. Peak events in the production process (e.g., high virus concentrations in irrigation water) may especially contribute the risk for outbreaks. To prevent these peak events, we need to identify their causes or risk factors (e.g., unusually heavy rainfall) and verify our hypotheses by intense monitoring studies [78].

Additional studies on the characteristics of food matrices and viruses that determine the risk of norovirus introduction and persistence in fresh produce chains are required. The performed risk assessment, for example, assumed 100% binding of norovirus to produce during drip irrigation. Further research on virus binding mechanisms to fresh produce [79-81], would contribute to data required to determine more reliable risk based-criteria for the quality of irrigation water. Norovirus binding preferences may also elucidate why certain fresh produce types are more often linked to outbreaks than others despite sharing comparable contamination sources and practices during production and consumption. Moreover, binding of noroviruses may not only be produce-specific but also strain-specific [81]. Currently, a greater persistence of NoV GI as compared to GII [82, 83] is widely considered to explain the frequent presence of NoV GI on food. Yet our studies did not confirm a greater persistence of NoV GI genomes. An additional explanation may be that NoV GI binds more efficiently to produce than NoV GII, as shown for shellfish [84].

Overall conclusions

The results of this thesis fill some of the knowledge gaps regarding the safety of fresh produce. Noroviruses were found to persist on fresh soft berries under current post-harvest conditions, supporting the need of a preventative food chain approach for safety assurance. A risk assessment approach confirmed the high relevance of infected food handlers and contaminated water as introduction sources of norovirus. In addition, the application of pesticides was determined to be a potential microbiological risk to public health, if contaminated water is used to dilute the pesticides. The efficiency of strategies to mitigate public health risk was evaluated, indicating that a robust 1 $\log_{10}$-unit reduction of norovirus levels in introduction sources and/or on the produce itself may considerably decrease the health risk of norovirus on fresh produce. Finally, the designed quantitative farm-to-fork risk assessment model provides a basis for food safety regulations, for example, by presenting a framework for deriving performance objectives like norovirus concentrations in irrigation water.

References

1. Berger, C.N., Sodha, S.V., Shaw, R.K., Griffin, P.M., Pink, D., Hand, P., and Frankel, G., *Fresh fruit and vegetables as vehicles for the transmission of human pathogens.* Environ Microbiol, 2010. 12(9):2385-97.
2. Lynch, M.F., Tauxe, R.V., and Hedberg, C.W., *The growing burden of foodborne outbreaks due to contaminated fresh produce: risks and opportunities.* Epidemiol Infect, 2009. 137(3):307-15.
3. Sivapalasingam, S., Friedman, C.R., Cohen, L., and Tauxe, R.V., *Fresh produce: a growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997.* J Food Prot, 2004. 67(10):2342-53.
4. Doyle, M.P. and Erickson, M.C., *Summer meeting 2007 - the problems with fresh produce: an overview.* J Appl Microbiol, 2008. 105(2):317-30.
5. CAC, *Guidlines on the application of general principles of food hygiene to the control of viruses in food*. 2012, Codex Alimentarius Commission.
6. Fallahi, S. and Mattison, K., *Evaluation of murine norovirus persistence in environments relevant to food production and processing.* J Food Prot, 2011. 74(11):1847-51.
7. Lamhoujeb, S., Fliss, I., Ngazoa, S.E., and Jean, J., *Evaluation of the persistence of infectious human noroviruses on food surfaces by using real-time nucleic acid sequence-based amplification.* Appl Environ Microbiol, 2008. 74(11):3349-55.
8. Escudero, B.I., Rawsthorne, H., Gensel, C., and Jaykus, L.A., *Persistence and Transferability of Noroviruses on and between Common Surfaces and Foods.* Journal of Food Protection, 2012. 75(5):927–935.
9. Butot, S., Putallaz, T., and Sanchez, G., *Effects of sanitation, freezing and frozen storage on enteric viruses in berries and herbs.* Int J Food Microbiol, 2008. 126(1-2):30-5.
10. Dawson, D.J., Paish, A., Staffell, L.M., Seymour, I.J., and Appleton, H., *Survival of viruses on fresh produce, using MS2 as a surrogate for norovirus.* J Appl Microbiol, 2005. 98(1):203-9.
11. Kurdziel, A.S., Wilkinson, N., Langton, S., and Cook, N., *Survival of poliovirus on soft fruit and salad vegetables.* J Food Prot, 2001. 64(5):706-9.
12. Mattison, K., Karthikeyan, K., Abebe, M., Malik, N., Sattar, S.A., Farber, J.M., and Bidawid, S., *Survival of calicivirus in foods and on surfaces: experiments with feline calicivirus as a surrogate for norovirus.* J Food Prot, 2007. 70(2):500-3.
13. Hamilton, A.J., Stagnitti, F., Premier, R., Boland, A.M., and Hale, G., *Quantitative microbial risk assessment models for consumption of raw vegetables irrigated with reclaimed water.* Appl Environ Microbiol, 2006. 72(5):3284-90.
14. FAO, *FAOSTAT database* 2013, Food and Agriculture Organization of the United Nations.
15. RIVM. *Dutch National Food Consumption Survey*. 2010,cited 2014; Available from: www.rivm.nl/vcp.
16. Paull, R.E., *Effect of temperature and relative humidity on fresh commodity quality.* Postharvest Biology and Technology, 1999. 15(3):263–277.
17. Saltveit, M.E. *Respiratory Metabolism* The Commercial Storage of Fruits, Vegetables, and Florist and Nursery Stocks. Agriculture Handbook Number 66 2004.
18. Robbins, J., Moore, P.P., and Patterson, M., *Fruit Respiration Rates and Firmness of red Raspberry and related Rubus Genotypes* Acta Horticulturae (ISHS), 1989. 262:311-318.
19. Singh, R.M. and Sharma, R.R., *Harvesting, Postharvest Handeling and Physiology of Fruits and Vegetables*. Postharvest Technology of Fruits and Vegetables: Handleling, Processing, Fermentation and Wastemanagment, ed. L.R. Vermer and V.K. Joshi. Vol. 1. 2010, New Delhi: Indus Publishing Group.
20. Fonseca, S.C., Oliveira, F.A.R., and Brecht, J.K., *Modelling respiration rate of fresh fruits and vegetables for modified atmosphere packages: a review. .* Journal of Food Engineering, 2002. 52:99-119.
21. Veraverbeke, E.A., Lammertyn, J., Saevels, S., and Nicola, B.M., *Changes in chemical wax composition of three different apple (Malus domestica Borkh.) cultivars during storage.* Postharvest Biology and Technology, 2001. 23(3):197-208.
22. Li, J., Predmore, A., Divers, E., and Lou, F., *New interventions against human norovirus: progress, opportunities, and challenges.* Annu Rev Food Sci Technol, 2012. 3:331-52.

23. Hall, A.J., Eisenbart, V.G., Etingue, A.L., Gould, L.H., Lopman, B.A., and Parashar, U.D., *Epidemiology of foodborne norovirus outbreaks, United States, 2001-2008.* Emerg Infect Dis, 2012. 18(10):1566-73.
24. Baert, L., Uyttendaele, M., Stals, A., van Coillie, E., Dierick, K., Debevere, J., and Botteldoorn, N., *Reported foodborne outbreaks due to noroviruses in Belgium: the link between food and patient investigations in an international context.* Epidemiol Infect, 2009. 137(3):316-25.
25. de Wit, M.A., Widdowson, M.A., Vennema, H., de Bruin, E., Fernandes, T., and Koopmans, M., *Large outbreak of norovirus: the baker who should have known better.* Journal of Infection, 2007. 55(2):188–193.
26. Liu, P., Escudero, B., Jaykus, L.A., Montes, J., Goulter, R.M., Lichtenstein, M., Fernandez, M., Lee, J.C., De Nardo, E., Kirby, A., Arbogast, J.W., and Moe, C.L., *Laboratory evidence of norwalk virus contamination on the hands of infected individuals.* Appl Environ Microbiol, 2013. 79(24):7875-81.
27. EFSA, *Scientific Opinion, An update on the present knowledge on the occurrence and control of foodborne viruses.* EFSA Journal, 2011. 9(7):2190-2286.
28. Mahr, D., McManus, P., Smith, B.R., Colquhoun, J., and Flashinski, R. *Strawberry and Raspberry Pest Management in Wisconsin*. 2009, January 2014; Available from: http://www.uncledavesenterprise.com/file/garden/fruit/Strawberry%20and%20Raspberry%20Pest%20Management%20in%20Wisconsin.pdf.
29. Fouche, C., Molinar, R., Canvari, M., Joshel, C., Mullen, B., and Weber, J. *Pesticides for specialty crops*. Small Farm Program 2000, January 2014; Available from: http://anrcatalog.ucdavis.edu/pdf/7253.pdf.
30. Verhaelen, K., Bouwknegt, M., Rutjes, S.A., and de Roda Husman, A.M., *Persistence of human norovirus in reconstituted pesticides--pesticide application as a possible source of viruses in fresh produce chains.* Int J Food Microbiol, 2013. 160(3):323-8.
31. Mokhtari, A. and Jaykus, L.A., *Quantitative exposure model for the transmission of norovirus in retail food preparation.* Int J Food Microbiol, 2009. 133(1-2):38-47.
32. Moe, C.L., *Preventing norovirus transmission: how should we handle food handlers?* Clin Infect Dis, 2009. 48(1):38-40.
33. Hall, A.J., Vinjé, J., Lopman, B., Park, G.W., Yen, C., Gregoricus, N., and Parashar, U., *Updated norovirus outbreak management and disease prevention guidelines.* Morbidity and Mortality Weekly Report, 2011. 60(3):1-18.
34. Liu, P., Yuen, Y., Hsiao, H.M., Jaykus, L.A., and Moe, C., *Effectiveness of liquid soap and hand sanitizer against Norwalk virus on contaminated hands.* Appl Environ Microbiol, 2010. 76(2):394-9.
35. Soon, J.M. and Baines, R.N., *Food safety training and evaluation of handwashing intention among fresh produce farm workers.* Food Control, 2012. 23(2):437–448.
36. CAC, *Code of hygienic practice for fresh fruits and vegetables. Adopted 2003. Revision 2010 (new Annex III for Fresh Leafy Vegetables).* 2010, Codex Alimentarius Commission.
37. Havelaar, A.H., Nauta, M.J., and Jansen, J.T., *Fine-tuning Food Safety Objectives and risk assessment.* Int J Food Microbiol, 2004. 93(1):11-29.
38. CAC, *Principles and guidelines for the conduct of microbiological risk management (MRM)*. 2007, Codex Alimentarius Commission.
39. Schijven, J.F., Teunis, P.F., Rutjes, S.A., Bouwknegt, M., and de Roda Husman, A.M., *QMRAspot: a tool for Quantitative Microbial Risk Assessment from surface water to potable water.* Water Res, 2011. 45(17):5564-76.
40. Rossum, C.T.M., Fransen, H.P., Verkaik-Kloosterman, J., Buurma-Rethans, E.J.M., and Ocke, M.C., *Dutch national food consumption survey 2007–2010. Diet of children and adults aged 7 to 69 years*. 2011, RIVM, National Institute for Public Health and the Environment: Bilthoven, The Netherlands.
41. de Roda Husman, A.M., Lodder, W.J., Rutjes, S.A., Schijven, J.F., and Teunis, P.F., *Long-term inactivation study of three enteroviruses in artificial surface and groundwaters, using PCR and cell culture.* Appl Environ Microbiol, 2009. 75(4):1050-7.
42. Rutjes, S.A., Lodder, W.J., van Leeuwen, A.D., and de Roda Husman, A.M., *Detection of infectious rotavirus in naturally contaminated source waters for drinking water production.* J Appl Microbiol, 2009. 107(1):97-105.
43. Tsai, Y.L., Tran, B., and Palmer, C.J., *Analysis of viral RNA persistence in seawater by reverse transcriptase-PCR.* Appl Environ Microbiol, 1995. 61(1):363-6.

44. Espinosa, A.C., Mazari-Hiriart, M., Espinosa, R., Maruri-Avidal, L., Mendez, E., and Arias, C.F., *Infectivity and genome persistence of rotavirus and astrovirus in groundwater and surface water.* Water Res, 2008. 42(10-11):2618-28.
45. Seitz, S.R., Leon, J.S., Schwab, K.J., Lyon, G.M., Dowd, M., McDaniels, M., Abdulhafid, G., Fernandez, M.L., Lindesmith, L.C., Baric, R.S., and Moe, C.L., *Norovirus infectivity in humans and persistence in water.* Appl Environ Microbiol, 2011. 77(19):6884-8.
46. El-Senousy, W.M., Costafreda, M.I., Pinto, R.M., and Bosch, A., *Method validation for norovirus detection in naturally contaminated irrigation water and fresh produce.* Int J Food Microbiol, 2013. 167(1):74-9.
47. Maunula, L., Kaupke, A., Vasickova, P., Soderberg, K., Kozyra, I., Lazic, S., van der Poel, W.H., Bouwknegt, M., Rutjes, S., Willems, K.A., Moloney, R., D'Agostino, M., de Roda Husman, A.M., von Bonsdorff, C.H., Rzezutka, A., Pavlik, I., Petrovic, T., and Cook, N., *Tracing enteric viruses in the European berry fruit supply chain.* Int J Food Microbiol, 2013. 167(2):177-85.
48. WHO, *Guidelines for the safe use of wastewater, excreta and greywater.* Wastewater use in agriculture, 2006. 2: http://whqlibdoc.who.int/publications/2006/9241546832_eng.pdf
49. da Silva, A.K., Le Guyader, F.S., Le Saux, J.C., Pommepuy, M., Montgomery, M.A., and Elimelech, M., *Norovirus removal and particle association in a waste stabilization pond.* Environ Sci Technol, 2008. 42(24):9151-7.
50. Mara, D. and Sleigh, A., *Estimation of norovirus and Ascaris infection risks to urban farmers in developing countries using wastewater for crop irrigation.* Journal of Water and Health, 2010. 8(3):572-576.
51. Mok, H.F., Barker, S.F., and Hamilton, A.J., *A probabilistic quantitative microbial risk assessment model of norovirus disease burden from wastewater irrigation of vegetables in Shepparton, Australia.* Water Res, 2014. 54:347-62.
52. Mara, D. and Sleigh, A., *Estimation of norovirus infection risks to consumers of wastewater-irrigated food crops eaten raw.* Journal of Water and Health, 2010. 8(1):39-43.
53. Kingsley, D.H., Vincent, E.M., Meade, G.K., Watson, C.L., and Fan, X., *Inactivation of human norovirus using chemical sanitizers.* Int J Food Microbiol, 2014. 171:94-9.
54. Li, D., Baert, L., and Uyttendaele, M., *Inactivation of food-borne viruses using natural biochemical substances.* Food Microbiol, 2013. 35(1):1-9.
55. Tuladhar, E., Hazeleger, W.C., Koopmans, M., Zwietering, M.H., Duizer, E., and Beumer, R.R., *Transfer of noroviruses between fingers and fomites and food products.* International Journal of Food Microbiology, 2013. 167:346–352.
56. D'Souza, D.H., Sair, A., Williams, K., Papafragkou, E., Jean, J., Moore, C., and Jaykus, L., *Persistence of caliciviruses on environmental surfaces and their transfer to food.* Int J Food Microbiol, 2006. 108(1):84-91.
57. Teunis, P.F., Moe, C.L., Liu, P., Miller, S.E., Lindesmith, L., Baric, R.S., Le Pendu, J., and Calderon, R.L., *Norwalk virus: how infectious is it?* J Med Virol, 2008. 80(8):1468-76.
58. Marks, P.J., Vipond, I.B., Carlisle, D., Deakin, D., Fey, R.E., and Caul, E.O., *Evidence for airborne transmission of Norwalk-like virus (NLV) in a hotel restaurant.* Epidemiol Infect, 2000. 124(3):481-7.
59. Caul, E.O., *Small round structured viruses: airborne transmission and hospital control.* Lancet, 1994. 343(8908):1240-2.
60. Atmar, R.L., Opekun, A.R., Gilger, M.A., Estes, M.K., Crawford, S.E., Neill, F.H., Ramani, S., Hill, H., Ferreira, J., and Graham, D.Y., *Determination of the 50% Human Infectious Dose for Norwalk Virus.* J Infect Dis, 2013.
61. Tuladhar, E., Hazeleger, W.C., Koopmans, M., Zwietering, M.H., Beumer, R.R., and Duizer, E., *Residual viral and bacterial contamination of surfaces after cleaning and disinfection.* Appl Environ Microbiol, 2012. 78(21):7769-75.
62. Group, F.I.W.S., *High-Dose Irradiation: Wholesomeness of Food Irradiated with Doses above 10 kGy* in *WHO technical report series*. 1999, World Health Organization (WHO): Switzerland, Geneva.
63. FAO and WHO, *Benefits and Risks of the Use of Chlorine-containing Disinfectants in Food Production and Food Processing* 2008, Report of a Joint FAO/WHO Expert Meeting USA.
64. Leon, J.S., Kingsley, D.H., Montes, J.S., Richards, G.P., Lyon, G.M., Abdulhafid, G.M., Seitz, S.R., Fernandez, M.L., Teunis, P.F., Flick, G.J., and Moe, C.L., *Randomized, double-blinded clinical trial for human norovirus inactivation in oysters by high hydrostatic pressure processing.* Appl Environ Microbiol, 2011. 77(15):5476-82.

65. Lou, F., Neetoo, H., Chen, H., and Li, J., *Inactivation of a human norovirus surrogate by high-pressure processing: effectiveness, mechanism, and potential application in the fresh produce industry.* Appl Environ Microbiol, 2011. 77(5):1862-71.

66. Kovac, K., Diez-Valcarce, M., Raspor, P., Hernandez, M., and Rodriguez-Lazaro, D., *Effect of high hydrostatic pressure processing on norovirus infectivity and genome stability in strawberry puree and mineral water.* Int J Food Microbiol, 2012. 152(1-2):35-9.

67. Anonymous, *Use of Irradiation to Ensure the Hygienic Quality of Fresh, Pre-Cut Fruits and Vegetables and Other Minimally Processed Food of Plant Origin* 2006, IAEA/FAO: Austria.

68. Wilkinson, V.M. and Gould, G.W., *Food Irradiation: A Reference Guide*. Vol. 2. 1998, Great Britain: Woodhead Publishing Limited.

69. Bidawid, S., Farber, J.M., and Sattar, S.A., *Inactivation of hepatitis A virus (HAV) in fruits and vegetables by gamma irradiation.* International Journal of Food Microbiology, 2000. 57(1-2):91-97.

70. Feng, K., Divers, E., Ma, Y., and Li, J., *Inactivation of a human norovirus surrogate, human norovirus virus-like particles, and vesicular stomatitis virus by gamma irradiation.* Appl Environ Microbiol, 2011. 77(10):3507-17.

71. Sanglay, G.C., Li, J., Uribe, R.M., and Lee, K., *Electron-beam inactivation of a norovirus surrogate in fresh produce and model systems.* J Food Prot, 2011. 74(7):1155-60.

72. Fernandez, A., Noriega, E., and Thompson, A., *Inactivation of Salmonella enterica serovar Typhimurium on fresh produce by cold atmospheric gas plasma technology.* Food Microbiol, 2013. 33(1):24-9.

73. Li, D., Baert, L., De Jonghe, M., Van Coillie, E., Ryckeboer, J., Devlieghere, F., and Uyttendaele, M., *Inactivation of murine norovirus 1, coliphage phiX174, and Bacteroides [corrected] fragilis phage B40-8 on surfaces and fresh-cut iceberg lettuce by hydrogen peroxide and UV light.* Appl Environ Microbiol, 2011. 77(4):1399-404.

74. Deng, M.Y. and Cliver, D.O., *Persistence of inoculated hepatitis A virus in mixed human and animal wastes.* Appl Environ Microbiol, 1995. 61(1):87-91.

75. Lees , D., *International Standardisation of a Method for Detection of Human Pathogenic Viruses in Molluscan Shellfish.* Food and Environmental Virology, 2010. 2:146–155.

76. Lowther, J.A., Avant, J.M., Gizynski, K., Rangdale, R.E., and Lees, D.N., *Comparison between quantitative real-time reverse transcription PCR results for norovirus in oysters and self-reported gastroenteric illness in restaurant customers.* J Food Prot, 2010. 73(2):305-11.

77. Lowther, J.A., Gustar, N.E., Hartnell, R.E., and Lees, D.N., *Comparison of norovirus RNA levels in outbreak-related oysters with background environmental levels.* J Food Prot, 2012. 75(2):389-93.

78. Westrell, T., Teunis, P., van den Berg, H., Lodder, W., Ketelaars, H., Stenstrom, T.A., and de Roda Husman, A.M., *Short- and long-term variations of norovirus concentrations in the Meuse river during a 2-year study period.* Water Res, 2006. 40(14):2613-20.

79. Esseili, M.A., Wang, Q., and Saif, L.J., *Binding of human GII.4 norovirus virus-like particles to carbohydrates of romaine lettuce leaf cell wall materials.* Appl Environ Microbiol, 2012. 78(3):786-94.

80. Gandhi, K.M., Mandrell, R.E., and Tian, P., *Binding of virus-like particles of Norwalk virus to romaine lettuce veins.* Appl Environ Microbiol, 2010. 76(24):7997-8003.

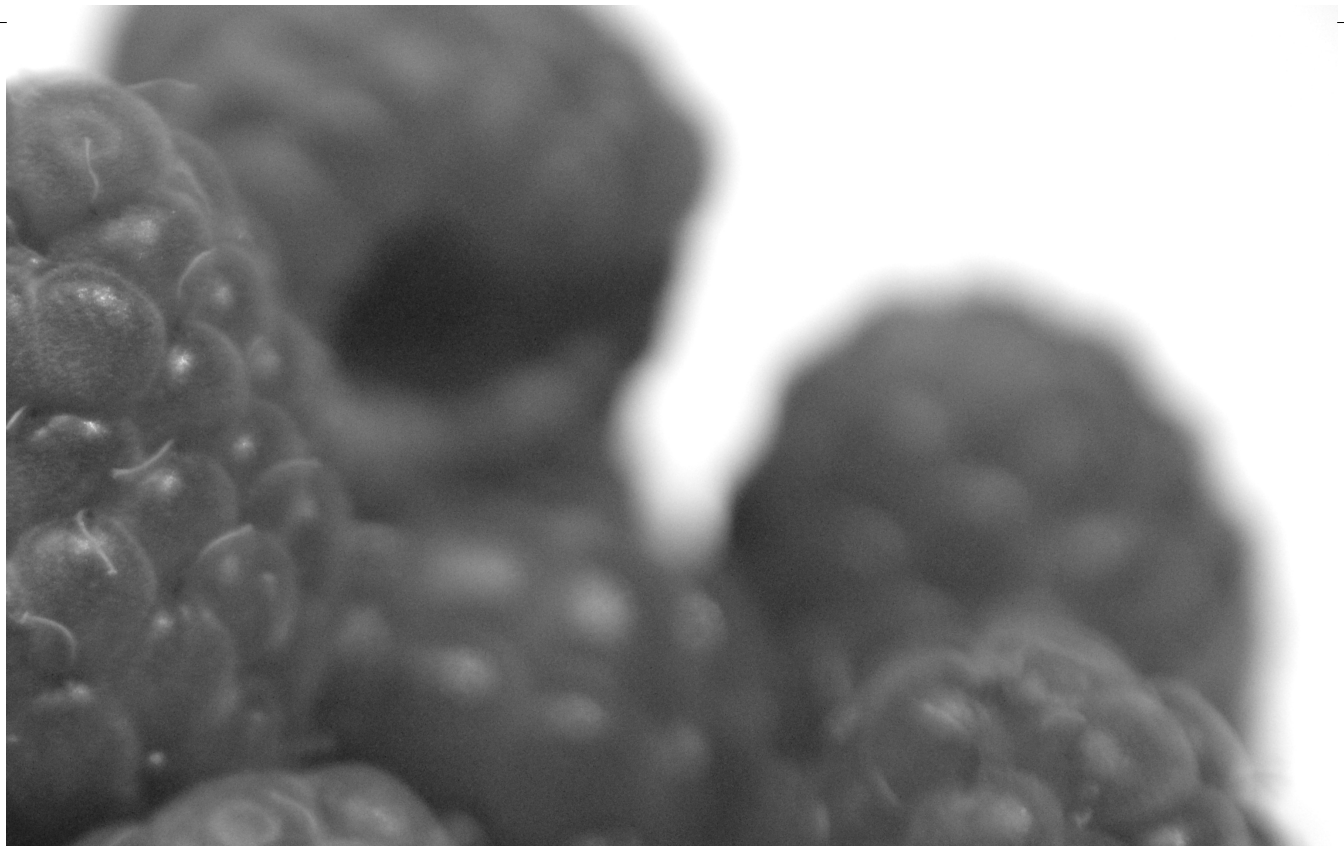
81. da Silva, A.K., Kavanagh, O.V., Estes, M.K., and Elimelech, M., *Adsorption and Aggregation Properties of Norovirus GI and GII Virus-like Particles Demonstrate Differing Responses to Solution Chemistry.* Environmental Science & Technology, 2011. 45(2):520-526.

82. Butot, S., Putallaz, T., Amoroso, R., and Sanchez, G., *Inactivation of enteric viruses in minimally processed berries and herbs.* Appl Environ Microbiol, 2009. 75(12):4155-61.

83. Hewitt, J., Rivera-Aban, M., and Greening, G.E., *Evaluation of murine norovirus as a surrogate for human norovirus and hepatitis A virus in heat inactivation studies.* J Appl Microbiol, 2009. 107(1):65-71.

84. Maalouf, H., Schaeffer, J., Parnaudeau, S., Le Pendu, J., Atmar, R.L., Crawford, S.E., and Le Guyader, F.S., *Strain-dependent norovirus bioaccumulation in oysters.* Appl Environ Microbiol, 2011. 77(10):3189-96.

8



Summary

Nederlandse samenvatting

Acknowlegdements

Curriculum Vitae

List of Pulbications

Summary

Human norovirus is a frequent cause of gastroenteritis worldwide, and viewed as the most common cause of foodborne disease. Norovirus outbreaks associated with fresh produce, especially soft-berries and lettuce are described, with up to thousands of cases. Risk management strategies need to be improved in order to reduce the norovirus disease burden associated with consumption of fresh produce. For this purpose, data on virus persistence, the relevance of norovirus contamination sources, and the efficacy of mitigation measures were compiled in this thesis. Since norovirus infectivity cannot be determined, our results are based on the detection of norovirus genomes and infectivity estimates derived from murine norovirus, a commonly used surrogate to study human norovirus persistence.

Noroviruses were found to persist during the shelf life of fresh raspberries and strawberries under commonly applied post-harvest storage conditions (chapter 2). Thus, post-harvest contamination and contamination during primary production, at least shortly before harvest, are likely to pose a public health risk upon fresh produce consumption. The study further showed that the food matrix affects viral persistence; however, the exact mechanism remained unknown. The identification of food-specific characteristics influencing norovirus persistence on foods requires further studies, for example, on the relevance of the respiration rate or antiviral substances of fresh produce on viral persistence.

Avoiding norovirus contamination, rather than reducing the presence of persistent viruses on produce, is the preferred approach for ensuring food safety. To this end, the relevance of diverse sources of contamination of fresh produce with norovirus was investigated using a risk-based approach (chapters 3, 5 and 7). In chapter 3, quantitative data on virus transfer proportions from hands to fresh produce and vice versa were determined to simulate the spread of noroviruses via fresh produce, and analyze the associated public health risk. The simulation showed that a single food handler picking raspberries may spread norovirus to a large number of raspberries and infect up to 290 consumers (chapter 7), given the model assumptions. The quantitative risk assessment model described in chapter 5 provided additional confirmation that the contact of hands with fresh produce, as compared with the other studied contamination sources, was one of the dominant sources of contamination, given the data and model used. Food handlers' hygiene is thus crucial for food safety, and already a 1 $\log_{10}$-unit reduction of noroviruses from hands by hand washing results in a substantial reduction of the public health risk, with a mean reduction of about 200 from 290 infections in the studied example (chapters 3 and 7). However, the compliance to hand washing procedures is a prerequisite.

In addition to food handlers, noroviruses may be introduced into food chains via contaminated surfaces. In chapter 6 the potential of wiping surfaces for the reduction of such cross-contamination was demonstrated. The persistence of noroviruses on standard viscose wipes and antiviral coated singlet oxygen producing wipes, and their contribution to the reduction of norovirus contamination on surfaces by wiping, was studied. Noroviruses were comparably persistent on the tested wipes, and cleaning wet spots with dry wipes efficiently eliminated 10^4 noroviruses from surfaces, and about 0.2% of the initial norovirus contamination was transferred to a clean surface through cross-contamination by wiping. Besides for high viral loads on surfaces (after, for example, a vomiting event), the risk of norovirus infections by cross-contamination from surfaces to fresh produce can likely be prevented by wiping surfaces (chapter 7).

Contaminated water used in the primary production of fresh produce, e.g., for irrigation, or for dilution of pesticides, presents another possible source for norovirus introduction. To investigate the potential of norovirus introduction by pesticide application, the persistence of viruses in diluted pesticides was studied in chapter 4. The results suggested that noroviruses may frequently (7 out of 8 tested pesticides) persist in the highest in practice applied concentrations of pesticides. Because viruses may remain infectious during the shelf-life of fresh produce (chapter 2), the application of pesticides, especially close to harvest, may pose a risk to public health if the microbiological quality of the water used to dilute the pesticides is not controlled.

Guidelines on the hygiene practices for fresh produce production emphasize the use of clean water in productions chains, but do not provide specific criteria on the required water quality. In chapter 7, performance objectives (critical pathogen concentrations) for the quality of irrigation water used to spray irrigate lettuce were estimated on the basis of the designed quantitative farm-to-fork risk assessment of chapter 5. A mean concentration of around 0.4 norovirus particles per L irrigation water was estimated to realize with 95% certainty an annual health target of less than 1 infection per 10,000 consumers of lettuce, as required for drinking water in the Netherlands. These calculations are specific to the lettuce chain described in chapter 5 and need to be reevaluated for other scenarios. The performance objective can also be used to estimate performance criteria for measures reducing norovirus contamination in irrigation water in which the critical norovirus concentration is exceeded. Depending on the type of water used, up to 10^3 norovirus genomes per L were detected in irrigation water, calling for intervention measures that reduce viruses by 3.5 $\log_{10}$-units, for the given example. Guidelines on water treatment processes applicable in agriculture are available, but a single treatment, e.g., waste stabilization ponds, or the addition of disinfectants, may not suffice to reduce virus concentrations to an acceptable health risk. A combination of water treatment

processes and postharvest measures such as drip irrigation, withholding periods, and produce washing is therefore recommended (chapter 7). In case of pesticide application, the addition of antiviral compounds to applied pesticides can be a supplementary mitigation strategy to reduce pathogen introduction, an approach that is independent of user compliance. A remaining challenge in the implementation of performance objectives and criteria for noroviruses is the absence of a methodology to determine infectious virus particles, as detected norovirus genomes not necessarily relate to infectious virus genomes.

Although a preventative food chain approach is preferred, additional measures reducing infectious norovirus loads on foods are required for adequate food safety, because a consistent and complete prevention of norovirus contamination in food chains is illusionary due to, e.g., non-compliance to good hygiene practice. Generally, mitigation measures are deemed efficient if they achieve at least a 3 $\log_{10}$-unit reduction of pathogens on produce. However, achieving such high reductions consistently with only one treatment step without causing produce deterioration is improbable; and most applicable treatments, such as washing, result in a 1 to a maximally 2 $\log_{10}$-unit reduction of noroviruses on fresh produce. Using a risk based approach we showed in chapter 7 that for the given scenario of norovirus introduction by food handlers, a 1 $\log_{10}$-unit reduction of norovirus on produce may reduce the risk of norovirus infection by 60 – 80%, depending on the initial virus concentration on the produce. Thus, even though not eliminating noroviruses from fresh produce, the implementation of mitigation measures achieving a robust 1 $\log_{10}$-unit reduction is advisable, especially if such measures present one of multiple links in the chain of risk management options to prevent and to reduce noroviruses in fresh produce chains.

Nederlandse samenvatting

Het norovirus is wereldwijd een frequente oorzaak van maagdarmklachten en wordt gezien als de meest voorkomende oorzaak van door voedsel overgedragen ziekten. Duizenden gevallen geassocieerd met norovirus uitbraken door consumptie van verse producten, in het bijzonder zachte fruit en sla, zijn beschreven. Risicobeheersmaatregelen moeten worden verbeterd om de norovirus ziektelast door de consumptie van verse producten te verminderen. Hiervoor werden gegevens over viruspersistentie, de relevantie van besmettingsbronnen van norovirussen, en de effectiviteit van maatregelen in dit proefschrift verzameld. Omdat norovirus infectiviteit vooralsnog niet kan worden bepaald, zijn onze resultaten gebaseerd op de detectie van norovirus genomen en schattingen van de infectiviteit afgeleid van gegevens voor het murine (muizen) norovirus, een veelgebruikt surrogaat om norovirus persistentie te bestuderen.

Norovirusen bleken te overleven gedurende de houdbaarheidsperiode van verse frambozen en aardbeien na de oogst onder algemeen toegepaste opslag condities (hoofdstuk 2). Hierdoor vormen besmetting na de oogst en gedurende de primaire productie, althans kort voor de oogst, bij consumptie van verse producten een mogelijk risico voor de volksgezondheid. Het onderzoek toonde verder aan dat de voedsel matrix invloed heeft op virale persistentie; echter, het exacte mechanisme bleef onbekend. De identificatie van voedsel-specifieke eigenschappen die norovirus persistentie op levensmiddelen beïnvloedt moet verder worden bestudeerd, bijvoorbeeld de relevantie van de respiratiesnelheid, of antivirale stoffen van verse producten op virale persistentie.

In plaats van het verminderen van de aanwezigheid van persistente virussen op producten, is het vermijden van norovirus besmetting de beste methode voor het waarborgen van de voedselveiligheid. Hiertoe werd de relevantie van diverse besmettingsbronnen van verse producten met norovirus, met behulp van een risico-gebaseerde aanpak onderzocht (hoofdstukken 3, 5 en 7). In hoofdstuk 3 werden kwantitatieve gegevens over virusoverdracht via de handen naar verse producten en vice versa bepaald om verspreiding van norovirussen op verse producten te simuleren en het daaraan verbonden risico voor de volksgezondheid te analyseren. De simulatie toonde aan dat, gezien de modelveronderstellingen, een enkele medewerker die frambozen plukt het norovirus kan verspreiden naar een groot aantal frambozen en tot 290 consumenten kan infecteren (hoofdstuk 7). Het kwantitatieve risicoschatting model beschreven in hoofdstuk 5 leverde aanvullend bewijs dat het contact van de handen met verse producten, in vergelijking met andere bestudeerde besmettingsbronnen, een van de dominante bronnen van besmetting was, gezien de gegevens en het gebruikte model. Hygiëne van medewerkers in de voedingsindustrie is dus cruciaal voor de voedselveiligheid. Een vermindering van slechts 1 $\log_{10}$-eenheid norovirussen op handen door het wassen van de handen resulteerde in een aanzienlijke vermindering van het risico op de volksgezondheid met een gemid-

delde verlaging van ongeveer 200 van 290 besmettingen in het bestudeerde voorbeeld (hoofdstukken 3 en 7). De naleving van handenwas procedures is echter een voorwaarde.

Naast medewerkers in de voedselbranche, kunnen norovirussen worden ingebracht in de voedselketen via besmette oppervlakken. In hoofdstuk 6 wordt de potentie van het schoonvegen van oppervlakken ten behoeve van vermindering van een kruisbesmetting bestudeerd. De persistentie van norovirussen op standaard viscose doekjes en doekjes gecoat met reactieve zuurstof voor antivirale werking, en de bijdrage van de doekjes aan de vermindering van norovirus besmettingen door het schoonvegen van oppervlakken werd onderzocht. Norovirussen waren vergelijkbaar persistent op de geteste doekjes. Het schoonmaken van natte plekken met droge doekjes verwijderde op een efficiënte manier 10.000 norovirussen van oppervlakken. Ongeveer 0,2% van de oorspronkelijke norovirus besmetting werd door kruisbesmetting door vegen overgebracht naar een schoon oppervlak. Behalve voor hoge virale belasting van oppervlakken (na bijvoorbeeld braken), kan het risico van norovirus infecties door kruisbesmetting van oppervlakken naar verse producten waarschijnlijk worden voorkomen door vlakken schoon te vegen met doekjes al dan niet met een antivirale coating (hoofdstuk 7).

Verontreinigd water dat wordt gebruikt in de primaire productie van verse producten, bijvoorbeeld voor irrigatie of voor het verdunnen van bestrijdingsmiddelen, vormt een andere mogelijke bron voor norovirus introductie. Om de potentiële introductie van norovirus door toepassing van pesticiden te onderzoeken werd de persistentie van virussen in verdunde pesticiden onderzocht in hoofdstuk 4. De resultaten suggereerden dat norovirussen vaak (bij 7 van de 8 geteste pesticiden) persisteerden in de hoogste in de praktijk toegepaste concentraties van pesticiden. Omdat virussen tijdens de houdbaarheidsperiode van verse producten (hoofdstuk 2) infectieus kunnen blijven (hoofdstuk 2), kan de toepassing van pesticiden, vooral vlak voor de oogst een risico vormen voor de volksgezondheid wanneer de microbiologische kwaliteit van het water dat gebruikt wordt om de pesticiden te verdunnen niet van voldoende kwaliteit is.

Richtlijnen voor een goede hygiëne bij de productie van verse producten benadrukken het gebruik van schoon water in productieketens, maar voorzien niet in specifieke criteria voor de benodigde kwaliteit van het water. In hoofdstuk 7 werden prestatiedoelstellingen geschat (kritische pathogeen concentraties) voor de kwaliteit van irrigatiewater dat wordt gebruikt om sla te besproeien op basis van de ontworpen kwantitatieve boerderij-tot-bord risicoschatting in hoofdstuk 5. Geschat werd dat een gemiddelde concentratie van 0,4 norovirus deeltjes per L irrigatiewater met 95% zekerheid een jaarlijkse gezondheidsdoelstelling van minder dan 1 besmetting per 10.000 consumenten van sla kan realiseren, zoals vereist voor drinkwater in Nederland. Deze berekeningen zijn voor de sla keten specifiek beschreven in hoofdstuk 5 en moeten opnieuw worden

geëvalueerd voor andere scenario's. De prestatie doelstelling kan ook worden gebruikt om prestatie criteria te schatten voor maatregelen die norovirus besmettingen in irrigatiewater waarin de kritische norovirus concentratie wordt overschreden beperken. Afhankelijk van het type water dat werd toegepast, werden tot 1000 norovirus genomen per L gedetecteerd in irrigatiewater, wat oproept tot interventiemaatregelen die virussen met 3,5 $\log_{10}$-eenheden kunnen verminderen, voor dit gegeven voorbeeld. Richtlijnen betreffende waterzuiveringsprocessen die toepasbaar zijn in de landbouw zijn beschikbaar, maar een enkele behandeling, bijvoorbeeld bezinkingsvijvers, of de toevoeging van ontsmettingsmiddelen, kunnen niet volstaan de virus concentraties tot een aanvaardbaar risico voor de gezondheid te reduceren. Een combinatie van waterzuiveringsprocessen en maatregelen na de oogst zoals druppelirrigatie, wachttijden, en het wassen van producten worden daarom aanbevolen (hoofdstuk 7). In het geval van toepassing van pesticiden kan het toevoegen van antivirale middelen aan de toegepaste pesticiden een aanvullende mitigatiestrategie zijn om pathogeen introductie te verminderen, een aanpak die onafhankelijk is van de naleving door de gebruiker. Een blijvende uitdaging in de uitvoering van de doelstellingen en criteria voor norovirussen is het ontbreken van een methodologie voor het bepalen van besmettelijke virus deeltjes, omdat gedetecteerde norovirus genomen niet noodzakelijkerwijs betrekking hebben op besmettelijke virus genomen.

Hoewel een preventieve aanpak de voorkeur verdient in de voedselproductieketen, zijn additionele maatregelen om besmettelijke norovirus belastingen op voedsel te verminderen vereist voor een adequate voedselveiligheid. Dit omdat een consistente en complete preventie van norovirus besmetting in de voedselketen een illusie is, als gevolg van bijvoorbeeld het niet naleven van een goede hygiëne. Over het algemeen worden risico beperkende maatregelen als doeltreffend beschouwd wanneer zij ten minste 3 $\log_{10}$-eenheden vermindering van ziekteverwekkers op producten bereiken. Echter, het bereiken van zo een hoge vermindering met maar een behandelingstap, zonder dat de productkwaliteit verslechterd is onwaarschijnlijk, en de meeste toegepaste behandelingen, zoals wassen resulteren in een 1 tot maximaal 2 $\log_{10}$-eenheden vermindering van norovirussen op verse producten. Met behulp van een op risico gebaseerde aanpak hebben we in hoofdstuk 7 laten zien dat voor het gegeven scenario van norovirus introductie door medewerkers in de voedselindustrie, een 1 $\log_{10}$-eenheid reductie van norovirus op producten het risico van norovirus infectie kan verminderen met 60 - 80%, afhankelijk van de oorspronkelijke virus concentratie op de producten. Hoewel dus het norovirus niet geëlimineerd wordt van producten, is de toepassing van mitigatie maatregelen die een robuuste 1 $\log_{10}$-eenheid vermindering bereiken aan te raden, vooral wanneer dergelijke maatregelen een van meerdere schakels in de keten van risicomanagement opties vormen om norovirussen in de productieketen van verse producten te voorkomen en verminderen.

Acknowledgements

Voltooid, Accomplished, Vollendet! Ik ben ontzettend blij en trots het project proefschrift eindelijk af te sluiten. Maar dit zou niet mogelijk geweest zijn zonder de hulp van een aantal mensen:

To start with, I wish to acknowledge Ana Maria. Thank you for your confidence in me during the last few years, for the freedom you offered me in doing my research, and for your valuable contributions to this thesis. Working with you improved both my research and my personal skills.

Arie, your structured way of working and your broad knowledge in the area of food safety made it a pleasure to work with you. Each meeting with you made me wiser, and I especially appreciate your helpful input to the thesis discussion. I wish you all the best for your new start in Florida.

Frans, our relationship was not characterized by frequent contact, but whenever I had a question or request, you and Christina would take a moment to answer. Thank you for that.

I am particularly grateful for the assistance of my dearest copromotor Martijn. Of all people involved in my thesis, you supported me the most, and you greatly improved my writing, data analysis, and general research skills. Working with you was instructive and fun, and I am deeply thankful that you invested so much time and effort in me. Je bent een heel aardige en slimme vent en ik vond het erg leuk om met jou samen te werken.

I would also like to express my gratitude and appreciation to Leen Baert, Rosina Gironés, Marion Koopmans, Marcel Zwietering, and Jack Schijven for agreeing to be part of my dissertation committee.

Saskia, I very much appreciated to work with you and learn from you. I could always pass by your office with a question and return with a helpful and practical answer, especially on issues related to my experimental set-ups.

Erwin and Era, you shared not only your norovirus knowledge with me, but also your virus stocks and cell lines – I really valued your help and assistance. Erwin, writing a paper with you was a pleasure.

Froukje, you had the sublime task of introducing me to the lab-world of viruses, and I might have challenged your patience from time to time. Nevertheless, as a result we

became a great team in the lab (and not only there), and thanks to you, long and repetitive days in the *flowkast*, became fun and memorable moments.

Dr. Willemijn Lodder, especially in the last period of my thesis you were a source of motivation to me. Being ahead of me in finishing your thesis, you always knew the answer to all my questions on "how", "why" and "where." Thank you for that and for your help and guidance during these years.

I also want to thank all my Z&O *lotgenoten*: Seta, Eri, Ankie, Ellen, Willemijn, Claudia, Jeroen and Frits. Thank you guys for fruitful discussions, for the (occasional) sharing of PhD frustrations, and for nice diners and drinks.

My special appreciation goes to Eri, Ankie and Ellen. I was very lucky to have had you as colleagues and to have gained you as my friends. I cherished our tea and cake parties, pizza evenings, chocolate and movie nights, and our trips to e.g. Gorinchem and Cologne. I count on you to perpetuate our carnival tradition and look forward to our trip to Copenhagen and Munich (subtle hint…).

Arno, as a newbie at the RIVM and in the Netherlands, times were challenging. You were the first friend I made and I want to thank you for your friendship, for being a source of good food and "beverages," for inspiring conversations, and of course for your contributions to this thesis. Together with Arnout, we had plenty of good laughs and nights out, and one highlight was definitely the trip to our former and much appreciated colleague Monika in Innsbruck. Thank you both for your encouragement, and for keeping me from time to time from surrendering.

Britta, ich bin sehr froh dich kennengelernt zu haben, wozu so ein Niederländisch Kurs doch gut sein kann. Du, Emile und Leana wart mir immer mein liebstes Zuhause in Utrecht und Rückzugsort für deutsche Kultur (gutes Essen und Tatort). Danke für Eure Unterstützung und Ablenkung in den letzten Jahren.

And my Catalan chicas: Ana and Anna. Ana, your character is certainly unique and you are very special to me. Living and working with you was terrific and together with Harold we had splendid times in Utrecht and Barca. Thanks to both of you for a great time and for your support. Anna, it was legen...dary to visit conferences with you, to participate in the VITAL project, and even to work together long days in the lab. Thank you for your visits in Utrecht, your good humor, your uplifting personality, and your contributions to this thesis.

Mohammed, you were the only student I worked with and you impressed me as a person and as a researcher. Especially because I had just started my PhD when you arrived, I often felt that I could learn more from you than you could learn from me. I very much enjoyed working with you.

De beste kamer op het RIVM: V3.49! Angelina, Wendy, Marieke, Ellen en Cecile, ik heb ontzettend genoten van onze kamer. Er was altijd een goeie sfeer en ik voelde mij op mijn gemak. Bedankt voor een hele goeie tijd en voor jullie steun!

Noël, Jeanette, Loes, Hennie en Ingrid (UU), bedankt voor jullie hulp met, bijvoorbeeld, personele zaken of het boeken van reizen. Een extra grote dank aan Noël voor het vertalen van mijn samenvatting. Roland, bedankt voor jouw hulp met het bestellen van lab spullen.

And there are so many more colleagues that were on hand with help and advice, for example, Manoj, Luc, Radi, Rob, Ingmar, Lianne, Rozemarijn, Annemarie, Marjolijn, Ton, Bas, Hein, Ciska, Arieke, Jurgen… Z&Oers, thank you for being such great colleagues.

Seta and Gudi, my dearest *paranimfen*, I am so fortunate to have you by my side. We form a great trio, and we shared pretty much everything in the last years: the tough times but also moments of great fun. You girls inspire me and make me progress, thank you for your support, encouragement, and our close friendship.

Mon chéri Baptiste, we have not met in an easy stage of my life, and I appreciate and admire your seemingly infinite patience with me. I want to thank you for supporting me in finalizing my thesis, for giving me the strength to continue at times when I just wanted to rest, for teaching me the French *joie de vivre*, for challenging my attitudes, and for making me a stronger and more self-confident person. Our long-distance relation can be demanding, but I am very grateful for all the marvelous moments we shared in Copenhagen, Paris, Utrecht, the Pyrenees, Sweden…, and now in Munich. I hope we will eventually manage to settle in the same country and city.

Und einen großen Dank an meine langjährigen Freunde aus dem hometown Goch, im Besonderen: Kristin, Lisbeth, Anna, Lena, Yennefa, Kathrin, Sara, Thomas & Cordula; und meine lieben Freunde aus meiner Zeit in Bonn: Markus & Kathrin (alias Uschi), Sascha, Almut, Gökçe und Stefan. Danke für Euren Beistand, Eure Hilfe, Euer Verständnis, Eure Geduld und Eure Freundschaft in den letzten 10 – 27 Jahren.

Elizabeth und Joachim, auch Euch möchte ich von ganzem Herzen für Eure fortwährende Unterstützung und Ermutigung danken – an dieser Stelle auch ein Dankeschön an Richard.

Den letzten, aber wichtigsten Platz der Danksagung räume ich meiner Familie ein. Mama, Papa, Alexander, ich bin sehr froh Euch als meine Familie zu haben und möchte mich für Euren Rückhalt, Eure Unterstützung und mein schönes Zuhause bedanken. Mit der Vollendung dieses Buches gehören Eure ständigen Fahrten von Goch nach Nijmegen und zurück endgültig der Vergangenheit an.

Curriculum Vitae

Katharina Verhaelen was born on February 25th 1983 in Kleve, Germany. She graduated from secondary school at the Gymnasium Goch in 2002. Afterwards she started studying food technology at the Rheinische Friedrich-Wilhelms-University Bonn, and graduated cum laude as a food technology engineer in March 2008. In the course of her diploma thesis, she visited Wageningen University in January 2007, to explore the use of *Rhizopus oryzae* cellulases in the bioethanol production. In December 2007, she traveled to New Zealand for an internship at AgResearch Ltd, Ruakura Research Centre, and investigated the potential of lactic acid bacteria for biopreservation of meat. In January 2009, she started working at the National Institute for Public Health and the Environment (RIVM), Centre for Infectious Disease Control, Laboratory for Zoonoses and Environmental Microbiology, on the research described in this thesis. The PhD project was a joint venture between RIVM and the Institute of Risk Assessment Sciences (IRAS), University Utrecht. Her research was embedded in a European project, with the acronym 'VITAL', on integrated monitoring and control of foodborne viruses in European food supply chains. Since May 2014, Katharina Verhaelen is employed as a research associate at the Bavarian Health and Food Safety Authority (LGL), and works on the establishment of an early warning system for food safety in Bavaria, Germany.

List of Publications

D'Agostino, M., Cook, N., Di Bartolo, I., Ruggeri, F.M., Berto, A., Martelli, T., Banks, M., Vasickova, P., Kralik, P., Pavlik, I., Kokkinos, P., Vantarakis, A., Söderberg, K., Maunula, L., Verhaelen, K., Rutjes, S., de Roda Husman, A.M., Hakze, R., Van der Poel, W., Kaupke, A., Kozyra, I., Rzeżutka, A., Prodanov, J., Lazic, S., Petrovic, T., Carratalà, A., Gironés, R., Diez-Valcarce, M., Hernandez, M., Rodríguez-Lázaro, D., 2012. *Multicenter Collaborative Trial Evaluation of a Method for Detection of Human Adenoviruses in Berry Fruit*. Food Analytical Methods, 5:1–7.

Verhaelen, K., Bouwknegt, M., Lodder-Verschoor, F., Rutjes, S.A., de Roda Husman, A.M. 2012. *Persistence of human norovirus GII.4 and GI.4, murine norovirus, and human adenovirus on soft berries as compared with PBS at commonly applied storage conditions*. International Journal of Food Microbiology, 160:137-144.

Verhaelen, K., Bouwknegt, M., Rutjes, S.A., de Roda Husman, A.M. 2013. *Persistence of human norovirus in reconstituted pesticides - pesticide application as a possible source of viruses in fresh produce chains.* International Journal of Food Microbiology, 160:323-328. – This study was elected as Elsevier Research Alert, resulting amongst other media attention in an article in the news forum of Environmental Health Perspectives, 121(5).

Verhaelen, K., Bouwknegt, M., Carratalà, A., Lodder-Verschoor, F., Diez-Valcarce, M., de Roda Husman, A.M., Rutjes, S.A. 2013. *Virus transfer proportions between gloved fingertips, soft berries, and lettuce, and associated health risks.* International Journal of Food Microbiology, 166:419-425.

Bouwknegt, M., Verhaelen, K., Rzeżutka, A., Kozyrac, I., Maunula, L., von Bonsdorff, C.H., Vantarakis, A., Kokkinose, P., Petrovic, T., Lazic, S., Pavlik, I., Vasickova, P., Willems, K.A., Havelaar, A.H., Rutjes, S.A., de Roda Husman, A.M. 2014. *Quantitative farm-to-fork risk assessment model for norovirus and hepatitis A virus in European leafy green vegetable and berry fruit supply chains*. Article in press in the International Journal of Food microbiology.

Verhaelen, K., Bouwknegt, M., Rutjes, S.A., de Roda Husman, A.M., Duizer, E. 2014. *Wipes Coated with a Singlet Oxygen Producing Photosensitizer are Effective Against Human Influenza Virus but not Against Norovirus*. Applied and Environmental Microbiology, 80(14).

Kokkinos, P., Bouwknegt, M., Willems, K., Moloney, R., de Roda Husman, A.M., Verhaelen, K., D'Agostino, M., Cook, N., Vantarakis, A. 2014. *Virological fit-for-purpose risk assessment in a leafy green production enterprise*. Article in preparation.